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DEVELOPMENT OF THE RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) TECHNIQUE TO MEASURE THE EFFECTS OF GENOTOXINS IN AQUATIC ORGANISMS

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**DEVELOPMENT OF THE RANDOM AMPLIFIED POLYMORPHIC DNA
(RAPD) TECHNIQUE TO MEASURE THE EFFECTS OF GENOTOXINS
IN AQUATIC ORGANISMS**

by

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in partial fulfilment for the degree of**

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Development of the random amplified polymorphic DNA(RAPD) technique to measure the effects of genotoxins in aquatic organisms

Franck André Atienzar

ABSTRACT

Studies were undertaken to evaluate the potential of random amplified polymorphic DNA (RAPD) to detect DNA effects (including DNA damage and mutations) in aquatic invertebrates, following their exposure to a variety of environmental contaminants under laboratory conditions. After rigorous optimisation of the RAPD method, the protocol, which used a high annealing temperature (50°C for 10-mer primers), was found to generate good-quality DNA profiles from groups of organisms belonging to the bacterial, plant and animal kingdoms. The RAPD method was initially used to detect benzo(a)pyrene [B(a)P] and copper-induced DNA effects in the water flea *Daphnia magna* and ultraviolet-mediated DNA effects in the marine alga *Palmaria palmata*. The results clearly showed that changes occurred in RAPD profiles obtained from the exposed populations when compared to controls. In these studies, the effect of the genotoxins at higher levels of biological organisation (e.g. Darwinian parameters and/or fitness parameters) were also investigated and were compared with genomic DNA template stability (GTS), a qualitative index representing clear changes in patterns compared to control RAPD profiles. The results from these experiments revealed that GTS could be more sensitive than growth parameters and showed at least equal or even greater sensitivity than other measures of fitness. Changes in RAPD profiles were believed to be the result of DNA effects, namely adduct formation, DNA breakage, oxidative damage and mutations and possibly other effects (e.g. variation in gene expression). Nevertheless, the nature and amount of DNA effects could only be speculated because diverse events may induce the same category of changes (i.e. variation in band intensity, appearance of bands, and disappearance of amplicons) in RAPD patterns. Further studies confirmed that RAPD had the potential to qualitatively detect oestrogen and xeno-oestrogen -induced DNA effects in barnacles. Additional experiments emphasised that oxygen radicals and variation in gene expression may induce significant changes in RAPD profiles.

To further understand the effects of DNA lesions and mutations on RAPD patterns, individual types of DNA damage were created *in vitro*. The results clearly indicated that B(a)P DNA adducts, DNA photoproducts, and DNA breakages had significant effects on RAPD profiles but that diverse types of DNA damage may induce the same category of changes in RAPD patterns which render the interpretation of the results difficult. It was also concluded that mutations could be detected provided they do not arise in a random fashion.

Finally, an attempt was made to determine the kinetics of DNA damage and DNA repair and whether changes in patterns obtained from B(a)P exposed *Daphnia magna* could be transmitted to successive generations. This strategy was developed to distinguish between mutations and DNA damage. The results showed that some bands obtained from the exposed populations were transmitted to the first and/or second generation but not to the third. It was concluded that the transmission of modified genetic material to the offspring was more likely to be the result of large genomic rearrangements and/or base methylation (epigenetic processes) rather than point mutations.

In conclusion, the results presented in this research project show the potential of the RAPD assay as a useful method for the qualitative assessment of DNA effects including genotoxicity and changes in gene expression. The main advantage of this technique is that it can be applied to any species without requiring any information about the nucleotide sequence. In the field of ecogenotoxicology, its main advantage lies in its sensitivity and speed to detect a wide range of DNA damage including DNA breakage, DNA adducts, oxidative damage as well as mutations (including point mutations and large rearrangements). On the other hand, RAPD only allows a qualitative assessment of the DNA effects and the nature of the changes occurring in profiles can only be speculated. Finally, a great deal of further experimentation and validation are required in order to assess the applicability of the technique to a variety of other species and pollutants, particularly under field conditions.

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Grayson TH, **Atienzar FA**, Alexander SM, Cooper LF, Gilpin ML. 2000. Molecular diversity of *Renibacterium salmoninarum* isolates determined by randomly amplified polymorphic DNA analysis. *Applied and Environmental Microbiology* 66:435-438.

Atienzar FA, Cordi B, Donkin ME, Evenden AJ, Jha AN, Depledge MH. 2000. Comparison of ultraviolet-induced genotoxicity detected by random amplified polymorphic DNA with chlorophyll fluorescence and growth in a marine macroalgae, *Palmaria palmata*. *Aquatic Toxicology* (in press).

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Signed 

Date.....9/7/2000.....

Abbreviations

%:	percent
µg:	microgram (10 ⁻⁶ g)
µl:	microliter
µm:	micrometre
µM:	micromolar
5-mC:	5-methyl cytosine
Ah:	aromatic hydrocarbon
ANOVA:	analysis of variance
AP-PCR:	arbitrarily primed polymerase chain reaction
ASTM:	American society for testing materials
ATP:	adenosine triphosphate
B(a)P:	benzo(a)pyrene
BKD:	bacterial kidney disease
bp:	base pair
BSA:	bovine serum albumin
Bt:	inter-brood time
C:	cytosine
CA:	chromosome aberrations
CHO:	Chinese hamster ovary
cm:	centimetre
CMC:	chemical mismatch cleavage
d:	day
DAF:	DNA amplification fingerprinting
dATP:	deoxyadenine triphosphate
dCTP:	deoxycytidine triphosphate
DGGE:	denaturing gradient gel electrophoresis
dGTP:	deoxyguanine triphosphate
DMSO:	dimethyl sulfoxide
DNA:	acid desoxyribonucleic
dNTP:	deoxynucleotide triphosphate
dsb:	DNA double strand breakage
dTTP:	deoxythymidine triphosphate
EC:	effect concentration
EDTA:	ethylenediamine tetra acetic acid
EROD:	7-ethoxyresorufin O-deethylase
g:	gram
G:	guanine
GTS:	genomic DNA template stability or genomic template stability
h:	hour
Hb:	hemoglobin
his:	histidine
hprt:	hypoxanthine phosphoribosil transferase
HSP 70:	heat shock protein 70 Kda
ICPEMC:	International commission for protection against environmental mutagens and carcinogens
ITS:	intergenic spacer
K:	thousand
Kbp:	kilobase pairs
Kda:	kilo dalton

kg:	kilogram (10^3 g)
L:	litre
LB:	Luria-Bertani
LC:	lethal concentration
LSD:	Least significant differences
l_x:	age-specific survival
m:	mixture
M:	molar
MFO:	mixed function oxygenase
mg:	milligram (10^{-3} g)
min:	minute
ml:	millilitre
mm:	millimetre
mM:	millimolar
MN:	micronucleus/micronuclei
mRNA:	messenger ribonucleic acid
MT:	metallothioneins
m_x:	age-specific fecundity
NADH:	reduced β nicotinamide adenine dinucleotide
NADPH:	reduced nicotinamide adenine dinucleotide phosphate
nm:	nanometer
NOEC:	no observed effect concentration
°C:	degree centigrade
OD:	optical density
p:	statistical probability
P450:	cytochrome P450
PAH:	polynuclear aromatic hydrocarbon
PCB:	polychlorobiphenyl
PCR:	polymerase chain reaction
PMS:	rat liver post-mitochondrial supernatant
PTT:	protein truncation test
RAPD:	random amplified polymorphic DNA
rDNA:	ribosomal DNA
RFLP:	restriction fragment length polymorphism
r_m:	intrinsic rate of natural increase
RNA:	ribonucleic acid
R_0:	net reproductive rate
RSM:	restriction site mutation assay
RT-PCR	reverse transcriptase polymerase chain reaction
s:	second
SCE:	sister chromatid exchange
SFG:	scope for growth
ssb:	single strand break
SSCP:	single-stranded conformation polymorphism
T:	thymine
TBE:	Tris-Borate-EDTA
TE:	Tris-EDTA
T_{min}:	minimum generation time
U:	uracil or units
UDS:	unscheduled DNA synthesis
UV:	ultraviolet
v/v:	volume/volume

V:	volts
w/v:	weight/volume
W:	watts
WHO:	World Health Organisation
x g:	acceleration due to gravity

Chapter 1

INTRODUCTION

Over the course of recorded history, human activities have dramatically altered the natural environment of this planet and its inhabitants. While the alarming levels of pollution in the major cities are often considered as unacceptable, the production, consumption and disposal of ever-increasing quantities of new chemicals continues. The number of organic compounds synthesised by man totals over 1.8 million and is growing at a rate of at least 250,000 formulations per year (Zhou *et al.*, 1996). Global production of these anthropogenic organics has been estimated to be 100-200 million tons per year and up to one third of this production may enter into the environment (Stumm and Morgan, 1981). In the United States, more than 384 million tons of waste are generated annually by the industry and billions of litres of industrial effluents are discharged daily into rivers, streams, harbours, and municipal sewage systems (Houk, 1992). Claxton *et al.* (1998) also reported that several million kilograms of genotoxic and/or carcinogenic industrial wastes are released into the United States environment each year. In addition to the continuous discharge all over the world, there have been accidents leading to exposures with tragic consequences for the environment. In 1984, a storage tank at a Union Carbide pesticide plant (Bhopal, India) exploded and released a cloud of methyl isocyanate killing 2,000 people and affecting the health of an estimated 200,000 more (Anonymous, 1984). In 1986, the largest radioactive release in history occurred in Ukraine where a reactor in a nuclear power station exploded at Chernobyl (Newman, 1998). It has been reported that the frequency of mutation significantly increased in human (Dubrova *et al.*, 1996) but not in rodents (Baker *et al.*, 1996*). In 1996, the grounding of the Sea Empress oil tanker resulted in the release of 72,000 tonnes of crude oil into Milford Haven, Wales, UK. It was reported that this contamination resulted in elevated levels of DNA adducts in vertebrate but not in invertebrate species (Lyons *et al.*, 1997; Harvey *et al.*, 1999). Unfortunately the list of such events is far from being exhaustive. However, such incidents could be considered of minor importance. Even during the heyday of major tanker incidents in the 1970's, these accidents contributed less than 30 % of the annual emissions of petroleum hydrocarbons to the marine environment (Evers *et al.*, 1997). Persistent chemicals have even accumulated in

* A retraction was printed by Baker *et al.* (1997, *Nature* 390:100) indicating that the mitochondrial cytochrome *b* mutation rates were incorrect as a result of miscoding mutations. The mutation rates in the voles near Chernobyl are higher than the control population but this difference is not statistically significant.

areas such as the Arctic and Antarctic far away from the source of release (Bacon *et al.*, 1992; Barrie *et al.*, 1992; Hargrave *et al.*, 1993). In this context, many organisms may not have the opportunity to either avoid contamination or adapt to the increasing number of chemicals released into the environment. Together with other anthropogenically induced disturbances such as deforestation, urbanisation, fisheries, agriculture, this may result in the extinction of local populations and even complete species at a rate previously unseen (Wilson, 1988; Ellis, 1989).

The risk of such widespread contamination events has led to the development of a new discipline of ecotoxicology or environmental toxicology. Ecotoxicologists have been charged to evaluate the impact of contaminants on the health of our environment and its inhabitants. The strategy consists in measuring endpoints at different levels of biological organisation in species sampled from the field. However, since the environment is contaminated by a large number of compounds the interpretation of the data can be quite difficult. Consequently, the ecotoxicologist has often exposed relevant species to environmental contaminants in the laboratory and measured endpoints at different levels of biological organisation to try to understand how the environmental contaminants can adversely affect the species in the environment. In the field of ecotoxicology, the evaluation of the effects of environmental contaminants on aquatic invertebrate species are particularly important because the aquatic environment which covers two thirds of the planet is inhabited by the majority of living species, more than 90% of which are invertebrates.

In recent years, considerable attention has been directed towards improving our understanding of the ways contaminants interact with genetic material. The genetic material which contains the information necessary to determine the development of an organism, is transmitted from parent to offspring. It consists of deoxyribonucleic acid (DNA), with the exception of some bacterial, plant and animal viruses in which it is ribonucleic acid (RNA). Environmental mutagens and carcinogens may have at least two main effects. They may cause mutation in germ cells resulting in the accumulation of heritable abnormal genes in the

population, and may also be responsible for mutation of somatic cells resulting in the formation of tumours in individuals (Nagao and Sugimura, 1978). Evidence for these effects have been widely reported in many studies using fish and mammals (Zarbl *et al.*, 1985; Barbacid, 1986; Van Beneden *et al.*, 1990; McMahon, 1994). However, the incidence of neoplasia in invertebrates is not as clear firstly because the number of investigations have been limited and secondly because there has been some controversy (Hesselman *et al.*, 1988; Gardner *et al.*, 1991; Kurelec, 1993; Depledge, 1994a; Van Beneden *et al.*, 1999). In the ecological context, exposure to genotoxins may have significant implications for the long-term survival of exposed populations (Wurgler and Kramers, 1992). Once released, the environmental contaminants have the capability not only to cause morbidity and/or mortality in the exposed organisms, but potentially may induce higher order changes such as alterations to population dynamics and changes to biological diversity at both intra- and interspecies levels. Such changes may initiate direct and catastrophic ecological consequences. However, whilst pollutant-DNA interactions can be readily demonstrated using diverse methodologies, there have been few direct experimental demonstrations of the wider relationships between DNA effects and the consequences at higher levels of biological organisation (Seiler, 1982; De Raat *et al.*, 1985; Wurgler and Kramers, 1992; Mohn and De Raat, 1993; Anderson *et al.*, 1994b; Anderson and Wild 1994; Depledge, 1994a; 1998; Jha, 1998).

The use of genotoxicity and mutagenicity assays for monitoring the environmental effects of mutagens and carcinogens is becoming increasingly important (De Flora *et al.*, 1991; Godet *et al.*, 1993; Jha *et al.*, 1996; 1997; 2000; Claxton *et al.*, 1998). These assays could be broadly classified as biochemical, molecular as well as cytogenetical assays. The biochemical assays allow, for instance, the detection of DNA adducts [e.g. ^{32}P -post labelling (Randerath *et al.*, 1981)], DNA strand break measurements [e.g. alkaline/neutral elution technique (Rydberg, 1984)] while the cytogenetical assays include, for instance, the determination of chromosomal aberrations (Natarajan *et al.*, 1996) and the induction of micronuclei (Cole *et al.*, 1979). Many of these assays, which were originally developed to

evaluate human health risk following exposure to physical and chemical genotoxins, have been adopted for detection of genotoxin exposure in natural biota. Other methods to detect and quantify DNA lesions induced by a wide variety of genotoxic agents include some physico-chemical techniques for analysis of biological samples, such as electrochemical (Park *et al.*, 1989) and spectroscopic (Giam *et al.*, 1989) methods as well as flow cytometry (Bickham, 1990), mass-spectrophotometry (Kaiser *et al.*, 1990), and microspectrofluorometry or autoradiography (Mori *et al.*, 1990). In addition, the detection of mutations has also been intensively studied. Many methods available involve the phenotypic expression of the mutation using bacterial, yeast and mammalian systems (Albertini *et al.*, 1990; Cole *et al.*, 1990; Steingrimsdottir *et al.*, 1996). Other mutation tests include, for example, the use of the polymerase chain reaction (PCR) (Cebula and Koch, 1991; Cortopossi and Arnheim, 1992), DNA fingerprinting (Jeffreys, 1987), transgenic animals (Dolle *et al.*, 1999), conformational analysis (Rossiter and Cashey, 1990). However, since most of the techniques characterise specific DNA damage or mutation, there is a real need to develop new powerful technologies to detect a wider range of DNA damage and mutation.

The PCR which was firstly described by Saiki *et al.* (1985) has revolutionised the molecular biology and has been used in diverse areas of research such as evolution, clinical medicine, forensics, pathogen detection, and molecular genetics. For instance, PCR was successfully used to study and quantify mutations (Cebula and Koch, 1991) as well as DNA damage (Murray *et al.*, 1992) in specific locations. New PCR based methods were subsequently developed. In particular, Williams *et al.* (1990) and Welsh and McClelland (1990) developed the random amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AP-PCR), respectively. Although RAPD and AP-PCR are very similar techniques, there are some procedural differences between the techniques (Meunier and Grimont, 1993). Both methods are based on the selective amplification of genomic sequences that, by chance, are flanked by adequate matches to an arbitrarily chosen oligonucleotide primer sequence. The RAPD and AP-PCR techniques have been used in genetic mapping, taxonomy, and

phylogenetic studies (Welsh and McClelland, 1990; Williams *et al.*, 1990; Welsh *et al.*, 1991). Since 1992, new applications of these methods have been developed. For instance, the AP-PCR method was successfully applied to detect γ -ray-induced DNA damage (Kubota *et al.*, 1992; 1995) and somatic genetic alterations in tumours of the colon, lung and rectum of human (Peinado *et al.*, 1992; Ionov *et al.*, 1993; Kohno *et al.*, 1994; Arribas *et al.*, 1997). In addition, the RAPD method was also successfully utilised to detect polycyclic aromatic hydrocarbons (PAHs)-induced DNA damage in rats (Savva *et al.*, 1994; Savva, 1996). Thus, both RAPD and AP-PCR techniques appeared to be useful procedures for the detection of DNA lesions and mutations. When the present study commenced, in February 1997, very little data were available on the possible applications of the AP-PCR and RAPD for the detection of DNA damage and mutation, and to the best of our knowledge, no studies had been carried out on invertebrates. Since 1997, the RAPD and AP-PCR were successively applied, mostly in non-invertebrates systems, to detect DNA damage and mutation (Shimada and Shima, 1998; Becerril *et al.*, 1999; Lopez *et al.*, 1999), as well as genetic diversity (Nadig *et al.*, 1998; Theodorakis *et al.*, 1998; 1999; Krane *et al.*, 1999; Liu *et al.*, 1999).

Although the RAPD method has been successfully applied in many areas of research, the technique has been criticised for its lack of reproducibility (Ellsworth *et al.*, 1993; Khanda *et al.*, 1997), and Mendelian inheritance (i.e. presence of non parental bands in patterns generated by offspring) (Riedy *et al.*, 1992; Scott *et al.*, 1992; Ayliffe *et al.*, 1994). The amplification of spurious products has also been reported to occur in RAPD reactions (Mullis, 1991). Nevertheless, after rigorous optimisation, the RAPD methodology has been reported as a robust assay with good reproducibility under defined conditions (Rothuizen and Wolferen, 1994; Benter *et al.*, 1995; Bielawski *et al.*, 1995).

In the light of aforementioned information, the aims of this research project were to:

- 1) evaluate the potential of the RAPD technique for the detection of DNA effects (including DNA damage and mutations) in aquatic invertebrates following exposure to a variety of environmental contaminants, under laboratory conditions,

- 2) examine the potential impairment of classical Darwinian fitness parameters (e.g. growth, reproductive output, viability of offspring) among the exposed population,
- 3) compare and link the effects measured at the DNA level by using the RAPD method with the effects determined at the population level.

The present paragraph gives an overview of the content of the research project. Chapters 2 and 3 describe literature review and materials and methods, respectively. In Chapter 4, the aim was to determine if the RAPD is a robust method using different species. In particular the objective was to investigate whether the problems associated with the lack of reproducibility and spurious amplifications could be solved by carefully studying the effect of each PCR component on RAPD profiles. In the subsequent chapters, the potential of the RAPD method was evaluated to detect benzo(a)pyrene (chapter 5) and ultraviolet (chapter 6) -induced DNA effects in the water flea *Daphnia magna* and the algae *Palmaria palmata*, respectively. The RAPD technique was also assessed for its ability to detect copper-induced DNA effects in *Daphnia magna* (chapter 6). In chapters 5-7, the potential effects of these genotoxins at population levels were also compared to genomic template stability (a parameter reflecting changes in RAPD profiles). Chapter 8 describes three different studies. The technique was used to detect the potential genotoxic effects of oestrogens and xeno-oestrogens in barnacle larva (study 1) and adult crabs (study 2). In the third study, the RAPD technique was assessed for the detection of DNA effects in *Daphnia magna* exposed to stressful environments without the use of anthropogenic contaminants. The aim of Chapter 9 was to determine the effects of specific types of DNA damage (e.g. DNA adducts, DNA breakages, DNA photoproducts) and mutations on RAPD profiles. In chapter 10, the RAPD method was evaluated as a tool to detect the kinetics of DNA effects, DNA repair and transgenerational effects in benzo(a)pyrene-exposed *Daphnia magna*. Finally, the thesis concludes with a general discussion (chapter 11).

Chapter 2

LITERATURE REVIEW

2.1 Ecotoxicology and eco-genotoxicology or genetic ecotoxicology

2.1.1 The origin of ecotoxicology

Hazardous substances are distributed widely in ecosystems due to diverse human activities such as energy usage, industrial enterprises, agriculture, and activities of the defence industry. Nevertheless, environmental problems continue to occur despite the increased awareness and complex regulations. For example, it has been estimated that 100-200 million tons of anthropogenic organics are produced each year and that up to one third of this production could enter into the environment (Stumm and Morgan, 1981). The recognition of the continual release of contaminants into the environment and its detrimental effects on the ecosystem has led to the development of the field of Ecotoxicology. Some definitions of ecotoxicology seem to exclude discussion of human except as the source of contaminants, but the original definition given to ecotoxicology by Truhaut (1977) also included the impact of pollutants on human life. He defined ecotoxicology as:

‘the branch of toxicology concerned with the study of toxic effects, caused by natural and synthetic pollutants, to the constituents of ecosystems, animals (including human), vegetable and microbial, in an integrated context’.

Since 1977, there have been numerous attempts to define the word ‘ecotoxicology’ (e.g. Cairns and Mount, 1990; Forbes and Forbes, 1994; Newman, 1998). It has been generally defined as the study of the harmful effects of chemicals upon ecosystems and their component parts (i.e. flora, fauna). It is an interdisciplinary subject which represents a synthesis of elements from the disciplines of chemistry, biochemistry, toxicology, physiology, population ecology and population genetics. In contrast to ‘classical’ toxicology, the ultimate concern is about effects at the population and community levels.

2.1.2 Eco-genotoxicology or genetic ecotoxicology

Of great concern to certain ecotoxicologists are the genotoxins. These are the chemical and physical agents capable of interacting with the genetic material in cells of living organisms. The release of genotoxins into the environment and their effects on the ecosystem has led to the development of eco-genotoxicology or genetic ecotoxicology (Würgler and Kramers, 1992) which can be defined as:

‘The study of chemical- or radiation-induced changes in the genetic material of natural biota. Changes may be direct alterations in genes and gene expression or selective effects of pollutants on gene frequencies (Anderson et al., 1994b).’

2.1.2.1 Terminology

The interaction of toxic chemicals with the genetic material may be divided into three types of effects: aneuploidisation, clastogenesis, and mutagenesis (Timbrell, 1991). Aneuploidy is the acquisition or loss of a complete chromosome. Clastogenesis is the loss, addition or rearrangement of parts of a chromosome. Mutagenesis is the loss, addition or alteration of a small number of base pairs. Genotoxins which have the potential to induce cancer are called carcinogen and those responsible for developmental malformations are called teratogen. A cancerous cell is a cell which has escaped normal controls regulating its growth and division, producing a clone of dividing daughter cells which invade adjacent tissues and may interfere with their activities (Abercrombie *et al.*, 1980). Cancer cells that proliferate but stay together form benign tumours; those that not only proliferate but also shed cells form malignant tumours, and cancer generally refers to a disease resulting from either. Among these, carcinomas are malignant tumours of epithelial cells; teratocarcinomas are carcinomas that can be cultured in serially grafted to other hosts; sarcomas are cancers of connective tissue; myelomas are malignant tumours of bone marrow (Abercrombie *et al.*, 1980). Druckrey (1973) reviewed the relationship between mutagenicity, carcinogenicity and teratogenicity. He concluded that ‘carcinogenesis

undoubtedly is far more complex than mutagenesis, and any generalisation as to a 'mutation theory of cancer' cannot be considered as satisfactory. Recent studies have also reported that the correlation between genotoxicity and carcinogenicity was fairly weak (White and Rasmussen, 1996; Fetterman *et al.*, 1997). Druckrey (1973) defined 'genotoxic' substances as 'any agent which, by virtue of its physical or chemical properties, can induce or produce heritable changes in those parts of the genetic apparatus that exercise homeostatic control over somatic cells, thereby determining their malignant transformation'. By using the word homeostasis he had already sensed the phenomenon of non-genotoxic carcinogenesis (Ashby, 1995).

2.1.2.2 State of the art

The situation in the evaluation of mutagenic risks to the environment is somewhat different from human risk assessment, in the sense that the objectives with regard to ecosystems and ecological compartments may be directed towards the maintenance of their integrity at the level of populations, rather than that of individuals (Seiler, 1982; Wurgler and Kramers, 1992; Mohn and De Raat, 1993). Although the detection of DNA damage has been greatly studied using a number of laboratory methods, to the best of our knowledge, the study of DNA lesions and their effects at higher levels of biological organisation have been rarely attempted (Anderson *et al.*, 1990; Anderson and Wild, 1994b; Jha, 1998). It has been postulated that toxicant-induced DNA damage can result in a cascade of effects at higher levels of biological organisation (McCarthy and Shugart, 1990). These effects could range from the impact on the physiological functions in individuals (Stein *et al.*, 1992), to alterations of gene pools (Gillespie and Guttman, 1993; Guttman, 1994; Woodward *et al.*, 1996; Tatara *et al.*, 1999) and community-levels changes (Seiler, 1982; De Raat *et al.*, 1985; Wurgler and Kramers, 1992; Stein *et al.*, 1992; Mohn and De Raat, 1993; Anderson *et al.*, 1994b; Depledge, 1998). Such changes may initiate direct and catastrophic ecological consequences. On the other hand, damaged DNA can also be repaired, or cells

replaced, before long lasting effects on fitness ever occur (Downes and Wilkins, 1994; Wood, 1996; Braithwaite *et al.*, 1999). In addition, epigenetic processes, such as the functioning of endocrine systems, can be disrupted by toxicants without any damage to DNA (Colborn and Clement, 1992). Epigenetic processes involve the study of changes in gene expression that occur during development, without any significant change in gene sequence.

To address the problem of DNA damage and the possible effects at higher levels of biological organisations, it is necessary to develop reliable genotoxicity assays, which may then be used in conjunction with traditional assays for detecting any impairment of classical Darwinian fitness parameters which include survival, reproductive output, and time to maturity between breeding (Forbes and Forbes, 1994). For example, Sadinski *et al.* (1995) offered support for this approach when they suggested that benzo(a)pyrene induced-DNA adducts and -micronuclei were sensitive measures of sub-lethal DNA damage, as well as possible short-term indicators of indirect effects on fitness in the amphibian *Xenopus laevis*. Furthermore, Anderson *et al.* (1994a) reported that genotoxic (cytogenetic effects) and developmental effects in the sea urchins (*Strongylocentrotus purpuratus*) were sensitive indicators of effects of three genotoxic chemicals; in contrast, fertilisation success was not considered to be the most sensitive end point. New approaches were also developed to measure fitness parameters such as mating success, fecundity, and locomotory activity in F₁ and F₂ generations of *Drosophila* after exposure to ethyl nitrosourea (Mohn and De Raat, 1993). The results indicated that fitness impairment in the offspring was not apparent below exposure levels that directly affected reproduction in the parental generations. Experiments with *Daphnia* also indicated clear reproductive effects, but no effects were observed in the F₁ generation (Mohn and De Raat, 1993).

2.2 The occurrence of cancer

2.2.1 The concept of tumour progression

The fundamental concept of tumour progression elaborated by Foulds (1975) and Nowell (1976) have as their central themes the idea that increasing genetic alterations generated by random somatic mutational events are responsible for heterogeneity and progression of tumour cells to increasingly and less responsive states. However, the concept of somatic mutation-induced tumour progression is perhaps too simplistic. Processes such as amplification, rearrangement, deletion, and methylation, which are components of normal cellular development (ontogeny), become increasingly attractive candidates for mediators of tumour cell progression. In addition, it is now well established that cancer occurs as a result of multiple events which includes initiation, promotion and progression (Heckler, 1987) with an important step being certainly the proliferation of cancerous cells (Preston-Martin *et al.*, 1990).

In higher animals such as mammals and fish, somatic mutations and chromosomal rearrangements may contribute to induction, promotion and progression of cancer by inactivating anti-oncogene or by activating oncogenes (Barbacid, 1986; McMahon, 1994; Ross and Nesnow, 1999). It is important to mention that the only guaranteed method to determine if a compound is a human carcinogen is to study it epidemiologically (Tomatis *et al.*, 1989). Epidemiological techniques have been particularly successful in cases where specific exposures to high concentrations of compounds have been involved. However, it is unlikely that the individual contribution of single chemicals in highly complex exposure situations will be fully understood by epidemiological studies (Farmer, 1994). Consequently, 'biomonitoring' or 'molecular dosimetry' techniques have been developed. Molecular dosimetry may be defined as the assessment of the internal dose of a toxic compound by measuring the extent of chemical interaction of the compound with biological macromolecules.

2.2.2 Contribution to cancer

2.2.2.1 DNA damage and mutations

DNA adducts: some carcinogens have the potential to react with DNA to form covalent binding. Adducts, if not repaired, can induce gene mutation and are believed to be necessary for the initiation of carcinogenesis. Mussarat *et al.* (1996) reported that the accumulation of 8-hydroxyguanine has a predictive significance for breast cancer risk assessment and is conceivably a major contributor in the development of breast neoplasia. Adducts formation at particular loci in DNA is known to lead to the activation of oncogenes (Zarbl *et al.*, 1985), and may also lead to inactivation of anti-oncogenes (Bressac *et al.*, 1991; Hsu *et al.*, 1991). Such events may lead to cancer.

DNA breakages: DNA single (ssb) and double (dsb) -strand breaks can lead to mutations (rearrangements but not point mutations) and consequently to biological effects such as cancer and cell death. Dsb are very mutagenic and lethal (Dugle *et al.*, 1976) and if unrepaired or misrepaired, result in loss and/or rearrangement of genetic material. Recently, Mallya and Sikpi (1999) suggested that *p53* (an antioncogene) participates in suppressing dsb rejoining following exposure of mammalian cells to ionising radiation.

Cytogenetic effects: cytogenetic anomalies are known to be of many types and to be induced by a variety of agents by several different mechanisms (Richold *et al.*, 1990; Tucker and Preston, 1996). Tumour cells have been examined in detail, and in almost all cases they contain structural and/or numerical chromosome alterations (Tucker and Preston, 1996). There is a clear association between chromosome rearrangements and cancer (Solomon *et al.*, 1991; Rabbitts, 1994). Although the relationship between micronuclei and cancer is not as well studied compared to chromosome aberration, it is conceivable that micronuclei can potentially lead to cancer. The importance of aneuploidy to adverse human health is well accepted, and the effects of aneuploidy include birth defects, spontaneous abortions, and infertility (Abruzzo and Hassold, 1995). Tumour cells

frequently have alteration in chromosome number (Oshimura and Barrett, 1986; Rew, 1994), but whether this is frequently the cause or the effect is not clear. Finally there is no direct association between induction of sister chromatide exchange with adverse cellular or health outcome (Tucker and Preston, 1996).

DNA mutations: it has been previously mentioned that gene mutation may be necessary for the initiation of carcinogenesis. DNA damage can manifest itself as mutations, recombinations and rearrangements, gross chromosomal abnormalities, or gene amplification (Bohr *et al.*, 1987). Point mutations which occur in the genomic DNA can be the result of replication of not repaired modified base such as 8-hydroxyguanine, thymine dimmers, bulky adducts. These results have been extrapolated from *in vitro* replication of DNA templates containing diverse DNA lesions using prokaryotic and eukaryotic polymerases. Results of these investigations revealed that DNA damage either blocked the replication process (Brown and Romano, 1991; Holler *et al.*, 1992; Hruszkewycz *et al.*, 1992; Huang *et al.*, 1993; Broschard *et al.*, 1995) or that the enzymes involved in the replication (i.e. DNA polymerase) bypassed the DNA lesion and incorporated the wrong base opposite the lesion (Taylor and Oday, 1990; Hruszkewycz and Dipple, 1991; Pillaire *et al.*, 1994; Chary and Lloyd, 1995; Hoffmann *et al.*, 1995; Nelson *et al.*, 1996a).

2.2.2.2 DNA repair

DNA is certainly more stable than RNA and proteins. This stability can be attributed to the double-helical structure, which carries the information in duplicate. Equally important for functional stability of DNA are the various DNA repair mechanisms. DNA repair has been mainly studied using mammalian or bacterial strains. Much less is known about DNA repair in invertebrates (Zahn *et al.*, 1983; James *et al.*, 1992). In direct DNA repair, the damaged base is simply reversed to the 'normal base'. DNA photolyase (Todo, 1999) and O⁶-methylguanine DNA transferase (Wood, 1996) are examples of this class of enzymes. Other repair pathways remove the modified nucleotides and rely on the

redundant information in the complementary strand to restore the duplex (Sancar and Sancar, 1988). In base excision repair, the base is first removed by a glycosylase, then the abasic sugar is removed by apurinic/aprimidic endonucleases and replaced by the normal base as a result of DNA polymerase action (Sancar and Sancar, 1988). In nucleotide excision repair, the modified bases are removed in the form of an oligonucleotide and the gap is filled in by polymerases (Sancar, 1996). In recombinational repair, the gaps that are generated during replication of damaged duplexes are filled in by strand transfer from an intact duplex (Sancar and Sancar, 1988). Although DNA repair is usually very efficient in deleting the DNA lesion and restoring the original sequence, it may also lead to mutagenesis principally when two opposite DNA lesions are close to each other (Witkin, 1976). In the case of nucleotide excision repair, one lesion will be excised with the surrounding non-damaged bases and the gap will be filled by using the sequence in the opposite strand (containing another DNA lesion) (Witkin, 1976). The bypass of the lesion may potentially lead to a mutation. A similar situation can occur in post-replication repair (i.e. recombinational repair) (Witkin, 1976).

2.2.2.3 Epigenetic processes

Epigenetic processes involve the study of changes in gene expression that occur during development, without any significant change in gene sequence. Epigenetic carcinogens encompass a diverse set of natural or man-made chemical compounds that may evoke carcinogenic processes such as enhanced cell proliferation, unregulated cell growth, and aberrant cell kinetics. These responses can be brought about by altered sensitivity to a wide variety of cytokines, hormones, growth factors, and other cell mediators that may operate *via* membrane receptors, signal transduction pathways, and intra-cellular communication processes (Williams, 1992; Klein and Costa, 1997a). Most epigenetic carcinogens require high level or long duration exposure to give rise to an increase in tumours. An important feature of epigenetic effects is that they are highly reversible even at

a stage of cellular alteration (Nera *et al.*, 1988). In contrast, with limited exposure to carcinogens of the type now known to be DNA-reactive, cancer occurrence increases with time (Williams and Watanabe, 1978).

There is a direct relationship between DNA methylation and gene silencing. However, hypomethylation is necessary but not sufficient for gene expression. An overall decrease in methylation is a frequent finding in carcinogenesis, and altered methylation may be a key factor in the transformation of a normal cell into a frank malignancy (Jones, 1986; Counts and Goodman, 1994; Zingg and Jones, 1997). Hypomethylation may be a non-genotoxic mechanism facilitating the aberrant expression of oncogenes involved in carcinogenesis. Alternatively, hypermethylation may turn off the expression of tumour suppressor genes.

2.2.2.4 Interference between genotoxic and epigenetic processes

Genotoxic responses have been shown to induce epigenetic processes. For example, single-stranded DNA has been suggested to signal *de novo* methylation (Christman *et al.*, 1995). Active oxygen species and DNA strand breaks have also been demonstrated to stimulate poly(ADP ribosylation) and protein kinase pathways affecting signal transduction that can lead to the modulation of the expression of essential genes for proliferation and tumour promotion (Cerutti and Trump, 1991; Singh and Aggarwal, 1995). The possible induction of proto-oncogenes by oxidative stress, establishes the importance of this process in the modulation of gene expression and its participating role in tumour promotion (Crawford *et al.*, 1995). In an *in vitro* experiment, the substitution of guanine with 8-hydroxyguanine in CpG islands was found to inhibit DNA methylation of adjacent cytosines and binding to the methyltransferase (Weitzman *et al.*, 1994; Cerda and Weitzman, 1997). Thus, oxidative damage in nascent DNA strands, as opposed to parental strands, inhibits DNA methylation. At present little is known about the efficiency of repair of the 8-hydroxyguanine lesion at methylated CpG sites and how this may affect the

preservation of normal methylation patterns. CpG sites may become hot spots for this oxidative lesion due to inefficient repair mechanisms, which could lead to mutations and altered methylation patterns, contributing to the carcinogenic process. Several carcinogens can affect DNA methylation either by modifying the target DNA or by inactivating the DNA methyl transferase (Boehm and Drahovsky, 1983; Wilson and Jones, 1984). Benzo(a)pyrene has been reported to inhibit the cytosine-5-DNA methylation enzyme (Wilson and Jones, 1984). Changes in DNA methylation can also render certain genomic regions unstable by altering the chromatin structure, leading to deletions, inversions and chromosomal losses. Finally, DNA methylation may lead to mutations via deamination-driven events [spontaneous deamination of 5-mCytosine (5mC) \rightarrow Thymine (T), and enzyme-mediated deamination of 5mC \rightarrow Uridine (U)] which could result in the inactivation of tumour suppressor genes or activate proto-oncogenes (Gonzalzo and Jones, 1997).

2.2.3 Cancer in invertebrates

In lower organisms incidence of tumours has not been widely reported (Kurelec, 1993). Consequently, there is a need to correlate DNA damage to some genotoxic endpoint other than cancer. In invertebrate, genetic damage is manifested primarily as alterations in phenotypic attributes and Darwinian fitness parameters rather than neoplasia, teratogenic effects and genetic diseases (Depledge, 1994a). Nevertheless, some studies have reported occurrence of neoplasia in aquatic invertebrates including for instance bivalve molluscs and arthropods (Harshbarger, 1969; 1977; Hesselman *et al.*, 1988; Gardner *et al.*, 1991; Van Beneden *et al.*, 1993; Van Beneden; 1994).

Stewart (1976) noted that years of study of hundreds of specimens are often required to sort out the morphology, behaviour, and biochemistry of neoplastic diseases. This may partly explain why cancer in invertebrates have not been widely reported.

Biochemical analyses, electron microscopy, molecular techniques, and other emerging biomedical technologies should be applied to the study of neoplasia in invertebrates to identify the etiologic agent(s) and to elucidate the mechanism(s) of disease (Peters *et al.*, 1994). It has been proposed that the survey of mutations in genes, such as *p53* or equivalent, might provide a genetic signature for specific chemical exposure in tissues of aquatic animals derived from environmentally damaged sites (McMahon, 1994).

2.3 Ecological stress

2.3.1 Definition

The word 'stress' has been widely used and redefined many times by biologists (Bijlsma and Loeschcke, 1997). For instance, Sibly and Calow (1989) defined stress as:

'an environmental condition that, when first applied, impairs Darwinian fitness'.

In this research project, 'stress' refers to any condition, whether environmental or man-made, which has a measurable impact, possibly but not necessarily at the DNA level and at higher level of biological organisation on any organism. In this context, the presence of xenobiotic in the environment is considered as a 'stress'. However, 'stress' has also been used in subsequent sections to describe variation in temperature or food.

2.3.2 Ecological significance of mutational stress

It has been already mentioned that mutations can lead to cancer. It has been suggested that, in addition, somatic mutations can result in reduced viability, which could lead to reduced survivorship and lower reproductive output (Bickham and Smolen, 1994). Ultimately these responses may result in ecotoxicological effects such as reduced population density which may ultimately lead to population extinction (Smith *et al.*, 1993). Mutations in germ cells may also lead to foetal death or teratogenic effects and may reduce

fertility (Kramers *et al.*, 1991). A mutation which is transmitted to the next generations may persist for a few or many generations (ICPEMC, 1983) and may have a significant effect at the population level and could also lead to population extinction (Lande, 1994; 1998; Lynch *et al.*, 1995). It is also important to consider that mutations, that can be induced by genotoxic agents, are a necessary ingredient of the process of maintaining life on earth (Wurgler and Kramers, 1992). Newly arising mutations which have a selective disadvantage are rapidly eliminated from the population (Wilson *et al.*, 1977; Seiler, 1982). Only neutral or beneficial mutations are expected to persist. However, a beneficial mutation for a given population may not be beneficial for other species and may increase instability in ecosystems (Wurgler and Kramers, 1992). Of economical importance are mutations which lead to the various forms of tolerance and resistance. In addition, recessive mutations in important crops could have disastrous consequences if for instance they become inferior in quality and quantity (Seiler, 1982). Moreover, the accumulation of mutations that alone may have a small or negligible effect, could result in a dramatic cumulative effect as a whole (Wurgler and Kramers, 1992).

In specific environmental stress situations (either natural or man-made) in a heterogeneous population, selection may favour genetic variants that were not advantageous in the absence of the stress and thus may lead to substantial changes in the genetic make-up of the population (Wurgler and Kramers, 1992; Anderson *et al.*, 1994b). In populations with a lower turnover, environmental stress may narrow the genetic diversity by killing the more sensitive variants. In addition, the adaptation of a species to a stressful environment is not without cost (Holloway *et al.*, 1990). For instance, in the case of pollution resistance, if the pollution is removed, the non resistant genotype may deal with the new environment in a better way than the resistant genotype. Many studies have unambiguously presented evidence that mutation frequencies increase under environmental stress conditions (not necessarily due to pollution) (Cairns, 1988; Hall, 1990; 1991;

Bridges, 1997; Foster, 1998). Bacteria in stationary-phase constitute a good model for the study of adaptive mutations in response to a stress (e.g. food limitation) because mutations which occur cannot be attributed to replication (MacPhee and Ambrose, 1996; Reddy and Gowrishankar, 1997; Rosenberg, 1997; Torkelson *et al.*, 1997). Although there was some controversy about the mechanism responsible for adaptive mutations (e.g. Cairns, 1988), Rosenberg (1997) reported that adaptive mutations are neither Lamarckian (i.e. selected genes are not the target) nor a peculiarity of bacterial sex, but rather they occur genome-wide in a hypermutable sub-population of stressed cells. Finally, Taddei *et al.* (1997) presented evidence from bacterial systems that stress could induce greatly elevated mutation rates by activating a mutagenic response and by inhibiting antimutagenic mechanisms like the mismatch repair system.

Beside the occurrence of mutations in somatic and germinal cells, there is another class of genetic effects resulting from exposure to pollutants. Specifically, changes in allele frequencies of population can occur as a result of population bottlenecks, inbreeding or selection at critical loci which may be critical for survival in polluted environments (Bickham and Smolen, 1994). Even non-mutagenic toxicants can have genetic effects if they alter allele frequencies of populations by this process. Thus, heritable effects can either be the result of direct interaction or of toxic stress not necessarily of a genotoxic nature. Ecological phenomena such as population bottlenecks, the disruption of social structure and other behavioural effects can lead to population genetic effects such as the reduction of genetic variability by inbreeding and genetic drifts (Anderson *et al.*, 1994b; Bickham and Smolen, 1994).

Recently, there has been an accumulation of evidence to suggest that environmental stress may have a considerable impact on the evolutionary and ecological processes that affect and shape the genetic structure and evolution of populations and individuals and may even play a significant role in the process of speciation (Bijlsma and Loeschke, 1997).

Forbes and Calow (1997) emphasised that studies of the response of biological systems to novel pollutants could provide significant information for addressing fundamental ecological and evolutionary issues, but, conversely, that ecological and evolutionary understanding is crucial for effective development of ecological tests and risks assessment models. It has also been reported that genetic stress and environmental stress both increase the extinction risk of small populations (Smith *et al.*, 1993; Lande, 1994; 1998; Lynch *et al.*, 1995) and, more important, that both stresses are not independent but can act synergistically (Bijlsma *et al.*, 1997). In addition, Zhivotovsky (1997) found that a population will not adapt to a foreign environment when the frequency of occurrence of this environment is rare.

2.4 Biomarkers

The key problem faced by ecotoxicologists is to recognise the damaging effects of pollutants on natural biota. With the exception of a few agents, such as asbestos fibres that inflict physical damage directly to cells and tissues, all toxicant effects begin as interactions with biomolecules. Consequently there is a possible cascade of effects in the following order: biochemical, subcellular, cellular, tissue, organ, individual, population, community, ecosystem, landscape, and biosphere levels of organisation. The identification of specific molecular, biochemical, physiological and behavioural changes in populations of animals and plants following pollutant exposure would greatly assist the recognition of the damaging effects of pollutants (McCarthy and Shugart, 1990; Depledge *et al.*, 1993; Walker, 1998a;1998b). The use of these so-called 'biomarkers' to evaluate pollution hazards has noticeably increased in the past few years. The biomarker approach has now attracted the attention of international regulatory agencies as a potentially powerful tool for detecting and documenting exposure to and the effects of environmental contamination

(McCarthy and Shugart, 1990). The use and understanding of biomarkers has been modified over recent years. An ecotoxicological biomarker can be defined as:

‘a biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms (either individuals or populations) that provides evidence of exposure to and/or effects of one or more chemical pollutants (and / or radiations)’ (Depledge, 1994b).

The biomarker approach aims to provide measures for evaluating the level of exposure of organisms to xenobiotics and also the consequences for the organism. Organisms may elicit responses at all levels of biological organisation, from the molecular level, where pollutants may cause damage to critical cellular macromolecules initiating detoxification and repair mechanisms, to the individual level (Figure 2.1). If the impact of a toxicant at, for example, the biochemical, cytological or physiological level is important enough to exceed compensatory responses, then its effect passes to successively higher level of biological organisation (Stebbing, 1985; Newman, 1998). Effects at the individual level may result in reduced growth or reproduction, which eventually may become evident at the population or community levels where changes in species diversity, density and genotypic frequencies may arise (McCarthy and Shugart, 1990). The temporal sequence shown in Figure 2.1 is important because responses to pollution at lower levels of organisation enable the ecotoxicologist to anticipate and predict effects that may occur later at higher levels of organisation. For example, biochemical responses such as DNA damage may provide an early warning signal of exposure to a particular toxicant. This is a great advantage because responses at higher levels such as the ecosystem are usually measurable only after significant or permanent damage has occurred (Newman, 1998). Utilising such responses at lower levels of organisation to predict possible effects that may occur at higher levels of organisation is a key feature underlying the rationale of the biomarker approach.

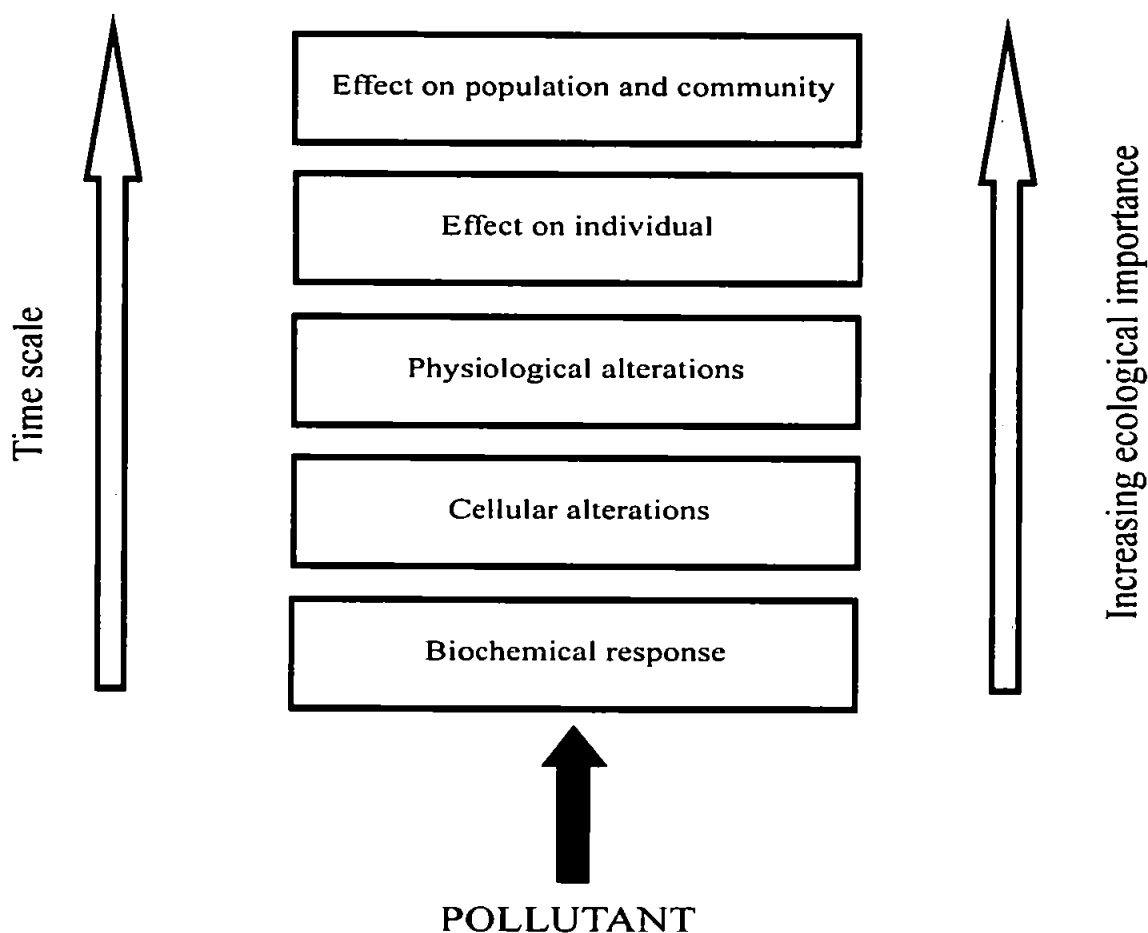


Figure 2.1 Relationship between responses to pollutants at different levels of biological organisation and their modifications along gradients of response time, toxicological and ecological relevance. Reproduced from Fossi and Leonzio, 1994, *Nondestructive biomarkers in vertebrates*, with permission from CRC Press.

2.4.1 The different categories of biomarkers

Depledge (1994b) proposed a division of ecotoxicological biomarkers into four classes:

- * The first class contains biomarkers of exposure, which signal exposure of an organism, a population, or a community to chemical pollutants. DNA adducts (La and Swenberg, 1996) and sister chromatid exchange (Albertini *et al.*, 1996) are considered to be biomarkers of exposure because these endpoints can be repaired.

- * The second class contains biomarkers of effect, which signal that an organism, a population, or a community has been affected by one or more pollutants. They do not necessarily provide information concerning the nature of the pollutant stress. In epidemiological studies chromosome aberrations and gene mutations have been considered to be biomarkers of effect (Albertini *et al.*, 1996) because these genotoxic endpoints are irreversible. Furthermore, a DNA adduct can potentially be considered to be a biomarker of effect. For instance, a DNA adduct, after replication (fixation), could lead to a point mutation (Loecher, 1995; Eckert and Opresko, 1999) and therefore can contribute to the development of cancer (Van Beneden *et al.*, 1990; McMahon, 1994) and other degenerative disease (Kirkwood, 1989). However, the major difficulty is to prove that a single DNA adduct/mutation is responsible for a disease or an effect on fitness.

- * The third class are the so called exposure/effect biomarkers. These biomarkers not only indicate exposure to one or several xenobiotics, but specifically link the exposure to an effect.

- * The fourth class, the latent effect biomarkers, deals with possible changes in the capacity of organisms to adapt to future environmental changes because of a previous pollutant exposure. The exposure of apparently normally functioning organisms is indicated by limitations in the ability to adapt or survive (e.g. scope for growth).

2.4.2 The ‘multiple response concept’

The ‘multiple response concept’ illustrated in Figure 2.2 has created a frame work for evaluating the health status of individuals and populations by using biomarkers to identify progressive changes in the physiological condition of organisms (Depledge *et al.*, 1993; Depledge, 1994b). A healthy individual exposed to a pollutant may experience a deterioration in health as it moves along the ‘physiological condition’ scale exceeding homeostasis at the point (h), from where it moves into a compensation zone. As the organism is exposed to increasing doses of a toxicant, compensatory biochemical and physiological responses are seen, with no obvious signs of disease. If the pollutant exposure exceeds the organism’s tolerance level (point c), a movement into the non-compensation zone with signs of overt disease will result. If point (r) on the curve is crossed, recovery through repair mechanisms is impossible and the organism will finally suffer from pathological processes leading to death (Depledge, 1994b).

Figure 2.2 shows a number of hypothetical biomarker responses compared to the health status curve. Particular biomarkers (B1-B5) may be present with more or less specific responses in each of the individual ‘zones’. Thus, there may be potential for determining *in situ* or in the laboratory, where individuals and whole populations samples lie on the health status curve (Depledge, 1994b). Early warning of potentially detrimental effects may be given by biomarker responses occurring in the compensation zone, which make these of special interest. Moreover, this figure also illustrates that the simultaneous use of more than one biomarker can offer greater possibilities for the detection of effects than do single biomarkers.

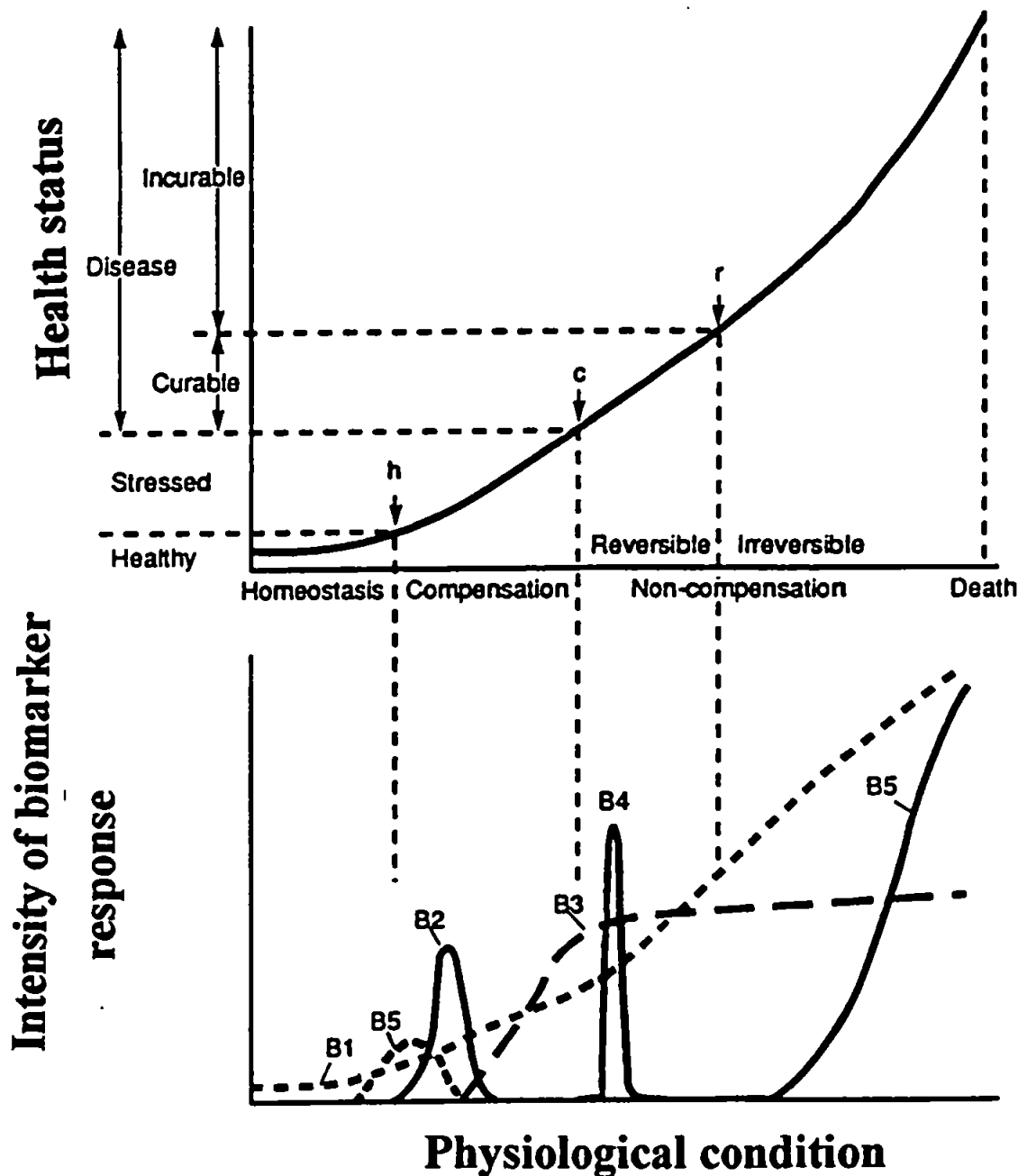


Figure 2.2 The relationship between health status and physiological condition in an organism. In the upper panel, changes in the physiological conditions are shown during progressive deterioration in health status. In the lower panel several biomarker responses are shown. Reproduced from Depledge, 1994b, in *Nondestructive biomarkers in vertebrates*, with permission from CRC Press.

2.4.3 Non-destructive biomarkers

The use of non-destructive biomarkers has proved particularly useful in research involving animals (Fossi and Leonzio, 1994; Leonzio and Fossi, 1994). However, many of the advantages for animals are also valid for research with other organisms. For example, sequential sampling on the same individual allows the individual to act as its own control and long-term studies of responses in individual are possible (Depledge, 1994b). Non-destructive sampling does not involve loss of individuals from the population, which is of special importance when rare or threatened species are investigated. In addition, the role of endogenous (sexual cycle, age, nutritional status, etc.) and exogenous (temperature, daylight, etc.) factors in variations in biochemical biomarkers can be investigated in the same individual, and thus exclude intraspecific variation (Fossi *et al.*, 1994).

2.4.4 Examples of biomarkers at different level of organisations

According to Walker *et al.* (1996), despite the importance of any changes at the population or community structure level, these changes are too general to be considered as specific biomarkers. In addition, responses to pollution at lower levels of organisation need to be measured to allow the ecotoxicologist to anticipate and predict effects that may occur later at population level.

2.4.4.1 Behavioural biomarkers

The behaviour of an organism represents the final integrated result of a diversity of biochemical and physiological processes (Walker *et al.*, 1996). Behavioural abnormalities include changes in preference or avoidance, activity level, feeding, performance, learning, predation, competition, reproduction, and a variety of social interactions such as aggression or mutual grooming (Newman, 1998). Very few studies have used this approach to evaluate the impacts of pollution (Kittredge, 1980; Gray, 1990) because of the difficulty in scoring and the inherent variability in behavioural data (Giattina and Garton, 1983). Walker

et al. (1996) reported that behavioural parameters are not especially sensitive to exposure to pollutants and that biochemical and physiological changes are usually at least as sensitive. In contrast, it has been suggested that behavioural changes are among the most sensitive response patterns yet available (Kinne, 1980).

2.4.4.2 Physiological biomarkers

Measures of a physiological function involving whole organism, such as respiration and ion regulation, have been used for the assessment of sublethal pollutant effects (Forbes and Forbes, 1994). In most of the cases, respiration is measured by determining the consumption of oxygen (Hutcheson *et al.*, 1985). Toxicant might either increase (McKenney and Matthews, 1990) or decrease (Forbes and Depledge, 1992) the oxygen consumption. Ion regulation has been shown to be disrupted by a variety of metals (Bjerregaard and Vislie, 1986) and organic pollutants (McKenney and Hamaker, 1984). Bayne (1980) claimed that physiological biomarkers may provide useful measures of pollutant effect, only if there is a reduction in fitness.

Scope for growth (SFG) is considered to be a good indicator of physiological stress and fitness. Measurements of growth (or the energy available for growth) have proven very useful as integrated measures of organism's performance (Forbes and Forbes, 1994). As growth can affect time to maturity, senescence and reproductive potential, it can have an important influence on population structure (Forbes and Forbes, 1994). Growth can change as a result of alterations in behaviour, metabolism or other physiological processes. SFG is the amount of energy taken into the organism in its food (A) minus the energy used for respiration (R) and excretion (U): $SFG = A - R - U$ (Cockerham and Shane, 1994). It reflects the energy available for growth and production of young (Newman, 1998). Thus when organisms are exposed to pollutants SFG may decrease as some energy is needed to repair some eventual damage at different level of biological organisation. For instance, SFG decreased in mussels exposed to a mixture of petroleum hydrocarbons and copper in

laboratory mesocosm experiments (Widdows and Johnson, 1988). Often mentioned disadvantages include the fact that SFG is a general measure of stress response, and that SFG can be quite seasonally variable (Bayne, 1980).

2.4.4.3 Histopathological biomarkers

Histopathology can be defined as the study of change in cells and tissues associated with communicable or non-communicable disease. Histopathological biomarkers are lesions that signal effects resulting from prior or ongoing exposure to one or more toxic agents (Hinton *et al.*, 1992). The histopathological biomarkers which integrate damage done at the molecular level (Hinton and Lauren, 1990), can be used as an early warning system for effects which are manifest at the level of individual and, sometimes, at the level of population (Newman, 1998). No other category of biomarker enables the researcher to examine so many potential sites so rapidly (Hinton *et al.*, 1992). This feature was particularly well illustrated by Wester and Canton (1986) who studied the medaka (*Oryzias latipes*) after long-term exposure to B-hexachlorocyclohexane, an isomer of the insecticide, lindane. Whilst histopathological analysis are particularly relevant to field investigations, there are two major disadvantages. Firstly, the variations in normal histology with season, diet, reproductive cycle, and other processes are not always well understood (Hinton and Lauren, 1990). Secondly, most histopathological studies are qualitative rather than quantitative (Jagoe, 1996).

2.4.4.4 Molecular biomarkers

In ecotoxicology, the evaluation of effect at the molecular level is determined to anticipate further consequences at higher levels of biological organisation. Molecular biomarkers are particularly useful when the target(s) of the environmental contaminant is/are identified. Consequently this allows better understanding of the mechanism of toxicity. However most of the molecular biomarkers described in the following sections are not of this type.

Cytochrome P4501A: all organisms possess biotransformation or detoxification enzymes which convert lipophilic organic xenobiotics to water-soluble and excretable metabolites. Such enzymes are present in most or all tissues, but in highest levels in the liver (vertebrates), or tissues involved in the processing of food, e.g. hepatopancreas in crabs (crustaceans) and pyloric caeca in starfish (echinoderms) (Livingstone, 1993). Enzymes of phase I metabolism (oxidases, reductases) introduce a functional group (-OH, -NH₂, -COOH) into the xenobiotic, to which the enzymes of phase II metabolism (conjugases) attach a large water-soluble moiety, such as the tripeptide glutathione or sugar derivative glucuronic acid (Timbrell, 1991). The presence of cysteine in glutathione provides a nucleophilic sulphhydryl group which reacts with electrophilic substances which are either produced as a result of metabolic processes or uptaken by the cells as a xenobiotic. The P450 complex is composed of two enzymes (cytochrome P450 and NADPH-cytochrome P450 reductase), NADPH, and molecular oxygen (Newman, 1998; James, 1990b). This system is often called the mixed function oxidase (MFO) (Timbrell, 1991). The cytochrome P450 system is often induced as a response to chemicals such as polycyclic aromatic hydrocarbons (PAHs) (Mitchelmore *et al.*, 1998a; Sparagano *et al.*, 1999), chlorinated hydrocarbons (Walker *et al.*, 1987), polychlorinated biphenyls (Brumley *et al.*, 1995), dioxins and dibenzofurans (Goksoyr and Forlin, 1992). Although cytochrome P4501A1 is part of the detoxification system, it can activate certain xenobiotics such as PAHs into mutagenic metabolites, and therefore its increased synthesis has consequences for carcinogenicity (Peters *et al.*, 1997). Thus, in protecting the organism, cytochrome P450 also endangers the organism (Bast, 1986). The most studied enzyme activities include the 7-ethoxyresorufin O-deethylase, benzo(a)pyrene hydroxylase, and aryl hydrocarbon hydroxylase (Walker *et al.*, 1996). However, great care must be taken when such enzymatic activities are measured. For instance, cytochrome P4501A1 status can change with species, reproductive condition and diet (Livingstone, 1993). In this context, such factors must be taken into consideration when using cytochrome P4501A1 as a biomarker.

Metallothioneins (MTs): they were first described by Margoshes and Vallee (1957) as cadmium-binding proteins in horse kidneys. They are widely-distributed, low molecular weight (6-7 Kda for vertebrates), cysteine-rich, metal-binding proteins, thought to be present in the tissues of most if not all animals, including fish and marine invertebrates (Hamer, 1986; Kille *et al.*, 1992). MTs bind metals in group Ib and IIb of the periodic table, via the cysteinyl residues, and usually contain displaceable metals such as Zn and Cu. MT's function is the uptake, internal compartmentalisation, sequestration, and excretion of essential (Cu, Zn) and nonessential (Ag, Cd, Hg) metals (Newman, 1998). MTs are also thought to function as free radical scavengers with some involvement in general scavenger and stress responses, e.g. responses to temperature stress (Livingstone, 1993). Induction of MTs has been observed for a wide range of metals, including Cd, Cu, Zn, Co, Ni, Bi, and Ag (Kille *et al.*, 1992; Stegeman *et al.*, 1992; Walker and Livingstone, 1992) and forms the basis of their use as biomarkers for metal exposure (Stegeman *et al.*, 1992). In addition, it has been shown that fluctuations in MT levels may be associated with processes such as moulting and reproduction (Roesijadi, 1992), or with hormone fluctuations such as glucocorticoids (Karin and Herschman, 1981). MTs have no known catalytic function and therefore quantitation is based on the measurement of the amount of protein itself. Although the usefulness of MTs as biomarkers for metal exposure is clear, very little is known of their role, if any, in the resultant pathophysiology of cell injury (Livingstone, 1993).

DNA adducts: many organic xenobiotics are metabolically activated to electrophilic metabolites which bind to nucleic acids and proteins, forming covalent adducts (Randerath *et al.*, 1981; Gupta *et al.*, 1982; Shugart, 1986; Shugart *et al.*, 1987; Livingstone, 1993; Walker, 1995). DNA adduct formation integrates xenobiotic uptake, metabolism and macromolecular repair, and is the initial event in chemical carcinogenesis (Loechler, 1995). Thus, it is currently being used in humans as a biomarker for exposure to

environmental carcinogens (Schoket, 1999; Farmer, 1999), and similar role as a biomarker of organic contaminant exposure and damage in aquatic organisms has been proposed (Kurelec *et al.*, 1989b; Chipman and Marsh, 1991; Jones and Parry, 1992). The formation of adducts with DNA and other macromolecules following experimental exposure to organic xenobiotics such as B(a)P and aromatic amines has been demonstrated or indicated for fish (Stegeman and Lech, 1991) and marine invertebrates (Kurelec *et al.*, 1989b; Venier and Canova, 1996; Canova *et al.*, 1998; Harvey *et al.*, 1999). Such adducts are persistent and dose-response relationships have been observed (Thomas, 1990). In other studies performed by Randerath and colleagues, it was shown that adducts may be rapidly lost or persist for several months (Randerath *et al.*, 1983; 1985a; 1985b; 1985c). Randerath *et al.* (1983) reported that persistent adducts may occupy specific genomic sites in quiescent cells where they may not be amenable to repair because of localised conformational alterations of DNA or shielding by associated proteins. In addition it was concluded that the bulk of the persistent adducts was present in dormant cells, that is in non-dividing cells (Randerath *et al.*, 1985a). DNA adduct formation has even been suggested to be associated with neoplasia in fish (McCarthy and Shugart, 1990). A commonly used method for the detection of DNA adducts is ³²P-postlabelling which has the potential to detect approximately 1 adduct in 10¹⁰ bases (Randerath *et al.*, 1981; Reddy and Randerath, 1986). Despite the widespread use of DNA adducts as biomarkers, there are some limitations. For instance, 'natural adducts' which have been found in various aquatic species appear to be species-specific and are not determined by the site of collection of the aquatic organism (Kurelec *et al.*, 1989a; Lyons *et al.*, 1997; Harvey *et al.*, 1999). In addition, artefacts have been shown to appear when the ³²P-postlabelling was used (Scates *et al.*, 1995).

Other molecular biomarkers: many other biochemical responses have been proposed over the years as biomarkers and used with varying degrees of success. Most have been indicators of a general stress response, rather than specifically associated with

contaminant exposure, e.g. steroid hormones (Thomas, 1990), phosphogens and adenosine phosphates (Carr *et al.*, 1991), free amino acid composition (Carr *et al.*, 1991), release of tissue enzymes (transaminases and lactate dehydrogenase) into the blood (Carr *et al.*, 1991), ATPase activity (Carr *et al.*, 1991), and inhibition of acetylcholinesterase activity (Carr *et al.*, 1991). Numerous other potential biomarkers have been proposed, including oncogene products (Moore and Simpson, 1992), expression of multidrug resistance genes (Kurelec, 1992), and stress proteins (Baldwin *et al.*, 1992).

Despite the usefulness of molecular biomarkers, researchers have to be aware of the possible limitations. It is important to understand the range of variation that a biomarker may exhibit under natural conditions. Rarely do we know enough about how and why physiological and biochemical processes vary in organisms in nature (Forbes and Forbes, 1994). For instance, Sole *et al.* (1995) reported that the mixed-function oxygenase system and antioxidant enzymes of the mussel were subjected to seasonal variation. In another study, it was shown that the level of stress protein varied as a function of the age of the daphniids (Bond *et al.*, 1993). In addition, many biochemical bioassays proposed for use in an environmental impact assessment face a number of other limitations, including the specificity of a response to a particular species or phylogenetic group, differences in responses between species, lack of consistency in responses, slow response time, low level of precision, and cost (Forbes and Forbes, 1994). It is therefore recommended that physiological and biochemical techniques are used in conjunction with other methods, such as histology, pathology, and behaviour for assessing organism health (Forbes and Forbes, 1994).

2.5 Detection of DNA damage and mutations

The model systems used for testing mutagenic or genotoxic effects now rely on the firmly established fact that the (multi-step) processes of mutagenesis share common properties in widely different organisms, ranging from bacteria to man. The genotoxicity of pollutants is directly related to their effects on the structure and function of DNA molecules which may be determined using a number of laboratory methods.

2.5.1 Detection of DNA damage

2.5.1.1 Biochemical and molecular assays

The detection of DNA adducts: the detection of DNA adducts provides a useful tool applicable to environmental monitoring (Shugart *et al.*, 1987; Forbes and Forbes, 1994; Newman, 1998). As previously mentioned, the ^{32}P -postlabelling method has been widely used in genetic-ecotoxicology mainly for its great sensitivity as it has the potential to detect approximately 1 adduct in 10^{10} bases (Randerath *et al.*, 1981; Reddy and Randerath, 1986). One possible disadvantage is that this method is not artefact free (Scates *et al.*, 1995) and that relatively large amounts of DNA (compared to PCR) are required (e.g. 5 μg). Immunoassays of DNA adducts have a potential advantage in that they are more applicable for routine and are relatively low-cost (Farmer, 1994; 1999; Li *et al.*, 1999). Knopp *et al.* (1999) developed an immunochemical assay for the detection of metabolites excreted in urine as the result of exposure to PAHs in human. Immunoassays are quite sensitive as 1 adduct per 10^8 unmodified nucleotides can be detected (Farmer, 1994; 1999). Once an adduct has been quantified, there are strong grounds for development of an immunoassay for its determination. One recent promising development of immunochemical procedures has been the use of the immunoslot blot assay (Farmer, 1999). The main advantage is that the time and labour involved in adduct detection is

reduced and that a large number of samples can be analysed. However, when immunochemical detection is performed, the quantitative demands for sample size may be relatively large (e.g. up to 500 µg) and cross reactivity is known to occur. Several other analytical methods proved useful for measuring the DNA adducts in organisms exposed to environmental contaminants. These include gas chromatography, gas chromatograph/mass spectroscopy (Giam *et al.*, 1989), fluorescence assays (Farmer, 1994), and electro-chemical detection (Park *et al.*, 1989). The techniques using fluorescence and mass spectrometry generally require hydrolysis of the DNA and are relatively sensitive as 1 adduct per 1.4×10^7 bases can be detected (Manchester *et al.*, 1988). The disadvantages of fluorescence assays for adduct analysis are that the number of adducts that can be analysed is limited and that cross-reactivity with other fluorophores can occur. The requirement for thermal stability and volatility of the analyte for gas chromatograph/mass spectroscopy determination is not always met by carcinogen-adducted nucleic acid components. However, the combination of liquid chromatography with electrospray mass spectroscopy holds considerable promise for DNA adduct detection (Farmer, 1999).

DNA Strand breakages: firstly, Rydberg (1975) used the alkaline/neutral elution technique to detect DNA breakage in irradiated mammalian cells. Then, the comet assay, also called the single cell gel assay or microgel electrophoresis, was introduced by Ostling and Johanson (1984) as a microelectrophoretic technique for the direct visualisation of DNA breakage in individual cells. The technique was later modified to achieve a substantial increase in sensitivity for the detection of radiation-induced DNA damage in human lymphocytes (Singh *et al.*, 1994). The comet assay is a sensitive and rapid technique for DNA strand break detection in individual cells (Fairbairn *et al.*, 1995). This method which has been successively applied in numerous studies (Singh *et al.*, 1989; Liepelt *et al.*, 1995; Klaude *et al.*, 1996; Alapetite *et al.*, 1996; Mitchelmore and Chipman, 1998a; 1998b; Mitchelmore *et al.*, 1998a; 1998b) is applicable to the detection of exposure to

agents which cause strand breakage either directly or during the process of DNA excision repair (Chipman and Marsh, 1991). If required, only extremely small samples containing from 1 to 10,000 cells can be used and results can be obtained in a single day (McKelvey *et al.*, 1991). As few as 1 DNA break per 10^{10} daltons can be detected in the assay (McKelvey *et al.*, 1991). However, limitations are also associated with this technique. For instance, Speit *et al.* (1999) reported that the results obtained with the comet assay are influenced by the temperature and alkaline treatment. It is also known that the protocol for the slide preparation needs to be optimised for each species. Other methods have also been used to detect DNA breakages. For instance, Theodorakis *et al.* (1994; 1997; 1999) electrophoresed genomic DNA in agarose gel under alkaline or neutral conditions. Electrophoresis performed under alkaline (pH 12.0) conditions separates the DNA into single strands. The average molecular length which can be determined with a scanning laser densitometer is inversely proportional to the number of DNA breaks. The average molecular length determined from alkaline electrophoresis is affected by both single- and double-strand breaks, while with neutral electrophoresis it is affected by double-strand breaks only. Koji (1996) used a method called *in situ* nick translation to detect DNA single-strand breaks. In the principle of *in situ* nick translation, the DNA strand with breaks is elongated in the presence of biotin-11-dUTP by *E. coli* DNA polymerase I at the nicked sites. The biotin moieties incorporated into the newly synthesised strand are visualised using an immunohistochemical technique. Leroy *et al.* (1996) assessed the ability of three methods, alkaline elution, nick translation, and single-cell gel electrophoresis, to detect DNA single-strand breaks in human peripheral blood lymphocytes exposed *in vitro* to three genotoxic agents: γ -rays, ethyl methanesulfonate and B(a)P diol epoxide. Compared to the other assays, the sensitivity of the single-cell gel electrophoresis assay was significantly higher. In addition, single-cell gel electrophoresis is a relatively simple method, not time-consuming and applicable to a large number of samples per working day.

2.5.1.2 Cytogenetical assays

Sister chromatid exchange (SCE): SCE could not be regarded as an indicator of DNA damage in the conventional sense since they are morphologically intact. Nevertheless, SCE occurs at site of mutational events including chromatid breakage and it is considered to be an indicator of mutagenesis (Carrano *et al.*, 1978). SCE has been successfully detected in different species of mussels following exposure to genotoxins (Dixon and Clarke, 1982; Harrison and Jones, 1982; Brunetti *et al.*, 1986; Jha *et al.*, 2000). SCE was also successfully used to detect genotoxicity in plant systems (Schubert and Rieger, 1994; Gomez-Arroyo *et al.*, 1997). These tests have been applied largely (but not exclusively) in laboratory exposures of test organisms (Jha *et al.*, 1996).

Chromosome aberrations (CA): CA include breakage, chromosomal rearrangements and loss of chromosomal segments. The analysis of CA requires arresting the cells in metaphase stage by the addition of a mitosis inhibitor, such as colchicine prior to visualisation of the chromosomes. Originally CA tests were developed *in vitro*. They usually utilise mammalian somatic cells, the most popular being human peripheral blood lymphocytes or Chinese hamster fibroblasts in long-term culture (Scott *et al.*, 1990). In the field of genetic ecotoxicology, most of the tests developed use an *in vivo* animal system. Al-Sabati and Kurelec (1985) reported that the occurrence of CA increased with PAH exposure in mussel, under laboratory conditions. Jha *et al.* (1996) developed an *in vivo* marine genotoxicity assay using the embryo-larval stages of the polychaete worm *Platynereis dumerilii* to detect SCE and CA. The assay was then validated in different studies including the evaluation of the genotoxicity of environmental samples (Jha *et al.*, 1997; 2000; Hutchinson *et al.*, 1998). The sea urchin, *Strongylocentrotus purpuratus* has also proved to be an useful species for evaluating relationships between cytogenetic responses in embryos and abnormal development (Anderson and Wild, 1994a). CA have

also been detected in plant assay following exposure to genotoxins (Rank and Nielsen, 1997).

Micronuclei: anomalies during cell division, such as chromosome damage and/or spindle dysfunction can produce micronuclei which can be defined as nuclear segments isolated in the cytoplasm from the nucleus (Tucker and Preston, 1996). The micronucleus test was developed with different animal species such as mussel (Majone *et al.*, 1987), *Xenopus* (Van Hummelen *et al.*, 1989), fish (Das and Nanda, 1986), plants (Degrassi and Rizzoni, 1982; Minissi and Lombi, 1997; Minissi *et al.*, 1998). Numerous studies have also shown the potential of micronuclei as a biomarker of exposure (Heddle *et al.*, 1983; Sadinski *et al.*, 1995; Venier *et al.*, 1997). Majone *et al.* (1987) reported that micronuclei persisted in the marine mussel after treatment with mitomycin C. The main advantage of the micronucleus test compared to other cytogenetic assays is that it does not rely upon chromosome number and size.

Aneuploidy: Dixon (1982) compared the occurrence of aneuploidy in mussels embryos from a polluted dock area and an unpolluted site. He found that embryos from the polluted site exhibited a higher incidence of aneuploidy. Despite the wide use of mammalian cells for studying chemically induced mutagenesis and chromosome breakage, aneuploidy studies with mammalian cells are limited (Oshimura and Barrett, 1986). In ecotoxicology, it seems as well that very little attention has been drawn to study potential aneuploidogens.

Limitations of cytogenetic assays: since most mutagens and genotoxic carcinogens are efficient inducers of chromosomal alterations, there represent important endpoints to look at (Natarajan *et al.*, 1996). However, cytogenetic studies (except micronucleus tests) present a number of disadvantages. Firstly, the karyotype needs to be determined (Jha *et al.*, 1995). Secondly, the cells need to divide to perform the study and the reduced size and large number of chromosomes can be a limiting factor. Finally, since

cytogenetic end points are cell cycle dependent, it is important to know the cell cycle kinetics of the cells used.

2.5.1.3 PCR based methods

Traditional PCR: PCR assays have been used to detect DNA damage because DNA polymerases are unable to synthesise across a damaged nucleotide residue (Moore and Strauss, 1979; Chan *et al.*, 1985; Malia and Basu, 1995). In the case of *Thermus aquaticus* (*Taq*) DNA polymerase, this has been demonstrated using a PCR-based ultraviolet footprinting assay (Axelrod and Majors, 1989). The fraction of gene segments which are spanned by PCR primers and bear one or more damaged nucleotides would be represented as a proportional reduction in the amount of amplified product. The advantage of the PCR assay is that it obviates the need for specific endonuclease complexes to recognise and cleave DNA adducts as previously required for analysing damage in specific genomic sequences (Jennerwein and Eastman, 1991). Studies have shown that a single photoproduct (Chan *et al.*, 1985; Govan *et al.*, 1990) or cisplatin adduct (Jennerwein and Eastman, 1991) was sufficient to fully block PCR amplification. Thymine hydrates have also been shown to significantly inhibit PCR reactions (Ganguly and Duker, 1992). However, Murray *et al.* (1992) suggested that whilst single adducts were insufficient to completely halt the *Taq* DNA polymerase cross-linked adducts were much more efficient at doing so. It has also been reported that the level of radiation required to bring an inhibition of the PCR assay was 600 fold higher than that required to detect gene-specific damage using T4 endonuclease V (Bohr *et al.*, 1986). This can be attributed to the smaller size of the target DNA which was analysed, 440 bp (base pair), compared to the 9-23 kbp (kilo base pair) fragments which were analysed in the endonuclease study. Such an enormous difference renders a direct comparison meaningless. PCR has been successively applied to evaluate quantitatively the extent of DNA damage and repair in specific genes (Wei *et al.*, 1995; Bingham *et al.*, 1996; Ploskonosova *et al.*, 1999; Braithwaite *et al.*, 1999). In

addition the ligation-mediated PCR assay has been successfully used for the detection of alkylguanine adducts and UV-induced cyclobutane pyrimidine dimers and (6-4) photoproducts (Pfeifer *et al.*, 1992; 1993). The sequence position of adducts can be mapped whenever it is possible to convert the adduct, either chemically or enzymatically, into a DNA strand break with a 5'-phosphate group. Fragments containing these ligatable breaks are amplified in a single-sided, ligation-mediated PCR reaction. Generally, PCR can be used to produce quantitative data. For instance, PCR has been successively applied to evaluate the extent of DNA damage and repair in specific genes (Wei *et al.*, 1995; Bingham *et al.*, 1996; Brandt and Aliosman, 1997; Ploskonosova *et al.*, 1999; Braithwaite *et al.*, 1999). Despite the advantages of using traditional PCR assay for detecting DNA damage, there are a number of potential difficulties. Firstly, the nucleotide sequence of the genomic DNA before and after the target region of interest needs to be determined. Secondly, the size of the PCR products plays a crucial role for the detection of DNA damage. The amplification of short fragments (e.g. less than 300 bp) may not be inhibited at all even in case of extensive DNA damage. Indeed, there are 10 times the number of opportunities for nucleotide damage to occur in 1000 bp compared to 100 bp. However, other features such as secondary structures and 'hot spot' motifs may also have a role in determining the susceptibility of a portion of DNA to be damaged. Thirdly, it is conceivable that any reduction in the intensity of PCR amplicons could be due to a partial inhibition of the PCR reaction caused by factors other than DNA damage such as residues of phenol remaining after DNA extraction. Alternatively, if an amplicon entirely disappears due to the presence of DNA damage, it could be argued that the PCR did not work at all.

Differential display messenger RNA analysis: this method was originally developed in cancer research (Liang and Pardee, 1992; Liang *et al.*, 1993). The basis of the mRNA differential is the systematic PCR-mediated amplification of cDNA derived from the total mRNA present in a target cell population. Brandt and Ali-Osmam (1997) used the

reverse transcriptase PCR (RT-PCR) to detect DNA damage in transcriptionally active genes. Its potential as a tool for ecotoxicological research lies in its ability to use the amplified mRNAs produced in further analyses such as molecular cloning, nucleotide base sequencing and as hybridisation probes (Depledge, 1998). The main advantage is related to the fact that no previous knowledge of the nucleotide sequence of the DNA or RNA is needed. In addition as gene expression is expected to be rapidly modulated as a response to the effects induced by the environmental pollutants, the differential display messenger RNA method is considered to be a sensitive assay. However, this test is not very specific as many parameters can modulate gene expression.

2.5.1.4 DNA repair assay

Unscheduled DNA synthesis (UDS): in non-replicative cells, the incorporation of the radioactive base is associated with the presence of DNA lesions which are removed and replaced by radioactively labelled nucleotides such as ^3H -thymidine. This phenomenon has been called UDS by Djordevic and Tolmach (1967). In the presence of DNA damage, cells try to efficiently repair the lesion using numerous tools such as nucleotides and base excision repair, recombinational repair, and direct repair (Sancar and Sancar, 1988; Modrich and Lahue, 1996; Sancar, 1996; Wood, 1996). This technique has been successfully used in numerous studies (Mitchell *et al.*, 1983; Walton *et al.*, 1984; Kelly and Maddock, 1985) because it is relatively easy to perform, and it measures DNA damage over the entire genome and not just at a specific locus (Mitchell *et al.*, 1983). It can also be used in a wide variety of cultured cell types. Diploid human fibroblasts and primary rat hepatocyte cells have been widely used (Mitchell *et al.*, 1983). However, UDS can also be performed on cells isolated from aquatic species such as oysters (Kelly and Maddock, 1985) and rainbow trout (Walton *et al.*, 1984). There are, however, some drawbacks associated with the technique. For instance, depending on the cell type, metabolic activation needs to be supplemented and ^3H -thymidine has been reported to degrade rapidly

(Kocal *et al.*, 1988). Generally this technique suffers from the fact that cells cultured under *in vitro* conditions are used (please see below).

***In vivo* rat hepatocyte DNA repair assay:** a further advance of the UDS test was the development of an *in vivo* rat hepatocyte DNA repair assay in which the chemical is administered to the animal and the resulting DNA repair is assessed in hepatocytes isolated from the treated animal (Mirsalis *et al.*, 1982). The advantage of *in vivo* assays is that they reflect the complex patterns of uptake, distribution, metabolism, detoxification and excretion that occur in the whole animal. Further, factors such as chronic exposure, sex differences, and different routes of exposure can be studied with these systems (Butterworth *et al.*, 1987).

2.5.1.5 Restriction enzymes

The methods rely on the fact that restriction enzymes such as DNA glycosylases (O'Connor and Laval, 1989), UvrABC nucleases (Tang *et al.*, 1989), T4 endonuclease V (Smith and Taylor, 1993), endonuclease III (Didzdaroglu *et al.*, 1993) can specifically recognise diverse types of DNA damage. Thus after digestion the extent of DNA damage can be estimated by electrophoresis. The higher the number of breaks, the higher the extent of DNA damage. The main advantage of these techniques is that they specifically recognise a type of DNA damage. Thus qualitative and quantitative data can be generated. However these techniques may be appropriated to experiments performed in the laboratory but not in the field unless chemical analyses have been previously carried out.

2.5.2 Detection of Mutations

2.5.2.1 *In vitro* mutational assays

It is sometimes considered that cytogenetic assays are equivalent to mutagenicity assays (Fielder *et al.*, 1992) because cytogenetic damage is indicative of the interaction of a test compound with DNA and consequently of its potential to induce other genotoxic

damage such as gene mutation. However, the mutagenicity assays described herein concern only the detection of mutation. Mutations arising at the nucleotide level include: base-pair substitutions (transitions, transversions), frameshifts resulting from small deletions or insertions, or large scale genomic rearrangements like deletions, insertions, duplications, and translocations.

Bacterial assays: the test receiving by far the most use and attention has been the *Salmonella* mutagenesis test (or Ames test) developed by Ames *et al.* (1973, 1975) because of its initial promise of high qualitative (yes/no) predictivity for cancer in rodents and, by extension, in humans. However, the relationship between mutagenesis and carcinogenesis is weak (Fetterman *et al.*, 1997). Some other tests have been developed such as the SOS chromotest (Quillardet *et al.*, 1982; White and Rasmussen, 1996) and the umu-test (Oda *et al.*, 1985; Oda *et al.*, 1993) to overcome the drawback of having to use several strains to detect various kinds of mutagens (Oda *et al.*, 1985).

Gene mutation assays in cultured mammalian cells: the origin of the most used cell lines come from the mouse, Syrian hamster, Chinese hamster and human (Cole *et al.*, 1990). Commonly used systems work by selecting for the loss of function of a gene product (enzyme) which is inessential for the survival of cells in culture. Three genetic loci [hypoxanthine phosphoribosyl transferase (*hprt*) (Chen *et al.*, 1990), thymine kinase, and the cell membrane Na^+/K^+ ATPase] are widely used for mutagenicity testing (Cole *et al.*, 1990). For instance, the cells resistant to 6-thioguanine result from lack of *hprt* enzyme activity (Li *et al.*, 1987) whereas those resistant to trifluorothymidine are due to a loss in the thymidine kinase enzyme activity (Clive *et al.*, 1987). One of the disadvantages is that rodent and human tumour cells *in vitro* are generally unstable and may drift in their sensitivity to mutagens (Fox, 1985). An additional limitation is the restricted metabolic capacity of many cultured cell lines (Cole *et al.*, 1990).

***In vitro* ecotoxicological assays:** the *Salmonella* bacterial assay has also been adopted for use with marine invertebrates. Rat liver post-mitochondrial supernatant (PMS) is substituted with subcellular fractions of tissues of aquatic organisms such as molluscs, crustaceans and echinoderms (Depledge, 1998). It has been reported that activation of B(a)P can be achieved using PMS of digestive gland of mussel (Michel *et al.*, 1992; 1993). It is important to mention that, in ecotoxicology, the information that can be obtained from *in vitro* bioassays whether performed on prokaryotic or eukaryotic cells has limited value for predicting the possible impact of genotoxic pollutants on complex organisms in the natural environment.

2.5.2.2 Tests for recombinogens

Assay systems are available with organisms of different genetic complexity to screen specifically for recombinagens: bacteria (Hoffmann, 1992), fungi (Zimmermann, 1992), *Drosophila* (Vogel, 1992), cultured mammalian cells (Bhattacharyya *et al.*, 1990) and the mouse *in vivo* (Fahrig and Neuhauser-Klaus, 1985). Such studies do complement the classical mutagenicity testing of chemicals and complex mixtures and make it possible to improve the testing in safety toxicology (Wurgler, 1992).

2.5.2.3 PCR based methods

The restriction site mutation assay (RSM): Parry *et al.* (1990) developed the RSM assay to detect and study DNA base change following mutagen exposure. The RSM assay has been applied in various species such as mouse, rat, *Xenopus*, flatfish and human cells (Jenkins *et al.*, 1999). The RSM assay allows the detection of mutations in restriction enzymes sites through selective PCR amplification of induced restriction enzyme-resistant sites and the concurrent destruction of wild-type sequences by enzymatic digestion. This method possesses a number of advantages. Any genes can be analysed for mutations in all type of tissues and silent mutations and phenotype altering mutations are equally detectable (Jenkins *et al.*, 1999). The main disadvantage is related to the fact that the RSM assay is

limited to analysing four to six bases and that nucleotide sequence must be known to perform PCR.

Restriction fragment length polymorphism (RFLP)/PCR: this technique is similar in principle to the RSM assay. In RFLP/PCR, mutations in restriction sites rendering them resistant to digestion and allowing their amplification by PCR are similarly detected (Chiocca *et al.*, 1992; Sandy *et al.*, 1992). The technique involves the isolation of the gene of interest by preparative restriction digestion, in order to enrich the DNA for the target gene. Then, the amplified mutants are cloned and identified by oligonucleotide probing. For instance, the RFLP/PCR technique has been employed to examine mutations in the *H-ras* gene (Sandy *et al.*, 1992) but mainly in the *p53* gene (Aguillar *et al.*, 1993) of cultured human cells. The main advantage lies in its very high sensitivity for the detection of mutation. However, this is a complex procedure (Steingrimsdottir *et al.*, 1995) which has only been described for two regions of human genomic DNA.

Example of some other tests: PCR amplification and DNA sequencing have been used to analyse benzo(a)pyrene-7,8-diol-9,10-epoxide (Chen *et al.*, 1990) and UV-induced (McGregor *et al.*, 1990) mutations in the *hprt* gene of human fibroblasts. All mutations analysed in these two studies were substitution mutations. Despite its usefulness this approach is not a desired approach for large tracts of genomic DNA. Several PCR-based techniques have been developed which are able to localise single base changes within rather large segments of genomic DNA (Cebula and Koch, 1991). ‘Multiplex’ PCR, whereby several exons are amplified simultaneously in a single reaction, has been used successfully to routinely screen for deletions within the large (>2Mb) Duchenne muscular dystrophy locus (Chamberlain *et al.*, 1988) and the *hprt* gene (Gibbs *et al.*, 1990). Ganguly and Prockop (1990) developed a method which uses a water soluble carbodiimide which modifies mismatched G and T residues. The advantage is that the polymerisation terminates at or near the modified base. PCR is extremely useful in the analysis of

mutational 'hot spots'. PCR amplification combined with allele specific oligonucleotide hybridisation can rapidly characterise frequent mutational events. For example, PCR- allele specific oligonucleotide analysis has been used to characterise mutations at different codons of *ras* (Verlaan De Vries *et al.*, 1986). Roest *et al.* (1993) have developed a rapid and sensitive method, the protein truncation test. It is based on a combination of reverse transcriptase-PCR, transcription and translation and selectively detects translation-terminating mutations. Recently, Youil *et al.* (1995) used the T4 endonuclease VII to consistently detect mismatches in heteroduplexes in the various PCR-derived products. They proposed that this method may be effective in detecting and positioning almost all mutational changes when DNA is screened for mutations.

2.5.2.4 DNA fingerprinting

Analysis of the vertebrates genome has revealed sequence elements known as minisatellite DNA that are subject to very high rates of germinal mutation (Jeffreys *et al.*, 1988). Minisatellite DNA sequences are tandemly repeated arrays of nucleotides, with units varying between 10 to 100 bp in length, that are spread throughout the genome of eucaryotic organisms. Minisatellite mutation rates are much higher than rates in unique sequence DNA regions considered to be hypervariable (Yauk, 1998). The hypervariability at minisatellite loci is maintained through a high germinal mutation rate producing new alleles resulting from changes in the number of repeated units. The technique consists of digesting the genomic DNA with a restriction enzyme, electrophoresing the digested products in agarose gel, and transferring the pattern to a membrane. A radioactive probe is then hybridised to the membrane and revealed by autoradiography. Mutations are identified as novel fragments present in the DNA fingerprints of offspring that cannot be ascribed to either parent. Alterations in banding patterns observed on DNA fingerprinting analyses reflect DNA alterations from single base change to complex chromosomal rearrangements (White *et al.*, 1990). DNA fingerprinting proved useful in the examination of cancer-

associated genetic alterations (Thein *et al.*, 1987; De Jong *et al.*, 1988; Fey *et al.*, 1988; Smit *et al.*, 1988; White *et al.*, 1990). Dubrova *et al.* (1996) reported that the rate of germline mutation increased in a population exposed to caesium-137 and possibly other environmental mutagens in the region of Chernobyl. In another study, Yauk and Quinn (1996) reported that multilocus DNA fingerprinting revealed high rates of heritable genetic mutations in herring gulls nesting in an industrialised urban site. Minisatellite DNA has several advantages for the detection of genotoxin-induced DNA damage (Yauk, 1998): minisatellite DNA has a much higher rate of mutation than unique sequence, mutations are predominantly of germline origin, and several loci are screened simultaneously. However, since minisatellites are predominantly in non-coding regions of the genome, mutations are not manifested as malformations or abortion.

2.5.2.5 Transgenic animals

Jaenisch and Mintz (1974), using purified simian virus 40 DNA, provided the first evidence that foreign DNA injected into mouse embryos could be found in tissues of the resultant offspring. Subsequent reports of high efficiency transformation of cultured mammalian cells by direct microinjection of DNA into the cell nucleus (Anderson *et al.*, 1980) suggested that nuclear injection of DNA might be effective with embryos. Transgenic animals such as mouse (Wight and Wagner, 1994) and fish (MacClean, 1998) can harbour bacterial reporter genes for the quantitative detection of mutations in diverse tissues (Boerritger *et al.*, 1995). The reporters LacZ (Gossen *et al.*, 1989; Douglas *et al.*, 1995; Dolle *et al.*, 1999), and lacI transgenic mice (De Boer, 1995) have been often used in such studies. For instance, treatment of adult female transgenic mice with *N*-ethyl-*N*-nitrosourea resulted in a dose-dependent increase of the frequency of mutated vectors isolated from brain DNA (Gossen *et al.*, 1989). Transgenic animals can be regarded as a sensitive, organ-specific, short-term mutagenicity assay. One of the major problems in working with transgenic animals is the difficulty and cost associated with maintaining a

large number of animals on hand while DNA study and/or expression is carried out (Wight and Wagner, 1994). In addition, a possible drawback is that mutational data has to be extrapolated from the foreign DNA inserted into the genome of the organism under study (Jenkins *et al.*, 1999).

2.5.2.6 Conformation analyses

Some of the recently developed methods used for mutation detection in genomic DNA rely on conformational changes in either double- or single-stranded DNA structure. Conformations can be distinguished by electrophoresis due to their different response to an electrical field. The rate of migration or mobility of DNA molecules through the electrical field depends not only on the shape of the molecules, but also on the ionic strength, viscosity, and temperature of the medium in which the molecules are moving (Drouin *et al.*, 1996a). Homoduplexes and heteroduplexes have distinctive melting properties, causing them to migrate differently when subjected to denaturing gradient gel electrophoresis (Fischer and Lerman, 1983). Orita *et al.* (1989) developed the single-strand conformation polymorphism technique. This is a rapid electrophoretic method for detection of point mutations which is based on mobility changes of single-stranded nucleic acids in non-denaturing polyacrylamide gels. The migration of single-stranded DNA is dependent not only on the size but also on the specific sequence of the DNA fragments. The main advantage of these methods is the high sensitivity and the rapidity to run the assays. However, the migration of DNA fragments is highly dependent on choice of the assay conditions and temperature. An empirical approach is recommended in establishing assay conditions (Cebula and Koch, 1991).

2.5.3 Comparison

2.5.3.1 Relative sensitivity of some of the assays previously described

A study was performed on 41 chemicals assayed with 4 *in vitro* tests [*Salmonella* assay, chromosomal aberrations (CA) in Chinese hamster ovary (CHO) cells, sister chromatide exchange (SCE) in CHO cells, and mutation in mouse lymphoma cells] (Benigni, 1992). The results suggested that there was a gradual increase in the sensitivity of the tests to detect genotoxic effects in the following order: mutation in mouse lymphoma cells > SCE in CHO cells > CA in CHO cells > *Salmonella* assay. In a comparative study, benzo(a)pyrene, cyclophosphamide, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were tested for their ability to induce genotoxic effects in the single cell gel test and the SCE test using human blood cells. While the range of concentrations which induced DNA migration or SCE was the same for *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and benzo(a)pyrene, much higher cyclophosphamide concentrations were necessary for a positive response in the single cell gel than in the SCE test (Hartmann and Speit, 1995). Perera *et al.* (1992) used a battery of biological markers to measure molecular and genetic damage in peripheral blood samples from residents of a highly industrialised region and from a control site, in Poland. The results showed that the exposure to environmental pollution was associated with significant increases in carcinogen-DNA adduct, SCE, and CA. Perera *et al.* (1992) found that DNA aromatic adducts were significantly correlated with chromosomal mutation. Rank and Nielsen (1994) compared the effect of 15 genotoxic chemicals in four different systems, namely the *Allium* CA test, the Ames test, the Microscreen assay, and the carcinogenicity tests in rodents. The results were as follow: carcinogenicity tests in rodents (100%) > *Allium* CA test (82%) > Ames test (73%) > Microscreen assay (45%). Finally, Tate *et al.* (1991) carried out a study on two populations occupationally exposed to ethylene oxide using different parameters. Blood samples were collected from 9 hospital workers and 15 factory workers engaged in sterilisation of medical equipment with

ethylene oxide and from matched controls. Blood samples were analysed for ethylene oxide DNA adducts, *hprt* mutants, CA, micronuclei (MN) and SCE. The relative sensitivity of endpoints for detection of ethylene oxide exposure was in the following order: ethylene oxide DNA adducts > SCE > CA > MN > *hprt* mutants.

2.5.3.2 *In vitro* versus *in vivo*

In vitro tests generally constitute miniaturised tools, rapid and easy to use, which are well-suited for large screening studies (Godet *et al.*, 1993). Another advantage of *in vitro* genotoxic bioassays is that they require only sample small volumes which is especially useful when analysing environmental water samples (Godet *et al.*, 1993). While microbioassays facilitate rapid testing, the results are often difficult to interpret in the broader context of human or animal health. In toxicology, *in vitro* eukaryotic cell cultures are considered to be more relevant than those using bacteria. However, the main criticism of *in vitro* tests (versus *in vivo* tests) is that it is difficult to extrapolate *in vitro* test results to higher levels of organisation in which the responses obtained integrate the effects of complex metabolising systems, hormonal regulation and immunological defences. In genetic ecotoxicology, the health of the ecosystem is of paramount importance. Therefore, while bacterial tests could still be used to detect the intrinsic genotoxic potential of contaminants, the *in vivo* tests are the ultimate choice.

2.5.4 The use of RAPD, AP-PCR and DAF to detect DNA damage and mutations

Advances in molecular biology have led to the development of a number of selective and sensitive assays for DNA analysis. The random amplified polymorphic DNA (RAPD), the arbitrarily primed polymerase chain reaction (AP-PCR) and DNA amplification fingerprinting (DAF) techniques are three semiquantitative methods which have been used in genetic mapping, taxonomy and phylogeny (Welsh and McClelland, 1990; Williams *et al.*, 1990; Welsh *et al.*, 1991; 1995b; Bassam *et al.*, 1992). In particular,

the RAPD method has been used to detect genetic polymorphism in studies examining genetic diversity (Campos *et al.*, 1994; Grayson *et al.*, 1999; 2000), pedigrees (Tinker *et al.*, 1993), the construction of genetic maps (Binelli and Bucci, 1994), the identification of cultivars (Koller *et al.*, 1993), the identification of pest resistance genes (Dax *et al.*, 1994), and sex markers (Hormaza *et al.*, 1994). RAPD, AP-PCR and DAF are a modification of PCR that provides an information-rich fingerprint of genomic DNA. These techniques are based on the selective amplification of genomic sequences that, by chance, are flanked by adequate matches to an arbitrarily chosen primer. If two template genomic DNA sequences are different, their arbitrarily primed PCR products display different banding patterns. Such differences can be exploited in ways largely analogous to the uses of RFLPs. Polymorphisms detected by these techniques can be used as taxonomic markers in population studies of a wide variety of organisms (Welsh *et al.*, 1992).

Despite the successful application of the RAPD in many areas of research, some studies have also criticised the technique for its lack of reproducibility (Ellsworth *et al.*, 1993; Khanda *et al.*, 1997), and Mendelian inheritance (Riedy *et al.*, 1992; Scott *et al.*, 1992; Ayliffe *et al.*, 1994). Spurious amplification has also been reported to occur in RAPD reactions (Mullis, 1991). Nevertheless after suitable optimisation, the RAPD methodology has been reported as a robust assay with good reproducibility under defined conditions (Rothuizen and Van Wolferen, 1994; Benter *et al.*, 1995; Bielawski *et al.*, 1995).

2.5.4.1 Description of RAPD, AP-PCR and DAF

Although RAPD, AP-PCR and DAF are very similar techniques often causing some ambiguity in terminology, there are some procedural differences which have been clearly defined by Meunier and Grimont (1993). Table 2.1 gives an overview of these procedural differences among these techniques.

Table 2.1 Procedural differences among AP-PCR, RAPD, and DAF.

	RAPD	AP-PCR	DAF
Primer length (bp)	10-12	20	5-15
Primer concentration (μM)	0.3-3	3	3-30
Annealing temperature	Low	Low and high	Low or high
Number of cycles	42	40	35
Separation	agarose	polyacrylamide	polyacrylamide
Visualisation	ethidium bromide	radiolabelling	silver staining
Resolution PCR product lower than 1Kb	intermediate/low	intermediate	high
Resolution PCR product higher than 1Kb	intermediate	intermediate/low	low

2.5.4.2 Detection of genetic diversity

The RAPD and AP-PCR techniques have also the potential to detect genetic effects that are not directly due to interactions of genotoxins with the DNA molecule. These represent the effects of chemical exposure on the genetic composition of the population, in terms of genetic variability, or the distribution of allele frequencies. Such effects come about as the result of pollution-mediated mortality or curtailment of reproduction, events that could lead to genetic bottlenecks or the introduction of a novel selective pressure (Nevo *et al.*, 1986; Guttman, 1994). Recently, the RAPD method was used to detect genetic diversity among populations which had been exposed to environmental contaminants, including well-known genotoxins (Nadig *et al.*, 1998; Theodorakis *et al.*, 1998; Krane *et al.*, 1999; Theodorakis *et al.*, 1999). Nadig *et al.* (1998) reported that fish populations in the contaminated sites were consistently less genetically distant from each other than they were from each of the reference sites. Krane *et al.* (1999) suggested that RAPD based measures of genetic diversity may be suitable for development as a sensitive means of directly assessing the impact of environmental contaminants upon ecosystems. Finally, Theodorakis *et al.* (1999) indicated that the probability of survival and degree of DNA strand breakage in radionuclide-exposed mosquitofish were dependent on RAPD genotype, and were consistent with the hypothesis that the contaminant-indicative RAPD bands were markers of loci which imparted a selective advantage in radionuclide-contaminated environments.

2.5.4.3 Detection of DNA damage and mutations

RAPD and AP-PCR have been used for the detection of mutations and DNA damage. For example, AP-PCR was used to detect γ -ray-induced genetic damage in fish embryos (Kubota *et al.*, 1992; 1995). Recently, Lopez *et al.* (1999) used the AP-PCR technique to analyse genomic damage in the mutagen-sensitive mus-201 mutant of *Drosophila melanogaster*. AP-PCR has been shown to be capable of detecting mutations (Welsh *et al.*, 1995b) that occurred in anonymous sequences in certain tumours (Arribas *et*

al., 1997). Such mutations included allelic losses or gains (Peinado *et al.*, 1992), deletion of one or a few nucleotides (Ionov *et al.*, 1993) and large deletions (Kohno *et al.*, 1994). Of special interest regarding the application of AP-PCR to cancer is that the amplified bands usually originate from unique sequences rather than from repetitive elements. Furthermore, the amplification is semi-quantitative, in that the intensity of an amplified band is proportional to the concentration of its corresponding template sequence (Welsh *et al.*, 1995a). The degree of aneuploidy of a tumour cell genome is reflected in the intensities of AP-PCR bands, compared to those from normal diploid genome from the same individual. In this context, by adjusting carefully the concentration of the template DNA, it is possible to detect losses or gains in the number of copies of a sequence by changes in the intensity of a band in the AP-PCR pattern (Welsh *et al.*, 1995a). This property is very useful in many studies such as the investigations of the genetic events that occur in the process of transformation from normal to cancer cell.

RAPD has also been successfully used to detect DNA damage and has been proposed as a biomarker assay in ecotoxicology (Savva, 1996; 1998). For instance, RAPD profiles generated from rats exposed to benzo(a)pyrene revealed the appearance and disappearance of bands in comparison to control patterns (Savva *et al.*, 1994). Savva (1998) concluded that changes observed in the fingerprints of exposed animals may be due to the presence of DNA adducts, mutations or DNA strand breaks. Recently, Becerill *et al.* (1999) used the RAPD technique to detect mitomycin C-induced genetic damage in fish cells.

2.5.4.4 Relative sensitivity of RAPD/AP-PCR/DAF for the detection of DNA damage and mutations

Although the sensitivity of the DAF technique is better than RAPD, the resolution of bands of a size greater than 1 Kbp is often poor. Thus, as the sizes of the PCR fragments plays a central role in detecting changes in amplicon profiles, DAF is probably not the ideal

tool for the detection of DNA damage. Because longer DNA fragments are more likely to possess regions which are prone to DNA damage, RAPD is the preferred method of analysis. To the best of our knowledge, DAF has never been used to investigate the effect of genotoxin on genomic DNA. Although the AP-PCR technique has proven to be a sensitive and useful means for detecting DNA lesions and mutation (see above) the maximum size of amplicon obtained is around 2 Kb and there is the inconvenient requirement for radioactive bases to be added to the reaction for the last 10 PCR cycles. In conclusion, the RAPD method has a broader range of application than DAF. Although AP-PCR has been more used than RAPD to detect DNA damage and mutation, both methods seem to have exactly the same potential.

2.6 Species selection criteria for ecotoxicological and eco-genotoxicological studies

More than 90% of the organisms in ecosystems are invertebrates. Of particular interest are the aquatic species which represent by far the highest proportion of invertebrates. In this context, relevant ecotoxicological studies should use either freshwater or marine species. Since each of the species has its own merits and demerits, it can be difficult to select a particular species for eco-genotoxicological studies. However, the following criteria should be taken into account to select a particular species. The invertebrate species should be ecologically relevant, widely distributed, easy to culture with a short life cycle. The karyotype of the species in question should be known when cytogenetic tests are carried out. Finally, the ideal invertebrates species should be well documented in terms of molecular biology, physiology, etc.

2.6.1 Example of some of the species used in *in vivo* ecotoxicological studies

There are a number of freshwater and saltwater vertebrates and invertebrates species including fishes, macroinvertebrates and amphibians (Table 2.2) which are recommended for performing acute toxicity tests (ASTM, 1975). These species were selected on the basis of availability, commercial, and ecological importance, past successful use, and ease of handling in the laboratory. Their use is encouraged to increase comparability of results and availability of much information about a few species rather than a little information about many species (ASTM, 1975). The most commonly used tests in ecotoxicology are certainly the *Daphnia* tests as described herein. A variety of phytotoxicity tests have also been conducted with freshwater green algae, duckweed, blue-green algae, diatoms and rooted macrophytes (whole plants and seeds) (Lewis, 1995). Several tests methods have been standardised for microalgae which are used primarily with chemicals, effluents, contaminated sediment elutriates and hazardous waste leachates (Lewis, 1995). However, phytotoxicity tests are conducted less frequently than acute toxicity tests with animal species.

2.6.2 Example of some of the species used in *in vivo* genotoxicological studies

There is a lack of adequately validated *in vivo* test methods which could be used effectively to evaluate genotoxicity in ecologically relevant organisms (e.g. aquatic organisms) under environmentally relevant conditions (Jha *et al.*, 2000). A few *in vivo* test systems have been developed for genotoxicity and ecotoxicological evaluations. These include aquatic models such as the amphibian *Xenopus laevis* (Sadinski *et al.*, 1995), the sea urchin *Strongylocentrotus purpuratus* (Anderson *et al.*, 1994a), the polychaete worm *Neanthes arenaceodentata* (Pesch *et al.*, 1981; Harrison and Anderson, 1989; Pesch, 1990) or *Platynereis dumerilii* (Jha *et al.*, 1996; 1997; Hutchinson *et al.*, 1998), and the mussel *Mytilus edulis* (Dixon, 1982; Dixon and Clarke, 1982; Al-Sabti, 1989; Jha *et al.*, 2000) and

Table 2.2 Species recommended by the American Society for Testing Materials (ASTM) for conducting acute toxicity tests. Reproduced from ASTM, 1975, EPA-660/3-75-009, Standard guide for conducting toxicity tests with fishes, macroinvertebrates, and amphibians, with permission of ASTM.

Species ^a
Freshwater:
Vertebrates:
Frog, <i>Rana</i> sp.
Toad, <i>Bufo</i> sp.
Coho salmon, <i>Oncorhynchus kisutch</i>
Rainbow trout, <i>Salmo gairdneri</i>
Brook trout, <i>Salvelinus fontinalis</i>
Goldfish, <i>Carassius auratus</i>
Fathead minnow, <i>Pimephales promelas</i>
Channel catfish, <i>Ictalurus punctatus</i>
Bluegill, <i>Lepomis macrochirus</i>
Green sunfish, <i>Lepomis cyanellus</i>
Invertebrates:^b
Daphnids, <i>Daphnia magna</i> , <i>D. pulex</i> , <i>D. pulicaria</i>
Amphipods, <i>Gammarus lacustris</i> , <i>G. fasciatus</i> , <i>G. pseudolimnaeus</i>
Crayfish, <i>Orconectes</i> sp., <i>Cambarus</i> sp., <i>Procambarus</i> sp., <i>Pacifastacus leniusculus</i>
Stoneflies, <i>Pteronarcys</i> sp.
Mayflies, <i>Baetis</i> sp., <i>Ephemerella</i> sp.
Mayflies, <i>Hexagenia limbata</i> , <i>H. bilineata</i>
Midges, <i>Chironomus</i> sp.
Snails, <i>Physa integra</i> , <i>P. heterostropha</i> , <i>Amnicola limosa</i>
Planaria, <i>Dugesia tigrina</i>
Saltwater:
Vertebrates:
Sheepshead minnow, <i>Cyprinodon variegatus</i>
Mummichog, <i>Fundulus heteroclitus</i>
Longnose killifish, <i>Fundulus similis</i>
Siverside, <i>Menidia</i> sp.
Threespine stickleback, <i>Gasterosteus aculeatus</i>
Pinfish, <i>Lagodon rhomboides</i>
Spot, <i>Leiostomus xanthurus</i>
Shiner perch, <i>Cymatogaster aggregata</i>
Tidepool sculpin, <i>Oligocottus maculosus</i>
Sanddab, <i>Citharichthys stigmaeus</i>
Flounder, <i>Paralichthys dentatus</i> , <i>P. lethostigma</i>
Starry flounder, <i>Platichthys stellatus</i>
English sole, <i>Parophrys vetulus</i>
Herring, <i>Clupea harengus</i>
Invertebrates:^b
Copepods, <i>Acartia clausi</i> <i>Acartia tonsa</i>
Shrimp, <i>Penaeus setiferus</i> , <i>P. duorarum</i> , <i>P. aztecus</i>
Grass shrimp, <i>Palaemonetes pugio</i> , <i>P. intermedius</i> , <i>P. vulgaris</i>
Sand shrimp, <i>Crangon septemspinosa</i>
Shrimp, <i>Pandalus jordani</i> , <i>P. danae</i>
Bay Shrimp, <i>Crangon nigricauda</i>
Mysid, <i>Mysidopsis bahia</i> , <i>M. bigelowi</i> , <i>M. almyra</i>
Blue crab, <i>Callinectes sapidus</i>
Shore crab, <i>Hemigrapsus</i> sp., <i>Pachygrapsus</i> sp.
Green crab, <i>Carcinus maenas</i>
Fiddler crab, <i>Uca</i> sp.
Oyster, <i>Crassostrea virginica</i> , <i>C. gigas</i>
Polychaete, <i>Capitella capitata</i>

Mytilus galloprovincialis (Al-Sabti and Kurelec, 1985; Brunetti *et al.*, 1986). Plants species have also been used to detect the effect of pollutants on DNA in *Vicia faba* root tips (Degrassi and Rizzoni, 1982; Gomez-Arroyo *et al.*, 1997; Minissi *et al.*, 1998), *Tradescantia* (Ma *et al.*, 1996), *Picea abies* (Schubert and Rieger, 1994), and *Allium cepa* (Rank and Nielsen, 1993; 1994; 1997). In most of these animal and plant tests, cytogenetic methods are used to detect chromosome aberration, and/or sister chromatide exchange, and/or micronucleus. Thus it seems important to develop new *in vivo* tests which are able to detect DNA damage not necessarily at the chromosomal level.

2.6.3 *Daphnia magna*

Although the karyotype of different species of *Daphnia* is known (Trentini, 1979; Beaton and Herbert, 1989), to the best of our knowledge, no cytogenetical analyses have been carried out on *Daphnia* possibly because of the reduced size of the chromosomes.

2.6.3.1 Generalities about *Daphnia magna*

D. magna (crustacea, cladocera) is a freshwater planktonic filter feeder. This species is distributed throughout the temperate regions of the northern hemisphere in ponds and lakes rich in small algae. *D. magna* plays an important role in freshwater food chains. They are often dominant consumers of phytoplankton and are important food for invertebrate and vertebrate predators.

D. magna exhibits two forms of reproduction, asexual and sexual. During most of the year, populations of *D. magna* consist almost exclusively of females, the males being abundant only in spring or autumn. Under favourable conditions females reproduce by diploid parthogenesis to produce genetically identical female offspring. Production of males appears to be induced principally by declining food quantity or quality, photoperiod and crowding conditions. These conditions also induce the females to produce two eggs which require fertilisation. When fertilised these are enclosed, as a pair, by a dense

brown/black case (the ephippium) which is released by the female at the next moult (Doma, 1979). These 'ephippial eggs' are resistant to adverse conditions such as drought and cold, and may remain dormant for a period of years. When hatched from the ephippia the young develop as females.

Laboratory cultures of *D. magna* are maintained in the asexual phase of reproduction. Under favourable conditions the first brood is released after approximately 8 days at a temperature of 20°C. Subsequent broods are produced every 3 days during the female life cycle.

2.6.3.2 The *Daphnia magna* tests

D. magna is the most commonly used test organism in freshwater ecotoxicology. Indeed, this species is sensitive to wide range of toxicants and is easy to handle and culture in the laboratory. In addition, it represents a major trophic level and it has a short generation time. In addition *D. magna* species are widely distributed. Two major types of tests are usually performed using *D. magna* (Adema, 1978). The 48-h acute toxicity test uses immobility as the end-point, producing a 48-h EC50 (effect concentration) value (i.e. the toxicant concentration resulting in 50% of the exposed individuals exhibiting the end-point effect). The effects of a toxicant on *D. magna* growth and reproduction, over a 21-day period, constitute the only common laboratory-performed study on population effects. The short life-cycle of *D. magna* makes it ideal for reproductive studies. The visibility of the eggs in the brood pouch allows toxicant effects on delaying egg production to be observed. The number of juveniles per adult is calculated at the end of the test, and the length of the surviving animals measured. Time-independent 'threshold' concentrations for immobility effects can be calculated. Other effects, such as the inability of the animals to moult, the presence of undeveloped eggs in moulted exoskeletons and the production of ephippia, may also be observed. These data are used to calculate EC50 values, the effect being the reduction in numbers of juveniles released, or the no-observed effect concentration

(NOEC): the highest toxicant concentration having no significant observable differences from the control population.

Toxicity tests based on *Daphnia* have been used in numerous studies. For instance, Arambasic *et al.* (1995) examined the acute toxicity of heavy metals to *Daphnia* and plant species. Based on the 50% inhibitory concentration values, the results showed varied sensitivity among the test organisms when the toxicity of phenol, Cu, Pb, Zn, Na₂SO₄, and NaCl was investigated. Another study compared the toxicity of 30 reference chemicals using *D. magna* and *D. pulex* (Lilius *et al.*, 1995). The results of both studies revealed that there was no difference in the overall sensitivity of the two species of *Daphnia*. Such a situation is not necessarily expected (see below). Experiments were also successfully conducted to determine the toxicity of chemicals such as boric acid (Lewis and Valentine, 1981), copper (Dave, 1984), and physical agents (Hessen, 1994) on *Daphnia*. However, there are some clear limitations. The main problem is that there is a considerable variation in interlaboratory bioassays using the same reference toxicant. Indeed the toxicity of a compound depends on diverse parameters such as medium quality, amount of food and the use of different clones of the same species (Meador, 1991; Soares *et al.*, 1992; Koivisto and Ketola, 1995). To improve consistency in the *D. magna* bioassay among testing laboratory, it seems essential to use the same culture conditions as well as the same clone (i.e. same genotype) (Baird *et al.*, 1989).

Chapter 3

GENERAL MATERIALS AND METHODS

Details of the formulation, preparation of reagents, solutions, growth media, technical details, and DNA markers can be found in appendix I at the end of this research project.

3.1 Experimental organisms

3.1.1 Daphnia magna

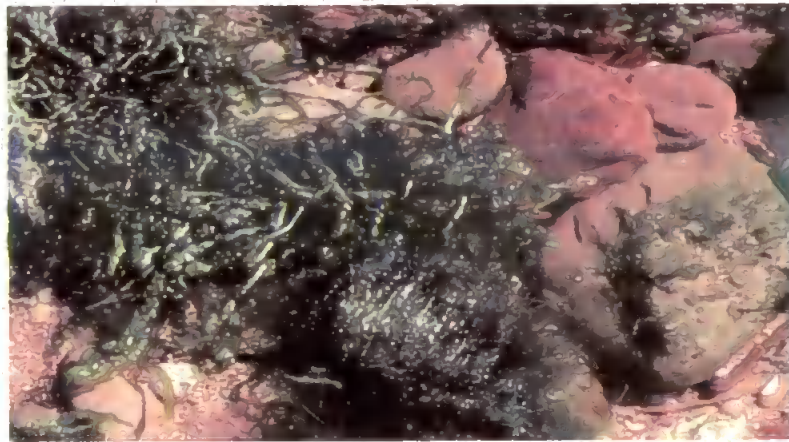
The water flea *D. magna* (clone 5) (Figure 3.1) was provided by Astra Zeneca's environmental laboratory at Brixham, Devon (UK), and was cultured at the University of Plymouth, UK. Initially, *D. magna* (clone b, provided by the University of Reading, UK) was also used but its utilisation was limited to some of the studies performed in chapter 4. The animals were maintained in Elendt's medium (Elendt and Bias, 1990), at a temperature of $20 \pm 2^{\circ}\text{C}$ with a photoperiod of 16 hour (h) light (1000 lux): 8 h dark. Populations consisting of a maximum of 50 individuals were maintained in 1 L of medium contained in 2 L tall-form glass beakers (Sigma, Poole, UK). The medium was changed 3 times per week (Monday, Wednesday, Friday). Animals were fed with the algae *Chlorella vulgaris* [$1.2\text{-}2.4 \times 10^7$ cells per daphnid per day (d)] and a booster solution of Frippack microencapsulated food (Salt Lake Brine Shrimp, Grantsville, USA). This extra source of carbon was incorporated into the culture medium at a ratio of 0.023 mg Frippack for every 1.2×10^7 algal cells. The Frippack microencapsulated food consists of proteins, lipids, phospholipids, minerals and vitamins.

3.1.2 Enteromorpha intestinalis

The green marine macroalgae *E. intestinalis* (Figure 3.1) was collected from Wembury beach near Plymouth, Devon (UK) which is considered to be an uncontaminated site. The thalli were harvested 24 h prior to the experiments and maintained in filtered sea



Daphnia magna (water flea) 0.5 mm

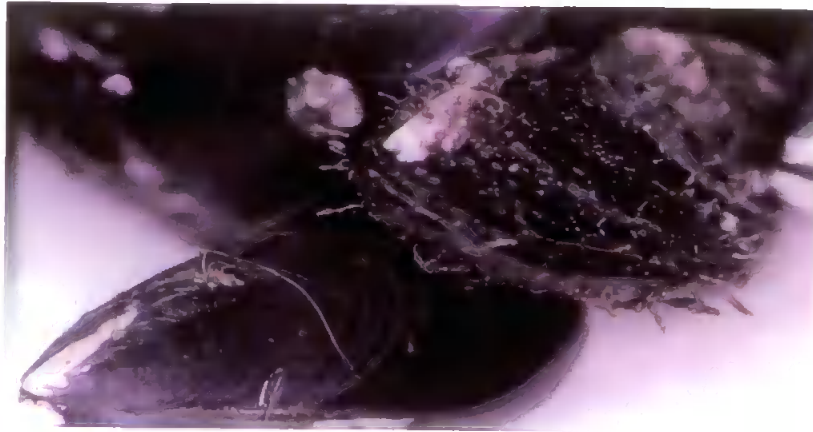


Enteromorpha intestinalis (gutweed) 3 cm



Palmaria palmata (red seaweed) 4 cm

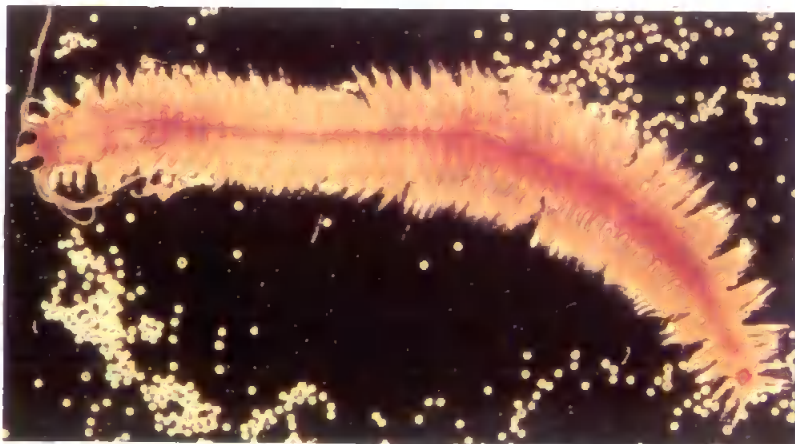
Figure 3. 1 Photographs of some of the aquatic species used in the studies. The latin



Mytilus edulis (common mussel) 1 cm



Carcinus maenas (common shore crab) 1.5 cm



Platynereis dumerilii (rag worm) 2 mm

and common name of each species plus the scale are indicated below each photograph.

water at constant temperature ($15 \pm 1^\circ\text{C}$) and in low light conditions ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$, fluorescent tubes, Philips TLD 32W/83 HF, Holland) in growth cabinets (Philips, Holland).

3.1.3 *Palmaria palmata*

The red marine macroalgae *P. palmata* (Figure 3.1) was collected from Wembury beach near Plymouth, Devon (UK) and was cultured under the same conditions as *E. intestinalis*.

3.1.4 *Mytilus edulis*

The marine mussel *M. edulis* (Figure 3.1) was collected from a clean site at Whitsand Bay, near Torpoint, Cornwall (UK). The mussels were maintained in aerated sea water (10 μm charcoal filters) at a temperature of $15 \pm 2^\circ\text{C}$. *M. edulis* was fed Liquifry® marine (Interpet, Surrey, UK) consisting of 70 % waterlife innertfood (a suspension of plankton and other marine derived proteins, vitamins and mineral salts), 28% liquifry marine (dextrin, pea flower, whole egg, yeast, and spinach), 1 % selco, and 1 % *Haematococcus* species (algae).

3.1.5 *Carcinus maenas*

The common shore crab *C. maenas* (Figure 3.1) was collected from a clean site at Bantam estuary, near Plymouth, Devon (UK). The crabs were maintained in aerated sea water (10 μm charcoal filters) at a temperature of $15 \pm 2^\circ\text{C}$ and were fed cooked mussel flesh every 4 d (1 mussel per animal). Water was changed every 2-3 d.

3.1.6 *Platynereis dumerilii*

The polychaete marine worm *P. dumerilii* (Figure 3.1) (provided by Dr Adriann Dorresteijn, University of Mainz, Germany) was maintained at $20 \pm 2^{\circ}\text{C}$ in filtered sea water (10 μm charcoal filters). The worms were fed spinach 3 times a week (Monday, Wednesday, Friday) and fish flake (Waltham[®], Elland, UK) twice a week (Tuesday, Thursday). 10 g of spinach was dissolved in 100 ml of distilled water; 20 ml of this solution was given in a tank containing approximately 100 animals. Medium was changed at least once a week.

3.1.7 *Elminius modestus*

Adult barnacles were obtained originally from Exmouth, Devon (UK) and have been cultured at the Marine Biological Association, Plymouth (UK). Adults were maintained in aerated, 1 μm -filtered seawater at $22 \pm 2^{\circ}\text{C}$. The seawater was changed every 2 d and the barnacles were fed on *Artemia* species. Nauplii were cultured in batches of about 5000, at 1 larva ml^{-1} , in aerated seawater at 28°C and on a 16:8 light dark cycle. They were fed daily on *Skeletonema costatum* (1 L of 2×10^5 cells ml^{-1}). Antibiotics were added following the procedure of Rittschof *et al.* (1992). Development of the cyprid stage took 7-8 d under these conditions.

3.1.8 *Escherichia coli* (C600, SMR 127, and SMR 346)

The strains C600, SMR346, and SMR687 were provided by Dr Susan Rosenberg (University of Alberta, Canada). After arrival the strains were kept at 4°C and later grown on plates [1.5 % agar in Luria-Betani (LB) medium] at 37°C . For more details please see chapter 9 (section 9.2.4.1) and appendice I.

3.2 DNA extraction and purification of genomic DNA

3.2.1 From *Daphnia magna*

3.2.1.1 Phenol/chloroform method

D. magna were homogenised in 400 µl of sperm lysis buffer and 4 µl RNase was added. The samples were kept at 37°C for 2 h. A volume of 480 µl of phenol (pH = 8; Fisher, Loughborough, UK) was added and the tubes were gently inverted by hand for 10 min. The samples were then centrifuged at maximum speed (18,000 x g) for 15 min and the upper aqueous layer was carefully collected and placed into a fresh tube. An equal volume of chloroform:isoamyl alcohol (1:1) (Sigma, Poole, UK) was added and the tubes were inverted by hand for 10 min. After centrifugation at maximum speed for 15 min, the upper aqueous layer was carefully collected and placed into a fresh tube. One tenth volume of sodium acetate (3 M, pH = 4.8) and 2 volumes of ice-cold ethanol were added. The tubes were inverted 10 times and incubated at -80°C overnight. After centrifugation at maximum speed for 15 min, the supernatant was very carefully discarded (the pellet can be loose) and the pellets were washed by adding 1 ml of 70 % ethanol. After centrifugation at maximum speed for 15 min, the 70 % ethanol solution was carefully removed. The tubes were centrifuged at maximum speed for a few seconds (s) and the remaining solution was removed (usually less than 10 µl) using the capillary action of a 10 µl tip. Finally the tubes were opened and were allowed to dry at 37°C for at least 1 h, and the genomic DNA pellet was dissolved in 100 µl Tris-EDTA (TE) buffer (1X).

3.2.1.2 Genie DNA extraction Kit

The kit was obtained from Immunogen International (IGi), Sunderland, UK. Individual *D. magna* were homogenised in 100 µl of sperm lysis buffer, and treated with 1 µl RNase at 37°C for 1.5 h. DNA extraction was performed using the protocol supplied by the manufacturer for the recovery of DNA from solution. A volume of 450 µl of gel

solution A was added to the sample as well as 10 µl gel resin per 1-2 µg of DNA to be recovered. The tubes were incubated at room temperature for 1 min, with frequent tube inversions, and centrifuged at maximum speed (18,000 x g) for 30 s. After the supernatant was carefully removed (at this stage the pellet is often translucent and difficult to see), 1 ml of 1X-wash solution was added. The pellet was resuspended and the tubes were centrifuged at maximum speed for 30 s. The wash solution was removed and the pellets were centrifuged at maximum speed for a few s to bring any remaining wash solution to the bottom of the tube. The capillary action of a yellow tip was used to remove any left-over wash. Finally, the samples were left with lids open to allow evaporation of any residual alcohol at either 55°C for 1 min or at room temperature for 5 min. A volume of 40 µl of TE buffer solution (1X) was added and after incubation at room temperature for 1 min, the tubes were centrifuged at maximum speed for 30 s and the eluted DNA sample was carefully removed to a fresh tube. The previous step was repeated to recover any remaining DNA and the second elution was pooled with the first one.

3.2.2 DNA extraction from other species (*Palmaria palmata*, *Enteromorpha intestinalis*, *Platynereis dumerilii*, *Mytilus edulis*, *Carcinus maenas*, *Elminius modestus*)

3.2.2.1 Phenol/chloroform procedure

Algal samples were subjected to a preliminary step contrary to the animal species. The algae was frozen in liquid nitrogen and ground with a mortar and pestle. From this point the procedure, which applies to every organism cited in the heading, is described in the section 3.2.1.1 except that the phenol step was repeated.

3.2.2.2 Caesium chloride DNA purification (*Palmaria palmata*)

After phenol chloroform extraction (see above), genomic DNA was purified by caesium chloride-ethidium bromide density gradient centrifugation according to Sambrook *et al.* (1989) or Maniatis *et al.* (1982). DNA molecules are separated from impurities

according to their density into discrete bands which can be readily harvested for use in a wide range of genetic manipulation techniques. The method described here is designed for use in the Beckman TL100 Ultracentrifuge (Beckman, Buckshire, UK) utilising 3.8 ml 'Quick-Seal' tubes in the TLA 100.3 rotor. A volume of 2.5 ml containing approximately 75 μ g genomic phenol-chloroform extracted DNA dissolved in TE buffer (final concentration = 1X) was mixed with 6 g of caesium chloride and 0.25 ml ethidium bromide (10 mg/ml). After gentle mixing, the sample was placed in the dark at ambient temperature overnight. During this time period, a complex of ethidium bromide-proteins was formed, and this unwanted material was then removed by centrifugation at 2,000 \times g for 20 min. The supernatant containing the DNA was collected using a Pasteur pipette, placed into the ultracentrifuge tube and centrifuged at 272 000 \times g for 20 h. After centrifugation, DNA bands were visualised using UV light and removed using a hypodermic syringe fitted with a large gauge needle. Ethidium bromide was removed from purified DNA solutions by organic phase extraction using isopropanol saturated with water. An equal volume of isopropanol was added to the DNA solution and the 2 phases were mixed. The phases were allowed to separate for 5 min and the upper phase consisting of ethidium bromide and isopropanol was removed. This extraction was repeated until all the pink colour had disappeared from both phases. The genomic DNA contained in the clear aqueous phase was then precipitated by adding 3 volumes of ice-cold ethanol in the presence of 0.1 volume of 3 M sodium acetate (pH = 4.8), incubated at -80°C for 15 min. After centrifugation at 14 000 \times g for 5 min, the supernatant was discarded and the pellet was washed in 1 ml of 70 % ethanol. At this stage the caesium chloride dissolved in the supernatant which was later discarded. The pellets were rewashed in 1 ml of 70 % ethanol, and the samples were centrifuged at 14 000 \times g for 5 min. After discarding the supernatant, the pellet was allowed to air dry and was dissolved in either sterile analytical grade water or 1X TE buffer.

3.2.3 DNA extraction from *Escherichia coli*

3.2.3.1 The Marmur's method

DNA was prepared as described by Marmur (1961) except that the bacteria were lysed with lysozyme and Triton-X-100 as described by Borck *et al.* (1976). The bacterial cells were centrifuged at $7\,500 \times g$ for 10 min at 4°C , and the pellet was resuspended in 860 μl of ice cold sucrose solution as well as 125 μl freshly prepared lysozyme. The samples were gently shaken at 37°C for 30 s and placed on ice for 5 min. A volume of 466 μl of ice-cold Na_2EDTA solution was added and after 5 min a volume of 967 μl of a Triton lysis solution was added and the samples were left on ice for 20 min. The lysate was next treated according to the Marmur procedure (1961). A volume of 610 μl of perchlorate solution (5 M) and 2 volumes of chloroform:isoamyl alcohol (1:1) were added. After shaking the preparation for 30 min, the samples were centrifuged at $3\,000 \times g$ for 5 min and the upper aqueous phase was carefully placed into a fresh tube. After addition of 2 volumes of chloroform:isoamyl alcohol (1:1), the samples were shaken for 5 min, centrifuged at $3\,000 \times g$ for 5 min and the upper aqueous phase was carefully placed into a fresh tube. Two volumes of 95 % ethanol were added and the threads of precipitate were 'spooled' and dissolved in 2.8 ml of dilute saline citrate solution. A volume of 0.3 ml of concentrated saline citrate solution was successively added along with 2 volumes of chloroform:isoamyl alcohol (1:1). The samples were shaken for 15 min, centrifuged at $3\,000 \times g$ for 5 min and the upper aqueous phase was placed into a fresh tube. This last procedure (chloroform:isoamyl alcohol, mixing, centrifugation and transfer) was repeated twice. Two volumes of 95 % ethanol were added and the treads of precipitate were 'spooled' and dissolved in 2.8 ml of dilute saline citrate solution. A volume of 0.33 ml of acetate-EDTA solution plus 2.7 ml of isopropanol were added. After vortexing for a few s, the precipitate was collected and placed in a 0.33 ml dilute saline citrate solution. A volume of 0.33 ml of acetate-EDTA solution was also added. Finally, two volumes of 95 % ethanol were added

and the samples were placed at -80°C overnight. After centrifugation at 18 000 x g for 15 min, the supernatant was discarded, and the DNA pellet, which was air dried, was dissolved in either analytical grade water or 1X TE buffer.

3.2.3.2 Puregene DNA isolation kit

The kit was obtained from Puregene (Minneapolis, USA). DNA extraction was performed using the protocol supplied by the manufacturer (gram-negative bacteria protocol/5 ml overnight culture preparation). The bacterial cell were centrifuged at 7 500 x g for 15 min and the pellet were stored at -20°C. A volume of 3 ml of cell lysis solution was added to the cell pellet and was 'pipetted' up and down to resuspend the cells. The samples were then incubated at 80°C for 5 min to lyse the cells and a volume of 15 µl of RNase solution was added to the cell lysate. The samples which were mixed by inverting the tubes 25 times were incubated at 37°C for 0.5 h and the tubes were allowed to cool at room temperature. A volume of 1 ml of protein precipitation solution was added and the samples were vortexed vigorously at high speed for 20 s. After centrifugation at 2 000 x g for 10 min, the precipitated protein formed a tight pellet. The supernatant containing the DNA was poured into a clean tube containing 3 ml 100 % isopropanol and the samples were mixed by inverting the tubes 50 times. After centrifugation at 2 000 x g for 3 min, the DNA was normally visible as a small pellet. The supernatant which was poured off was added into a volume of 3 ml 70 % ethanol and the samples were inverted several times to wash the DNA pellet. After centrifugation at 2 000 x g for 1 min, the ethanol was carefully discarded and the pellets were allowed to air dry for 15 min. Finally, 500 µl of 1X TE buffer solution was added and the DNA was allowed to rehydrate overnight at room temperature.

3.2.4 Other genomic DNA used

Caesium chloride DNA extracted from *Escherichia coli* (strain B; reference D4889), calf thymus (reference D4764) and human placental tissue (reference D4642) were purchased from Sigma (Poole, UK) and used in some of the studies presented in this research project.

3.3 Estimation of DNA concentration

3.3.1 Agarose gel electrophoresis

A rapid way to estimate the amount of DNA is to utilise the UV-induced fluorescence emitted by ethidium bromide molecules intercalated into the DNA. Because the amount of fluorescence is proportional to the total mass of DNA, the quantity of DNA in the sample can be estimated by comparing the fluorescence yield of the sample with that of a series of standards (Sambrook *et al.*, 1989). As little as 1-5 ng of DNA can be detected by this method. Usually a minimum of 10 (e.g. 1, 2.5, 5, 10, 20, 30, 60, 100, 200, 300, 400, 500, and 750 ng/15µl) known amounts of single stranded Lambda phage DNA (Sigma, Poole, UK) were loaded in 1.2 % agarose gel. After electrophoresis, the agarose gel was exposed to UV light using a transilluminator (UV Products Inc., California, USA). The DNA concentration of unknown samples was estimated by comparing the fluorescent yield with that of the standard. This was performed either by eye or by computer (image of the gel was captured using a digital camera) or both. Quality of the samples could be estimated in terms of protein contamination and ribonucleic acid (RNA) as well as nucleic acid degradation. Most of the time, this method was preferred compared to the spectrophotometric measurements.

3.3.2 Spectrophotometric measurements

Alternatively, if the samples are pure (i.e. without significant amounts of contaminants such as protein, phenol, agarose, or other nucleic acids), spectrophotometric measurement of the amount of UV irradiation absorbed by the bases is simple and accurate (Sambrook *et al.*, 1989). The readings were taken at wavelengths of 260 and 280 nm. The readings at 260 nm allowed calculation of the concentration of nucleic acid. An optical density (OD) of 1 corresponds to approximately 50 µg/ml for double-stranded DNA, 40 µg/ml for single-stranded DNA and RNA, and 20 µg/ml for oligonucleotides (Sambrook *et al.*, 1989). The ratio between the readings at 260 and 280 nm (OD_{260}/OD_{280}) provided an estimation of the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} of 1.8 and 2.0, respectively. If there is a contamination with protein, phenol, or with other UV absorbing substances the OD_{260}/OD_{280} will be not accurate. In addition when the DNA concentration is pretty low (< 250 ng/ml) the spectrophotometric measurements may not be accurate.

3.4 PCR reactions

The buffer, $MgCl_2$, H_2O , eppendorf tubes, filter tips, pipettes were systematically exposed to UV (254 nm) for at least 30 min in a PCR cabinet (Holten LaminAir, Gydevang, Denmark). Further precautions (e.g. the use of a set of micropipettes only dedicated to PCR, the aliquots of the PCR reagents, the use of filter tips) were also taken to avoid DNA contamination, and cross-contamination.

All PCR reactions were performed with reagents obtained from IGI (Sunderland, UK). Oligonucleotide primers were also obtained from Operon Technologies (Southampton, UK) or MWG Biotech (Milton Keynes, UK). Reactions were carried out in

a final volume of 25 μ l, overlaid with mineral oil (Sigma, Poole, UK) in a Perkin Elmer 480 DNA thermal cycler (Roche, Lewes, UK).

3.4.1 Optimised conditions

The RAPD PCR assay was performed using PCR buffer (1 X), 0.33 mM dNTP, 2 μ M primer, 5.11 mM $MgCl_2$, 2.5 μ g bovine serum albumin (BSA), 2-20 ng genomic DNA template and 2 or 2.8 units of *Taq* DNA polymerase. Most of the reactions were performed using 20 ng genomic DNA as well as 2.8 units of PCR enzyme except when otherwise mentioned. The PCR thermal cycling conditions consisted of a template denaturation step of 95°C for 4 min, followed by 40 cycles of 95°C for 1 min (denaturation), 50°C for 1 min (annealing) and 74°C for 1 min (extension). Polymerisation was augmented by a final 10 min extension step.

3.4.2 10 mer-primers used in the different studies

The sequence of the primers used in the different studies is given in Table 3.1.

3.5 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA was carried out using gel electrophoresis apparatus supplied by Pharmacia (Milton Keynes, UK). Agarose gels were prepared by adding the desired percentage of agarose (molecular biology grade, Igi, Sunderland, UK) to 250 ml of 1X Tris-Borate-EDTA (TBE) buffer. The solution was heated in a 800 watts microwave for 3 min at maximum power. Molten agarose was cooled down using running tap water for 1.5 min after which gels were cast by pouring the agarose solution into the gel former supplied with the electrophoresis apparatus. The comb was quickly inserted and the gels were allowed to set for at least 40 min before use and most of the time the gels were allowed to dry overnight. A volume of 25 μ l of the PCR reaction was mixed with 5 μ l of

Table 3.1 Sequence of the main 10-mer primers used in the different studies (except in chapter 9). The sequence of each primer is given from 5' to 3'. The sequence can also be obtained from <http://www.operon.com>.

<u>OPA9</u> GGGTAACGCC	<u>OPB1</u> GTTTCGCTCC	<u>OPB 5</u> TGCGCCCTTC	<u>OPB6</u> TGCTCTGCCC
<u>OPB7</u> GGTGACGCAG	<u>OPB8</u> GTCCACACGG	<u>OPB10</u> CTGCTGGGAC	<u>OPB11</u> GTAGACCCGT
<u>OPB 12</u> CCTTGACGCA	<u>OPB14</u> TCCGCTCTGG	<u>OPB17</u> AGGGAACGAG	

gel loading buffer (5 X) and 12-15 μ l were subjected to electrophoresis alongside molecular size markers in a 1.2 % (w/v) agarose gel, using 1X TBE buffer system. Three molecular markers were used namely the 1Kb ladder [Gibco BRL (BRL), Paisley, UK], the 1Kb ladder (Igi, Sunderland, UK), and the 100bp DNA ladder plus (Igi, Sunderland, UK). The molecular size (in Kb) of each bands of the three markers is presented in appendix I. After electrophoresis of either the PCR product (RAPD amplicons) at 80-100 volts (V) for 6 h, or the genomic DNA at 100 V for 2 h, gels were stained using ethidium bromide [0.015% (v/v)], visualised under UV illumination and photographed using a Polaroid CU5 camera system (Eastman Kodak, New York, USA). The Kodak Digital Science™ 1 D analysis software (Eastman Kodak, New York, USA) allows the size of each band displayed in the RAPD profile to be determined by comparison with the DNA ladder as well as the relative band intensity.

3.6 Measurement of parameters at the population and molecular levels

(*Daphnia magna*)

3.6.1 Measurement of growth, reproduction, and mortality

The length of *D. magna* (apex to base) was measured by video capture and image analysis using a Quantimet 570 image analyzer (Cambridge Instruments, Cambridge, UK). During experiments, neonates were counted and recovered at daily intervals. Once pooled, each batch of neonates was snap frozen in liquid nitrogen and stored at -80°C prior to DNA extraction and RAPD. In addition, the number of dead animals was determined on a daily basis.

3.6.2 Calculation of fitness parameters

The intrinsic rate of natural increase of the *D. magna* population r_m , was calculated using Lotka's equation: $\sum l_x m_x e^{-r_m x} = 1$ (Lotka, 1925; Krebs, 1978). For a cohort of animals observed from birth to death at regular intervals; x is the age in days; l_x is the age-specific survival (number of living females on day x /number of females at start of life table); m_x is the age-specific fecundity (number of new-born individuals produced on day x /number of living females on day x). Realised fecundity (U_x) was also calculated for the test populations ($U_x = l_x m_x$). Using these data, the net reproductive rate (R_0) can also be calculated, as R_0 represents U_x summarised over the entire test period ($R_0 = \sum l_x m_x = \sum U_x$). Minimum generation time (T_{min}) was calculated by measuring the time that elapsed between birth and the deposition of the first batch of offspring. The inter-brood time (Bt) was measured as the time (in d) between clusters or broods.

3.6.3 Estimation of genomic template stability

In order to compare the changes in RAPD profiles with the fitness parameters, each separate DNA effect observed in RAPD profiles (disappearance of bands, appearance of new bands and variation in band intensities) was given the arbitrary score of +1, and the average calculated for each experimental group of animals with the different primers used. The genomic template stability (GTS) (%) was calculated as '100-(100a/n)' where 'n' is the number of bands detected in control DNA profiles and 'a' the average number of changes in DNA profiles. The GTS gives a qualitative evaluation of the DNA effects (including DNA damage and mutation) induced by genotoxins. An example for the calculation of GTS is given in appendix II.

3.6.4 Transformation of the data

To compare the sensitivity of each parameter (GTS, l_x , m_x , r_m and R_o), changes in these values were calculated as a percentage of their control value (set to 100 %).

3.6.5 Statistical analyses

The LC50 value and 95 % confidence limit was determined either by using the method of Finney (1971) (Chapter 5) or the probit method (Wardlaw, 1985) (Chapter 7). LC50 value and 95 % confidence limit were calculated using the package software SPSS 6.1 for Windows for Chapter 5 and 7, respectively.

Differences among growth rates were calculated using multiple regression analysis. For more details refer to Conradi and Depledge (1998). Briefly, data were log-transformed to homogenize the variances. Because the number of surviving animals decreased as the toxic effect increased, the regressions were weighted with $n^{1/2}$ (where n is the number of the surviving animals). Correlation and analysis of variance (ANOVA) were performed using the computer software package Statgraphics (Statgraphics plus for Windows version 2.1, Statistical Graphics, USA).

To compare the sensitivity of each parameter, changes in GTS and key fitness parameters [age-specific survival (l_x), age-specific fecundity (m_x), net reproductive rate (R_o) and intrinsic rate of population increase (r_m)] were statistically tested using ANOVA (Statgraphics plus for Windows version 2.1, Statistical graphics, USA). The LSD (least significant differences) test was used to reveal statistical differences.

3.6.6 Experimental approach

Figure 3.2 gives an overview of the experimental approach which was adopted to compare the GTS with the parameters measured at the population level. Briefly, after having exposed an organism to an environmental contaminant the DNA was extracted,

DNA concentration was determined and PCR was performed. After electrophoresis the GTS was calculated and was compared to parameters measured at higher level of biological organisation (fitness and Darwinian parameters).

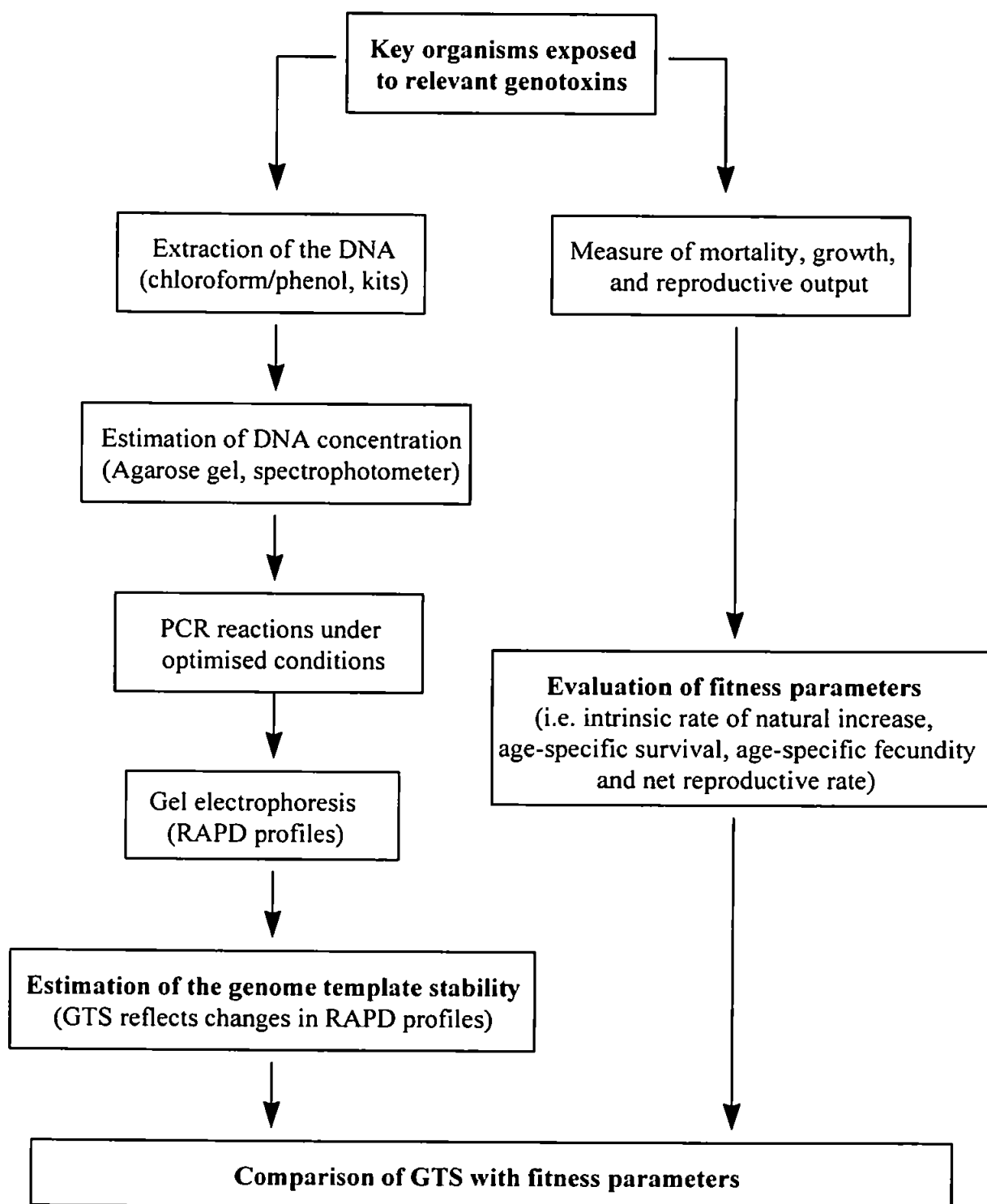


Figure 3.2 Brief diagrammatic representation of the experimental protocol followed in some of the studies presented in this research project.

Chapter 4

OPTIMISATION OF THE RAPD REACTIONS

FOR THE DETECTION OF

DNA DAMAGE AND MUTATIONS

4.1 Introduction

Before evaluating the potential of the RAPD technique to detect toxicant-induced DNA effects in aquatic organisms it was essential to verify whether this methodology is a robust assay. Indeed the use of the RAPD method has often been questioned mainly due to its lack of reproducibility.

Despite having shown great utility for many applications, problems may occur in RAPD which diminish its discriminative ability. The problems include the presence of spurious amplification products in negative control reactions not containing template DNA (Ruano *et al.*, 1989; Mullis, 1991), and a lack of reproducibility (Khandka *et al.*, 1997; Ellsworth *et al.*, 1993) amongst amplification patterns in positive controls. In addition, the use of the RAPD technique for paternity assessment has been questioned since non-parental bands were detected (Scott *et al.*, 1992; Riedy *et al.*, 1992). It was demonstrated that the non-parental band present in some RAPD reactions may consist of a heteroduplex molecule formed between two allelic sequences of different size (Ayliffe *et al.*, 1994). Another restriction is related to the fact that homozygotes cannot be distinguished from heterozygotes (Gwakisa *et al.*, 1994). In addition, profiles may reveal a poor efficiency in amplification and to some extent the absence of PCR products, as may occur with any PCR assays. It has been even suggested that the *Taq* DNA polymerase could be contaminated with DNA (Bottger, 1990) and protocols have been proposed to avoid false positives with PCR (Kwok and Higuchi, 1989; Kitchin *et al.*, 1990). It is clear that among the possible problems, the main concern about RAPD has certainly been the lack of reproducibility. The arbitrary nature of the DNA polymerisation catalysed in the RAPD reactions has often drawn criticism because of the low annealing temperature (34-36°C) used with short primers.

After robust optimisation of the RAPD reactions, many studies have also reported that reproducibility was achieved (Rothuizen and Van Wolferen, 1994; Benter *et al.*, 1995;

Bentley and Bassam, 1996; Caetano-Anolles, 1998). Those converted to the method claim that, with practice and care, repeatability is actually high (Hedrick, 1992). However, it is also well established that many conditions of the RAPD reaction procedure may influence the results. For instance, the use of different thermostable DNA polymerase, or thermal cyclers can generate variable RAPD profiles (MacPherson *et al.*, 1993; Meunier and Grimont, 1993; Schierwarter and Ender, 1993). In this context, a comparison of RAPD profiles among laboratories should not be attempted unless RAPD reactions are performed under strictly identical conditions.

The aim of this chapter was to identify and solve the potential problems associated with the technique. This implicated the study of each parameter (physical and chemical) on the RAPD profiles using different species belonging to the bacterial, vegetal, and animal kingdoms.

4.2 Materials and methods

4.2.1 Effect of UV treatment of DNA, *Thermus aquaticus* DNA polymerase, primer OPA9, and dNTP on RAPD profiles

Genomic DNA was extracted from *D. magna* (clone b, see section 3.1.1) using a phenol-chloroform extraction procedure as described in section 3.2.1.1. The extracted DNA was electrophoresed in 1.2 % agarose gels at 100 V for 2 h and the DNA concentration of unknown samples was determined by comparison with known amounts of Lambda phage DNA (see section 3.3.1). Genomic DNA (10 ng), *Taq* DNA polymerase, primer OPA9, and dNTP were exposed to UV in turn and used to perform RAPD reactions. The UV box contained 3 UV-C tubes (254 nm) and materials to be treated were placed at a distance of 5 cm which corresponded to an energy of 550 μ watts/cm. PCR reactions were carried out under the following conditions (condition 1). RAPD reactions were performed

with 0.22 mM dNTP, 2.18 mM MgCl₂, 10 ng DNA, 2.8 units of *Taq* DNA polymerase (Promega, Madison, USA), 1 X buffer (appendix I) and 0.56 µM primer OPA9 (see section 3.4.2). The thermocycling conditions were 5 min denaturation at 95°C, following by 10 cycles with an annealing temperature of 32°C, and 30 cycles with an annealing temperature of 35°C. Each cycle also included 1 min at 74°C for extension [except for the last cycle (10 min)] and 1 min at 95°C for denaturation. PCR products were electrophoresed in 1.2 % agarose gel at 90 V for 6 h, stained with ethidium bromide and visualised under UV light. A photograph was also taken and the image of the gel was saved electronically for further analysis when necessary (see section 3.5).

4.2.2 Effect of annealing temperature on RAPD profiles

Genomic DNA was extracted from *D. magna* (clone b, see section 3.1.1) using a phenol-chloroform extracted procedure as described in section 3.2.1.1. DNA concentration was estimated as described in section 4.2.1. The effect of different annealing temperatures, 35, 37, 39, 41, 43, 45, 50, 52, 54, 55, and 60°C on RAPD profiles were investigated under the following conditions: 4 min denaturation at 95°C followed by 40 cycles with a denaturation step at 95°C (1 min), an annealing temperature of X°C (1min), extension temperature of 74°C [(1 min); the final extension period was adjusted to 10 min]. RAPD reactions were performed under the following conditions (condition 2) with 0.22 mM dNTP, 2.18 mM MgCl₂, 10 ng DNA, 2.8 units of *Taq* DNA polymerase (Promega, Madison, USA), 1 X buffer (appendix I) and 2 µM primer OPA9 (see section 3.4.2). Reactions were also performed in the absence of genomic DNA template. PCR products were electrophoresed, gels stained, and the results analysed as described in section 4.2.1.

4.2.3 Effect of every PCR reagent concentration on RAPD profiles

Genomic DNA was extracted from *D. magna* (clone 5, see section 3.1.1) and the alga *P. palmata* (see section 3.1.3) as well as *E. intestinalis* (see section 3.1.2) using a phenol-chloroform extracted procedure as described in sections 3.2.1.1 (*D. magna*) and 3.2.2.1 (*P. palmata* and *E. intestinalis*). Experiments were also carried out using caesium chloride extracted DNA from *E. coli*, calf thymus, and human placenta (Sigma, Poole, UK; see section 3.2.4). DNA concentration was estimated as described in section 4.2.1. Every PCR reagent concentration was subjected to variation as follows:

- Magnesium: 1, 2, 3, 4, 5, 6, 7 and 8 mM.
- dNTP: 0.11, 0.22, 0.33, 0.44, 0.55, 0.66, 0.77 and 0.88 mM.
- 10-mer primer: 0.25, 0.5, 1, 1.5, 2, 3 and 4 μ M.
- DNA: 0.001, 0.002, 0.003, 0.007, 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 ng (except when otherwise mentioned).
- *Taq* DNA polymerase: 0.25, 0.5, 1, 1.5, 2, 3.5, 5 and 7.5 units.
- Buffer: 0.25, 0.5, 1, 1.5, 2 and 3 X.

Each PCR reaction was run in duplicate. When the concentration of a PCR chemical was varied, the remaining PCR chemical and physical parameters were as stated in the optimised PCR conditions (see section 3.4.1) using some of the primers described in section 3.4.2. PCR products were electrophoresed, gels stained, and results analysed as described in section 4.2.1.

4.2.4 Effect of DNA preparation on RAPD profiles

D. magna (clone 5, see section 3.1.1) was used in this experiment. The effect of DNA purity on RAPD profiles was determined by using different DNA preparations as follow. Single *D. magna* was either boiled for 15 min in 50 μ l of analytical grade water or

homogenised in 100 µl of analytical grade water and diluted 200 times in analytical grade water. In both procedures, 4 µl was used in PCR reactions. Genomic DNA was also extracted and purified using a phenol/chloroform extraction procedure or a Genie DNA extraction kit as described in sections 3.2.1.1 and 3.2.1.2, respectively. DNA concentration was estimated as described in section 4.2.1. RAPD profiles were generated using 10-mer primers OPA9 (see section 3.4.2) under the optimised conditions as described in section 3.4.1. PCR products were electrophoresed, gels stained, and results analysed as described in section 4.2.1.

In a second set of experiments, genomic DNA was extracted from *P. palmata* (see section 3.1.3) using a phenol/chloroform extraction procedure as described in section 3.2.2.1. Some of the extracted DNA was further purified by equilibrium centrifugation caesium chloride ethidium bromide gradients (see section 3.2.2.2). DNA concentration was estimated as described in section 4.2.1. Genomic DNA obtained from both procedures was resuspended in 100 µl of analytical grade water and 50 µl of this volume was transferred to a fresh tube and concentrated TE buffer was added to obtain a final concentration of 1X. RAPD profiles were generated using 10-mer primers OPA9, OPB1, OPB5, and OPB8 (see section 3.4.2) under the optimised conditions as described in section 3.4.1. PCR products were electrophoresed, gels stained, and results analysed as described in section 4.2.1.

4.2.5 RAPD profiling of genomic DNA templates obtained from different species

The applicability of RAPD profiling to genomic templates isolated from different organisms [*D. magna* (clone 5, see section 3.1.1), *E. coli*, calf thymus and human placenta (see section 3.2.4)] was determined. Genomic DNA was extracted from *D. magna* using a phenol/chloroform extraction procedure as described in section 3.2.1.1. DNA concentration was estimated as described in section 4.2.1. Caesium chloride purified genomic DNA from *E. coli*, calf thymus and human placenta was purchased from Sigma (Poole, UK). RAPD

profiles were generated using 10-mer primers OPA9, OPB1, OPB5, OPB6, OPB7, OPB8, OPB10, OPB11, OPB12, OPB14, OPB17 (see section 3.4.2) under our optimised conditions (see section 3.4.1). PCR products were electrophoresed, gels stained, and results analysed as described in section 4.2.1.

4.2.6 Inter-Individual reproducibility of RAPD profiling

The reproducibility of the RAPD profiling method was evaluated using genomic DNA from individuals of *D. magna* (clone5; see section 3.1.1), *P. palmata* (see section 3.1.3), *P. dumerilii* (see section 3.1.6) and *M. edulis* (see section 3.1.4). Genomic DNA of these 4 species was extracted from the whole organism (*D. magna*, *P. palmata* and *P. dumerilii*) and from the gill (*M. edulis*) using a phenol/chloroform extraction procedure as described in section 3.2. DNA concentration was estimated as described in section 4.2.1. Profiles were generated using 10-mer primers OPA9, OPB1, OPB5 OPB8 and OPB14 (except that primers OPA9, OPB7, and OPB10 were used for *D. magna*) (see section 3.4.2) on four replicate templates under the optimised conditions as described in section 3.4.1. In the case of unreproducible profiles, the presence/absence of amplification products and hence the fidelity of the PCR amplification was determined by using DNA concentrations of both 5 and 20 ng. PCR products were electrophoresed, gels stained, and results analysed as described in section 4.2.1.

4.2.7 Influence of time of DNA extraction and PCR on RAPD profiles

Forty *D. magna* (clone 5, see section 3.1.1), *E. intestinalis* [see section 3.1.2, mixture of 3 individuals (approximately 1 g)] and *P. palmata* [see section 3.1.3, mixture of 3 individuals (approximately 1 g)] were homogenised in 1ml of sperm lysis buffer (appendix I) and 3 samples (3 X 100µl) were phenol/chloroform extracted (see sections 3.2.1.1 and 3.2.2.1) on day i. After the first extraction, the samples (7 X 100µl) were kept at -20°C

until DNA extraction (3 X 100µl) was performed on days ii and iii. DNA concentration was estimated as described in section 4.2.1. RAPD reactions were also carried out on days iv and v under the optimised conditions (see section 3.4.1) using 10-mer primers OPA9 and OPB5 (see section 3.4.2). PCR products were electrophoresed, gels stained, and results analysed as described in section 4.2.1.

4.3 Results

4.3.1 RAPD under non optimised conditions

Figure 4.1A and B reveal that reproducibility between individual and mixture of *D. magna* (clone b) was not satisfactory when RAPD reactions were performed using non optimised conditions. Bands of molecular weights higher than 1 Kb were not always amplified and the relative intensities between bands appeared to be different (e.g. Figure 4.1B the highest molecular weight band for individual number 6 and 7). In addition, amplified bands may appear in reactions not containing any DNA template (Figure 4.2A and 4.2B). Negative controls are used to ascertain the purity of PCR reagents with respect to DNA contamination. Results clearly showed that the reproducibility of amplified bands in the absence of DNA target was poor (Figure 4.2A and 4.2B).

4.3.2 Effect of UV treatment of DNA, *Thermus aquaticus* DNA polymerase, primer OPA9, dNTP and negative control on RAPD profiles

Figure 4.3 clearly shows that after 20 min of UV-C irradiation, 10 ng of DNA could not be amplified. The number of bands decreased with the time of exposure and the amplification of the longer fragments was more inhibited than that of the shorter fragments. The *Taq* DNA polymerase was also affected after 20 min of exposure but to a lesser extent. In contrast, UV C radiations did not have any effect on the dNTPs and primer OPA9.

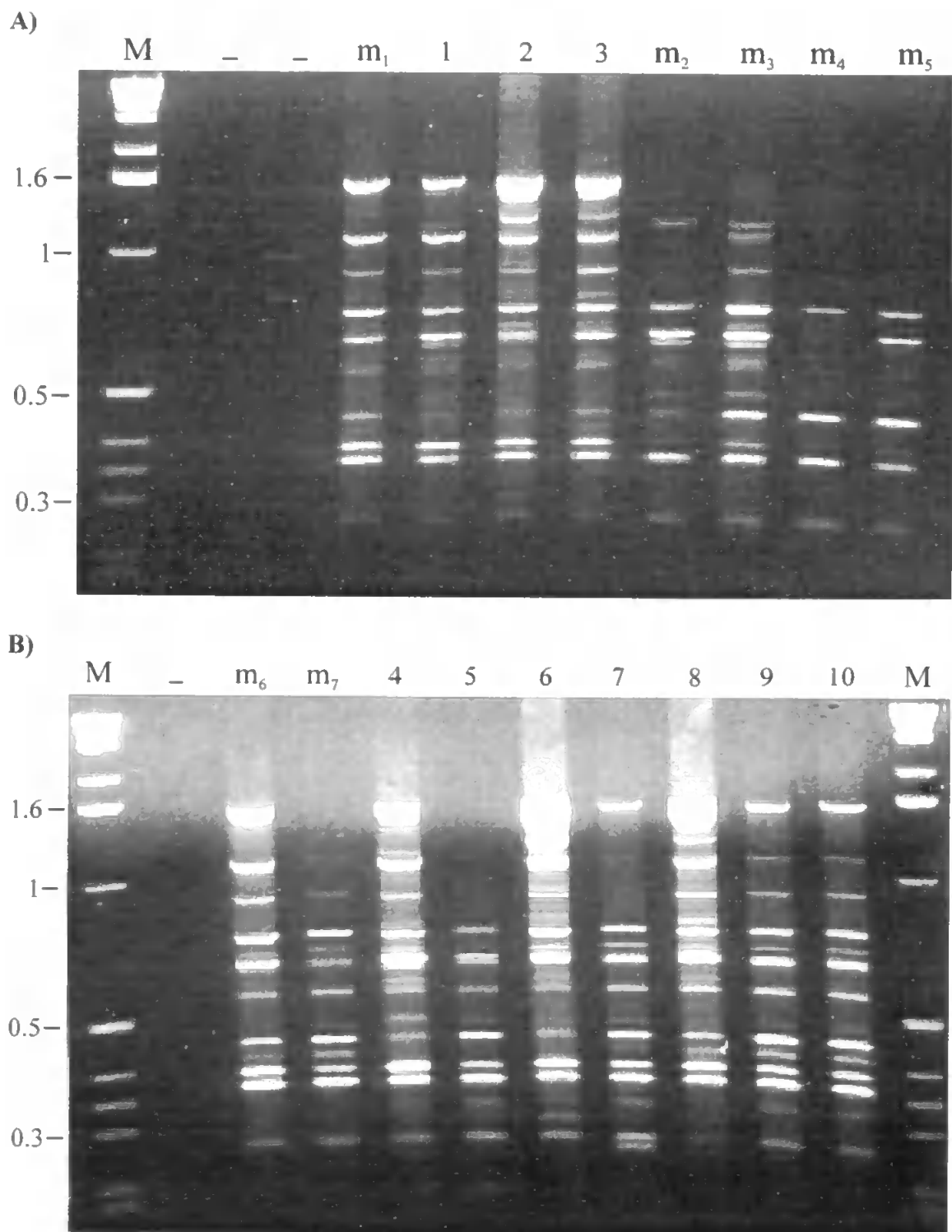


Figure 4.1 Problem of reproducibility among RAPD profiles generated by individuals (from 1 to 10) and mixtures (from m_1 to m_7) of *Daphnia magna* (clone b). - : no DNA control (negative control). M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left of each gel. PCR reactions were performed under condition 1 with primer OPA9.

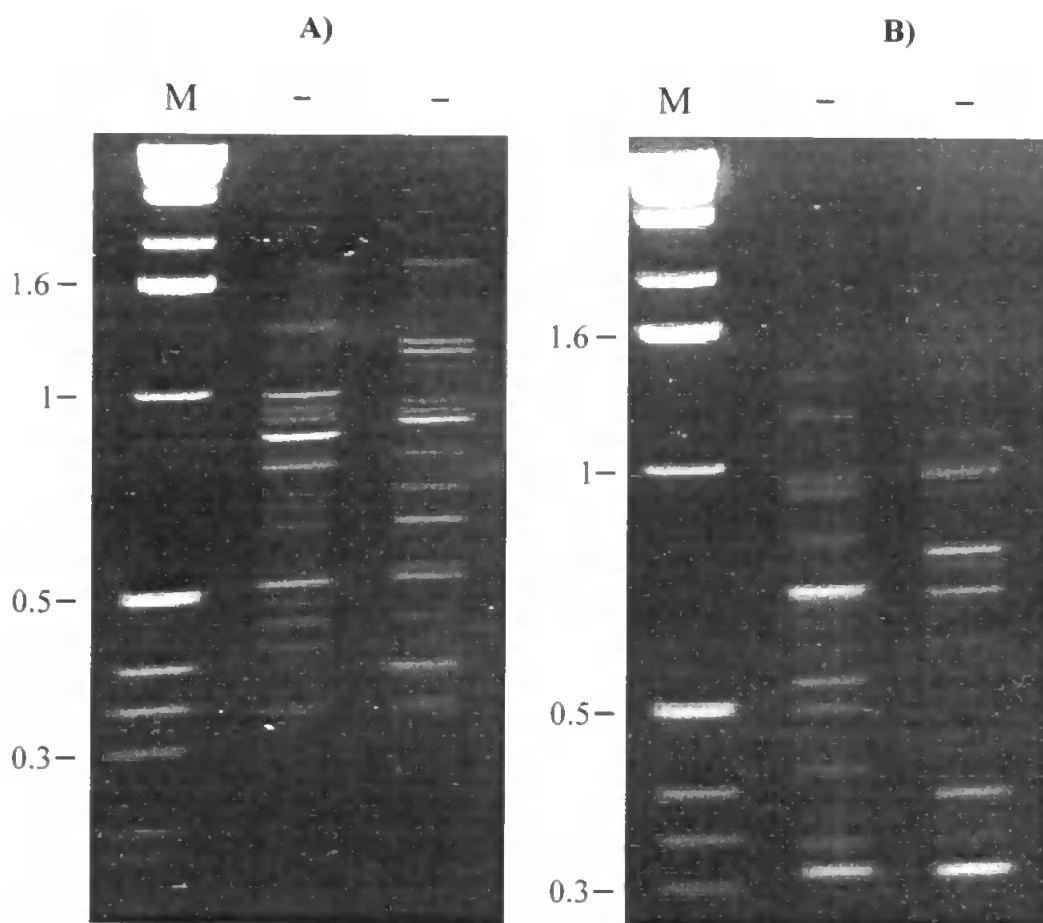


Figure 4.2 Spurious amplification in absence of genomic DNA target. -: no DNA control. M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left of each gel. PCR reactions were performed with primer OPA9 under condition 1 and 2 for Figures 2A and 2B, respectively.

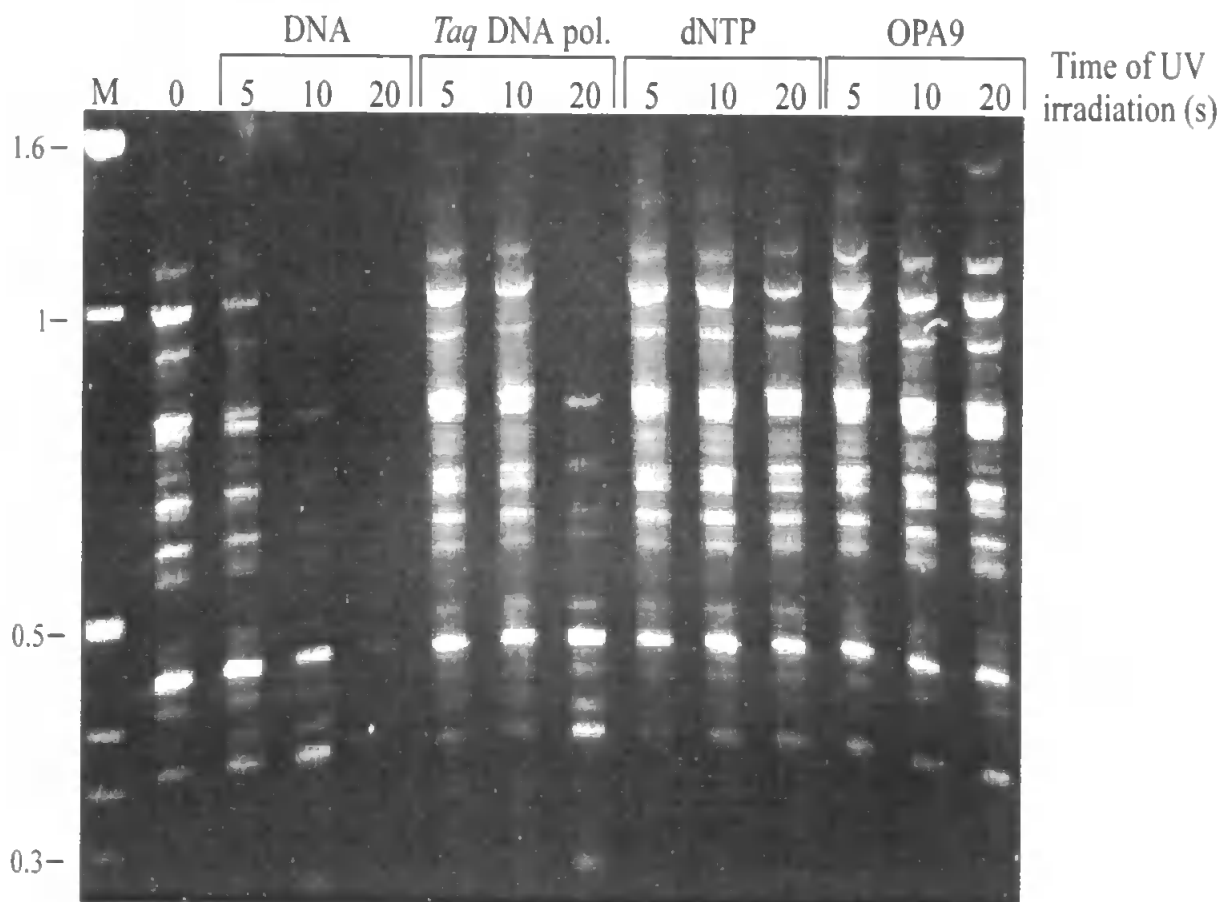


Figure 4.3 Effect of UV irradiation of genomic DNA (*Daphnia magna*), *Thermus aquaticus* DNA polymerase, dNTP, and primer OPA9 on RAPD profiles. M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left. Time of exposure to UV-C irradiation (s) and irradiated chemicals as indicated at the top of the gel. PCR reactions were performed with primer OPA9 under condition 1.

Another set of experiment was carried out to investigate the effect of UV radiation and primer concentration on negative controls. Figure 4.4. revealed that PCR products were present in both UV-treated and untreated reactions. In addition, it was particularly clear that the number of bands present in negative control was proportional to the concentration of the primer.

4.3.3 Optimisation of the RAPD reactions

4.3.3.1 Stringency of the PCR conditions

Different annealing temperatures were tested from 35 to 60°C in positive (Figure 4.5A) and negative (Figure 4.5B) controls. At high annealing temperatures such as 55 and 60°C respectively, RAPD profiles were either partially or completely affected in both negative and positive controls. The results showed that in absence of DNA template spurious amplifications occurred even under stringent conditions. An important feature was the fact that the reproducibility in negative controls was very poor in contrast to positive controls. Since RAPD profiles could be generated at high annealing temperature we have selected an annealing temperature of 50°C to conduct the optimisation work.

Figure 4.6 revealed that the use of DMSO reduced the efficiency in both positive and negative controls; the inhibition of the PCR reactions was consistent with the increase in DMSO concentration. However, a clear negative control could not be generated in combination with a high quality positive RAPD profile.

4.3.3.2 Influence of DNA preparation on RAPD profiles

The reproducibility of RAPD patterns was satisfactory when DNA was purified using either the phenol/chloroform extraction method (E₁) or the kit (E₂) (Figure 4.7A). However, the repeatability was affected when *D. magna* was boiled (B) and it was very poor when the water flea was homogenised and diluted in analytical grade water (H). The intensity and number of bands decreased when compared to RAPD profiles obtained with

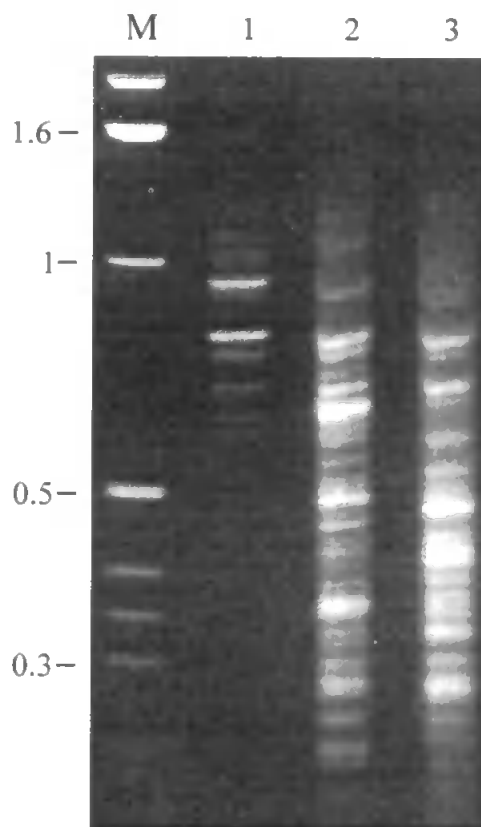


Figure 4.4 Effect of UV treatment and primer concentration on RAPD negative controls. 1: UV treated, 0.2 μ M primer, 2: UV treated, 2 μ M primer, 3: not UV treated, 2 μ M primer. M: 1 Kb DNA ladder (BRL). Every PCR reagent (except the *Taq* DNA polymerase) was exposed to UV-C radiation for 20 min. The UV box contained 3 UV-C tubes (254 nm) and materials to be treated were placed at a distance of 5 cm which corresponded to an energy of 550 μ watts/cm. The molecular sizes (Kb) are indicated on the left. PCR reactions were performed with primer OPA9 under condition 2 except for lane 1 (primer concentration was 0.2 μ M).

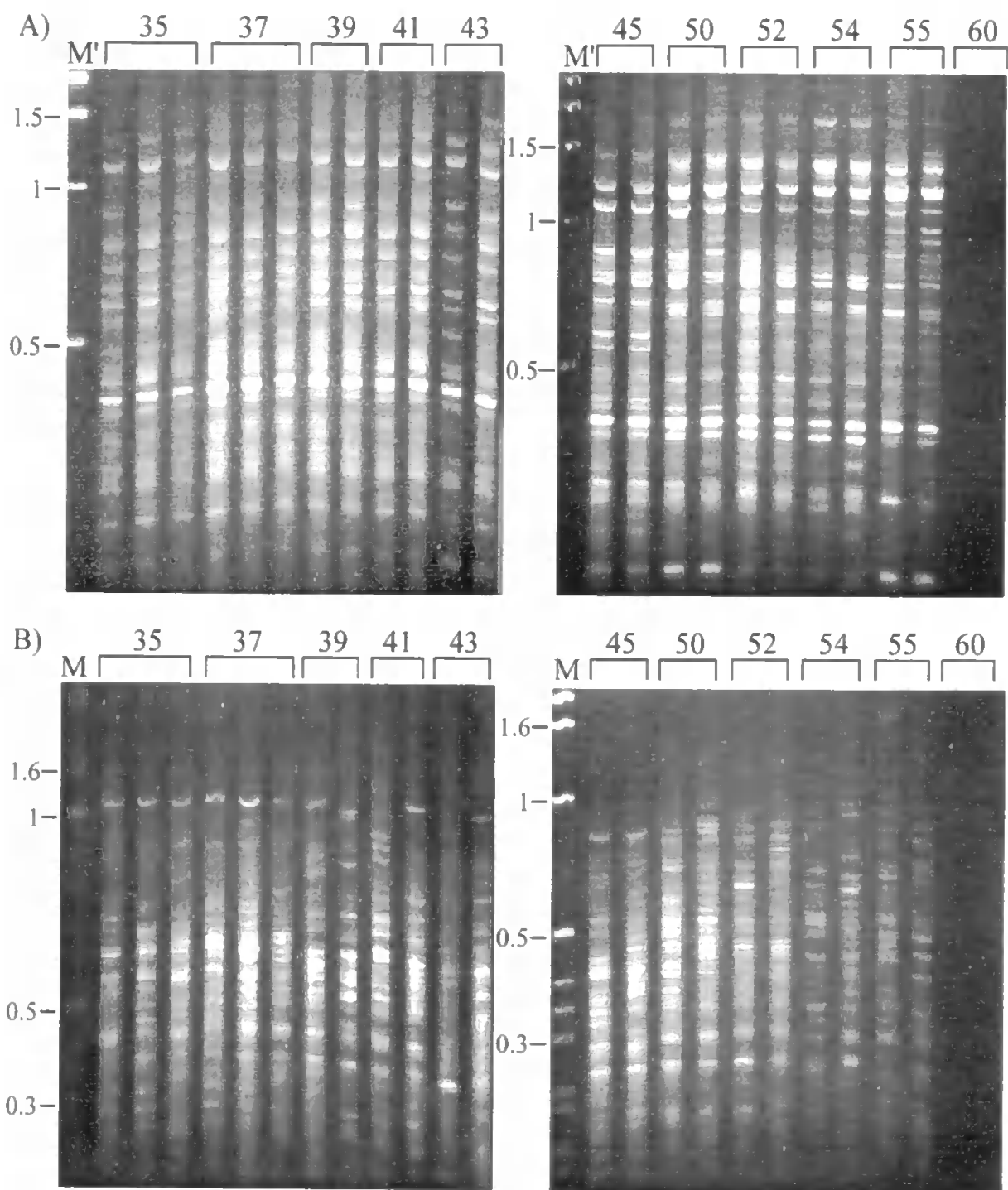


Figure 4.5. Effect of annealing temperature on RAPD profiles generated by positive (A) and negative (B) controls. Annealing temperature as indicated at the top of each gel. M and M': 1 Kb DNA ladder from BRL and Igi, respectively. The molecular sizes (Kb) are indicated on the left of each gel. PCR reactions were performed with primer OPA9 under condition 1. *D. magna* (clone b) was used as positive control.

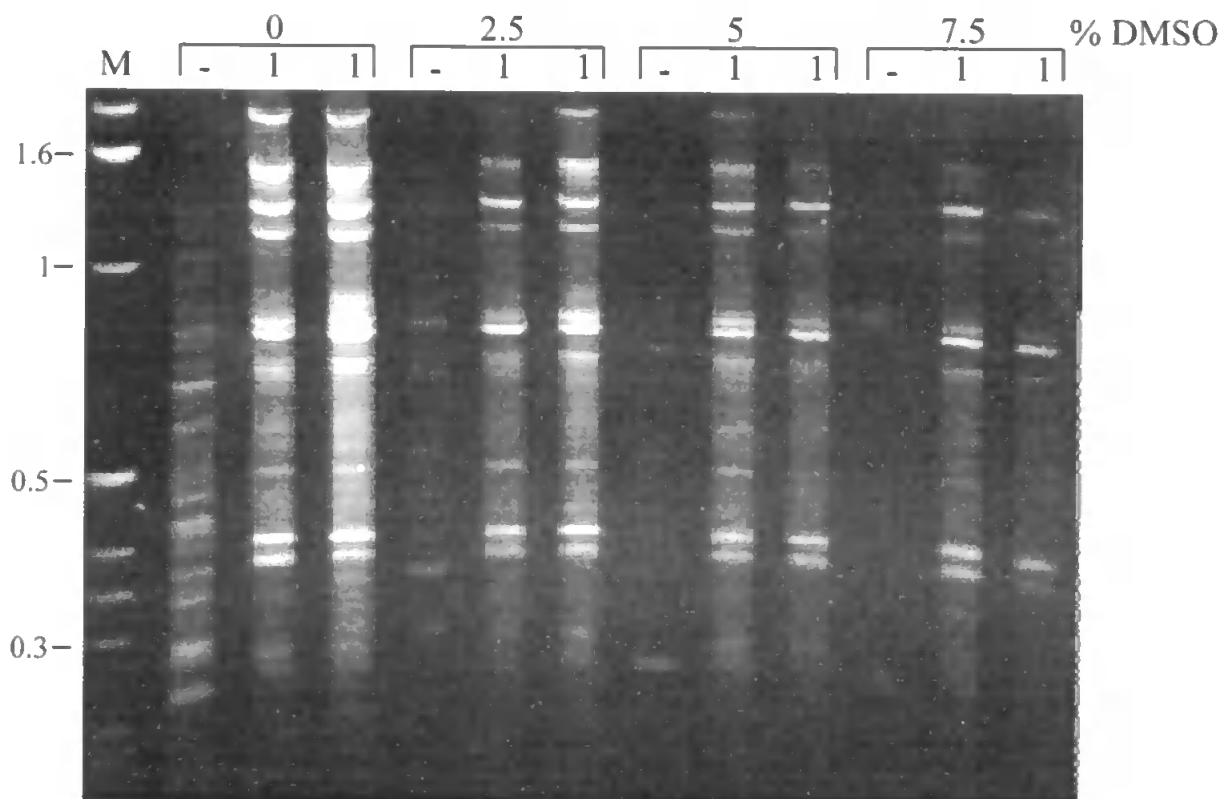
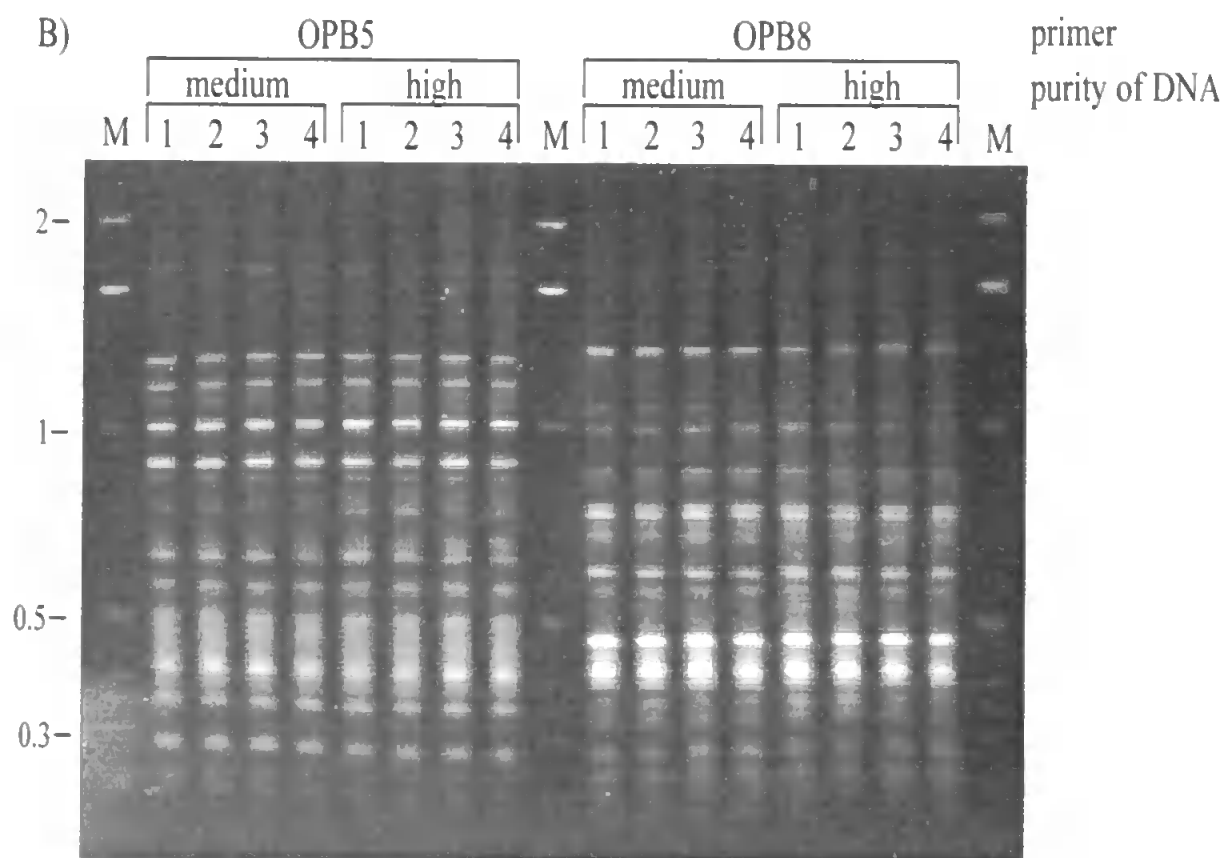
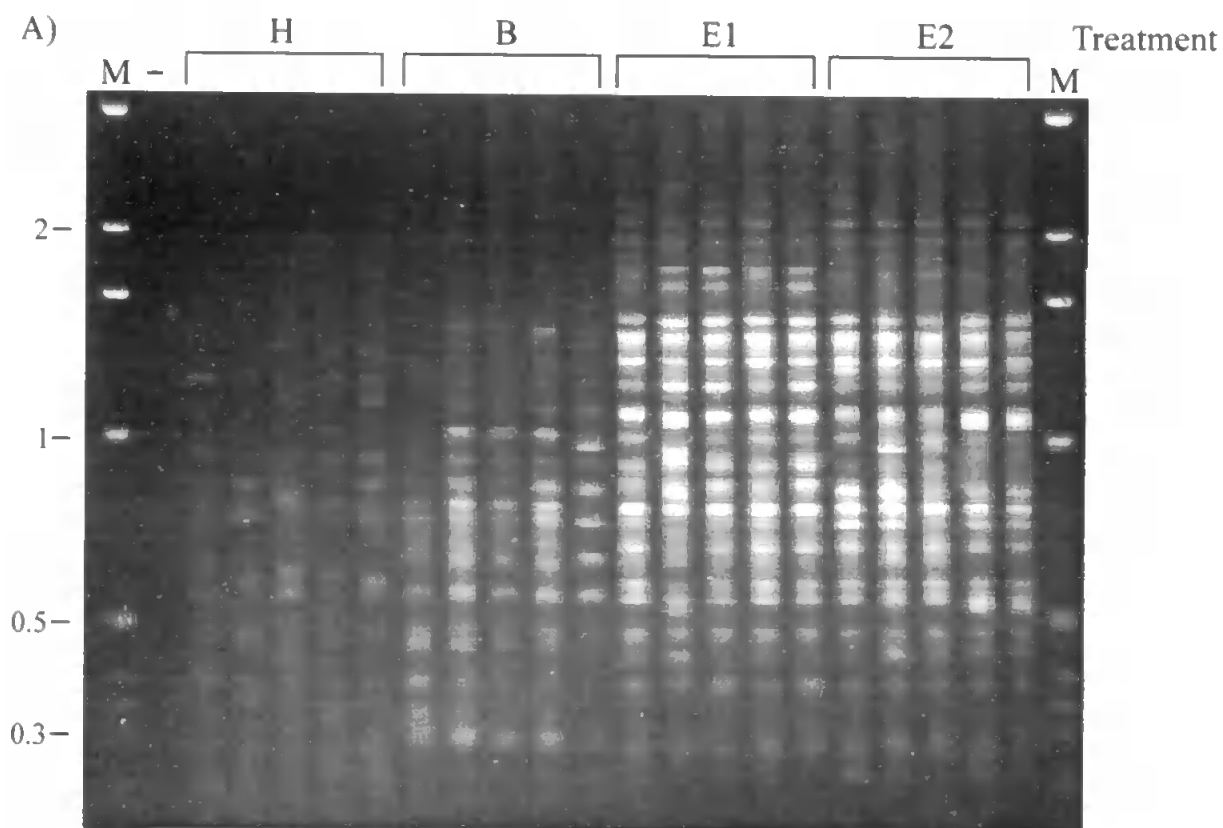


Figure 4.6 Effect of DMSO on RAPD profiles. Percentage of DMSO as indicated at the top of the gel. -: negative control. 1: 20 ng DNA [*D. magna* (clone b)]. M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left. PCR reactions were performed with primer OPA9 under condition 2.

Figure 4.7 Effect of DNA preparation on RAPD profiles. - = no DNA control. M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left of each gel. PCR reactions were performed under the optimised conditions (see section 3.4.1) with primer OPA9 (Figure 4.7A) and as indicated at the top of Figure 4.7B. A) Individual *D. magna* (clone 5) were subjected to different treatments. H: homogenised in 100 µl of analytical grade water, B: boiled in 50 µl of analytical grade water for 15 min (volume used = 4 µl for both treatments), E1: phenol/chloroform extracted DNA, E2: kit extracted DNA. B) DNA from *P. palmata* was extracted using two methods of purification: phenol/chloroform extracted DNA (medium quality DNA) and caesium chloride extracted DNA (high quality DNA). Lanes 1 and 2: 20 and 5 ng DNA dissolved in analytical grade water, respectively; lanes 3 and 4: 20 and 5 ng DNA dissolved in 1X TE buffer, respectively.



extracted DNA. In addition, both DNA extraction procedures generated similar RAPD profiles.

In another experiment presented in Figure 4.7B, PCR was performed using DNA which had been extracted from *P. palmata* with phenol/chloroform and caesium chloride diluted in either sterile analytical grade water or 1X TE buffer. The results showed that RAPD profiles were identical using 2 primers and 2 concentrations of DNA. The use of 2 other 10 mer-primers led to the same conclusion (appendix III).

4.3.3.3 Effect of DNA concentration on RAPD profiles

PCR reactions were performed using variable amounts of genomic DNA from *D. magna* (from 7 pg to 256 ng) and *P. palmata* (from 1 pg to 256 ng) (Figure 4.8.). The RAPD profiles showed high levels of reproducibility and discrimination in the range of 250 pg to 64 ng and 30 pg to 256 ng DNA for *D. magna* and *P. palmata*, respectively. Although other experiments suggest that the optimum concentration of genomic template varies according to species and priming oligonucleotide (appendix III), the overwhelming majority of high quality DNA profiles were produced in the range 50 pg to 100 ng.

4.3.3.4 Effect of primer sequence and concentration on RAPD profiles

As expected, RAPD profiles were variable depending on the specific priming oligonucleotide utilised in the PCR reactions. With regard to the amount of primer per reaction, RAPD patterns were generated using *D. magna*, *P. palmata*, and human placenta DNA templates and oligonucleotide concentrations of 0.25 to 4 μ M (Figure 4.9 and appendix III). The overall effect of increasing primer concentration, was to increase the number and intensity of DNA bands seen on electrophoretic profiles. The clearest profiles were produced using primers at a concentration of 1 to 4 μ M. In addition, even at high primer concentration, non specific PCR products could not be found (Figure 4.9). These results were confirmed with other primers (appendix III).

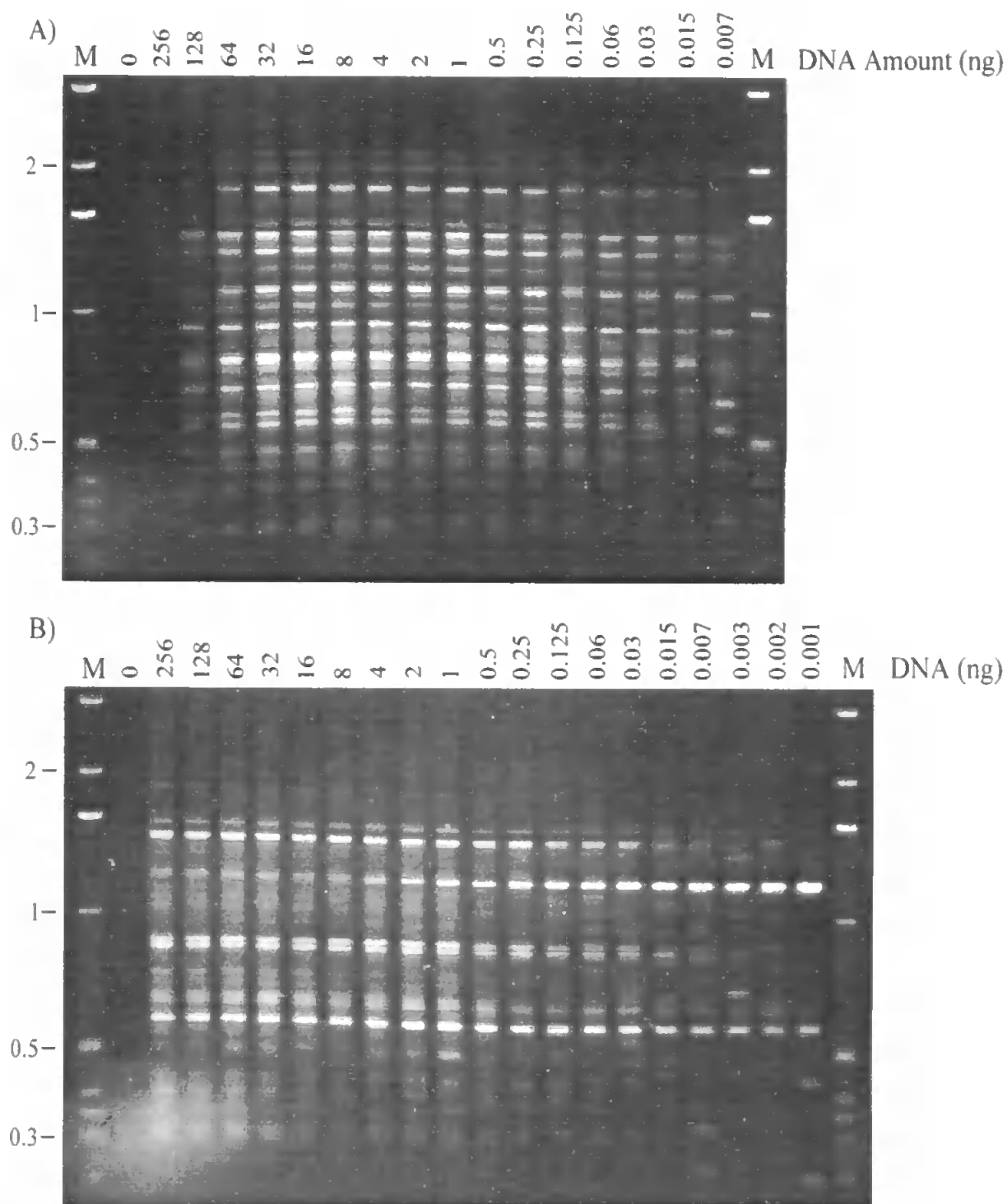


Figure 4.8 Effect of DNA concentration on RAPD profiles. M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left of each gel. PCR reactions were performed with primer OPA9 (A) and OPB1 (B) using genomic DNA from *D. magna* and *P. palmata*, respectively, under the optimised conditions (see section 3.4.1) except for the DNA concentration (as indicated at the top of each gel).

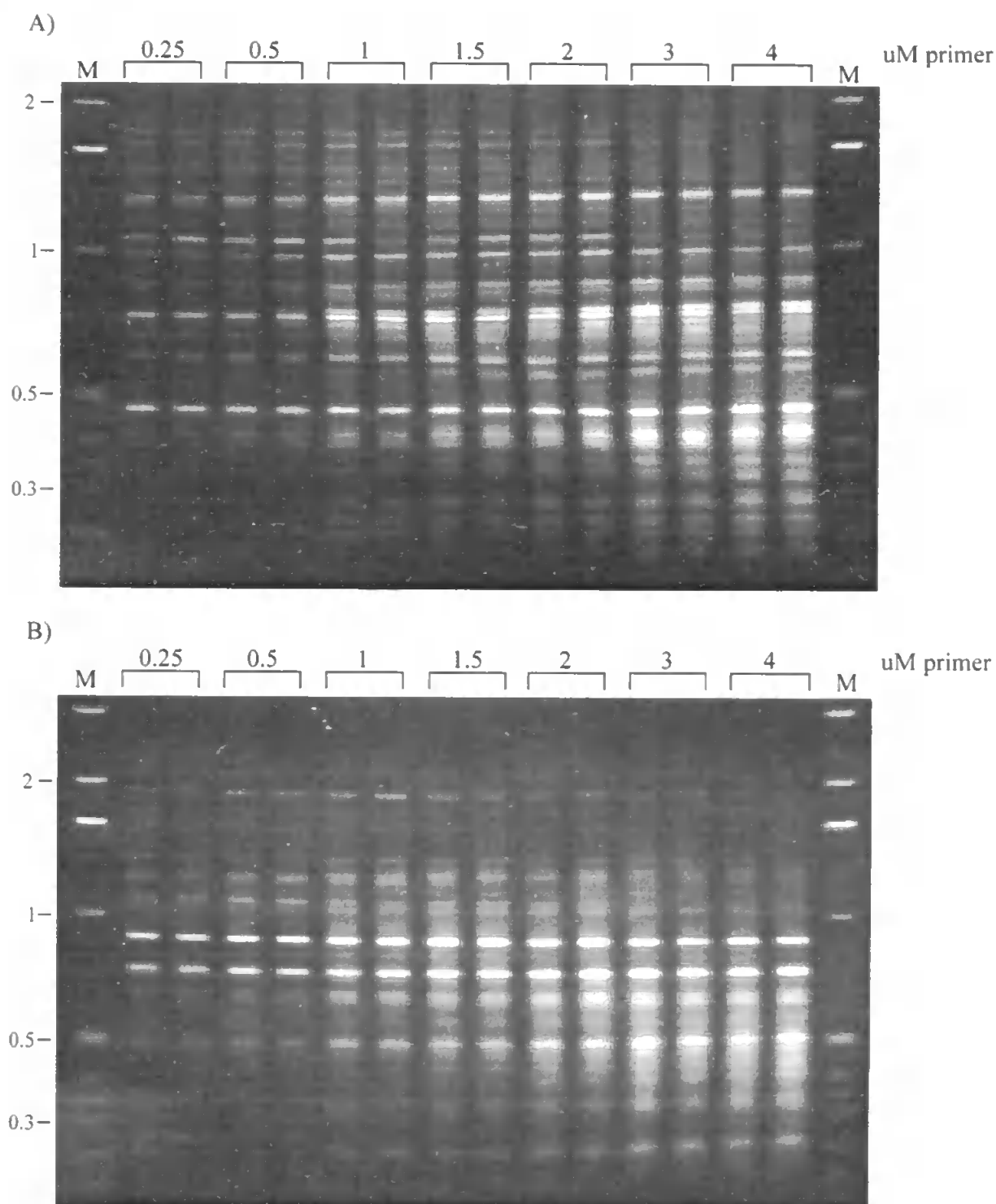


Figure 4.9. Effect of primer concentration on RAPD profiles. M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left of each gel. PCR reactions were performed with primer OPB8 using genomic DNA from *P. palmata* (A) and human placenta (B), under the optimised conditions (see section 3.4.1) except for the primer concentration (as indicated at the top of each gel).

4.3.3.5 Effect of magnesium ion concentration on RAPD profiles

The optimum concentration of magnesium ions for inclusion in the RAPD reaction mixture was determined using amounts in the range of 1 to 8 mM with *D. magna* and *E. intestinalis* genomic DNA (Figure 4.10 and appendix III). RAPD patterns could not be generated or were significantly affected at 1 and 2 mM Mg^{++} , respectively. In the range 3-6 mM, DNA profiles of high quality were generated. Above these concentrations, reproducibility and discrimination were affected.

4.3.3.6 Effect of dNTP concentration on RAPD profiles

dNTP concentration was subjected to variation within the range 0.11 to 0.88 mM using *D. magna* and *E. intestinalis* genomic DNA (Figure 4.11 and appendix III). Reproducible RAPD profiles were found using amounts between 0.22 and 0.66 mM. The discrimination of the pattern was affected at low (0.11 mM) and high (0.77 and 0.88 mM) dNTP concentrations. Of greater significance however, was the effect of dNTP concentration on the appearance of spurious amplification products in negative control reactions. At dNTP concentrations of 0.33 mM and above, spurious amplification were rarely found (see negative controls which were performed in the different studies).

4.3.3.7 Effect of *Thermus aquaticus* DNA polymerase concentration on RAPD profiles

The concentration of *Taq* DNA polymerase used in RAPD reactions was varied within the range 0.25 to 7.5 units using *P. palmata*, *E. coli*, and calf thymus genomic DNA (Figure 4.12 and appendix III). With increasing enzyme concentration an increase in the number of amplified bands was observed, up to a maximum amount in reactions catalysed by 2.0-3.5 units. Above this concentration, profiles were not improved, and below this concentration they were impoverished with respect to the number of visible DNA bands.

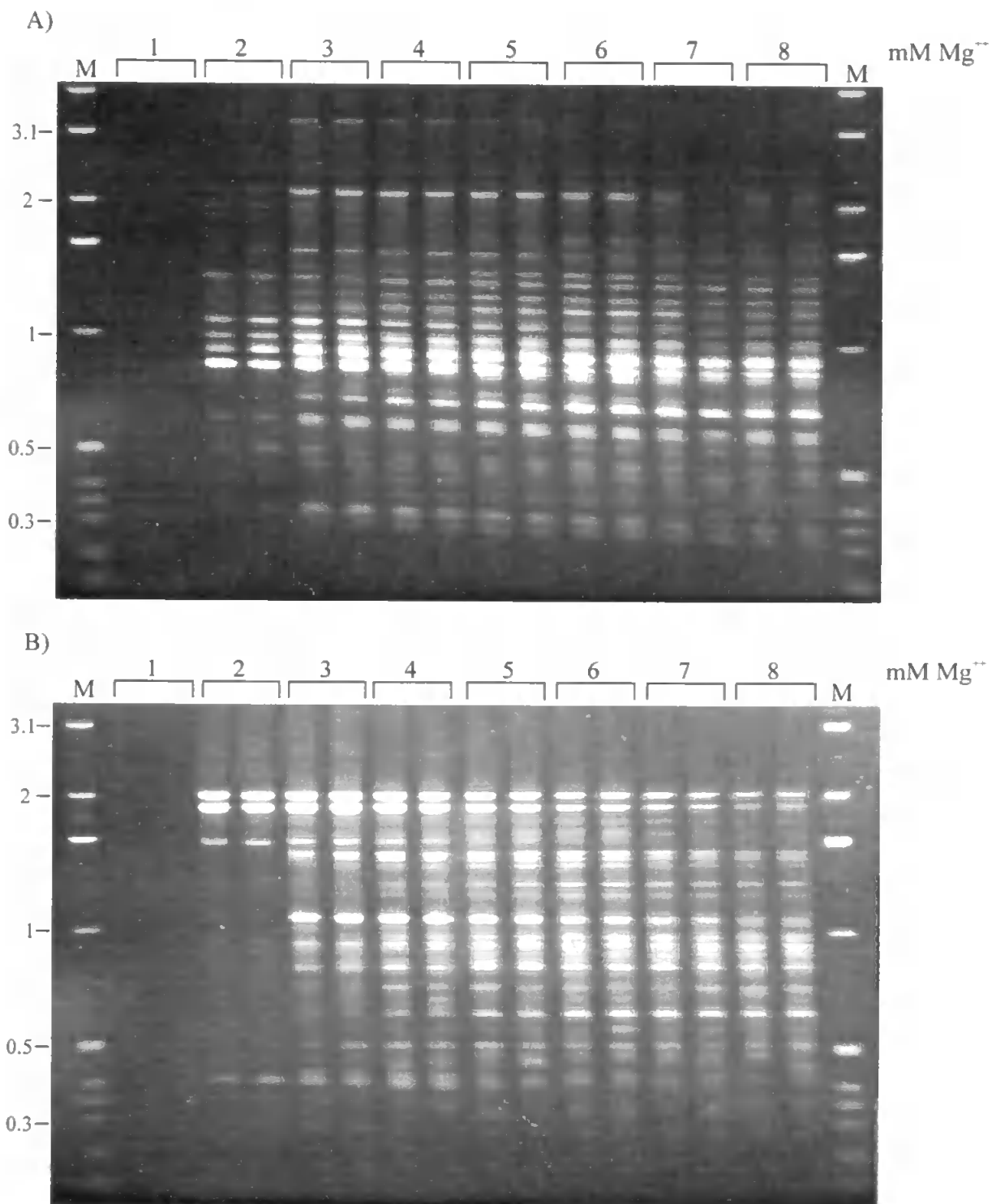


Figure 4.10 Effect of magnesium concentration on RAPD profiles. M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left of each gel. PCR reactions were performed with primer OPB5 using genomic DNA from *D. magna* (A) and *E. intestinalis* (B), under the optimised conditions (see section 3.4.1) except for the magnesium concentration (as indicated at the top of each gel).

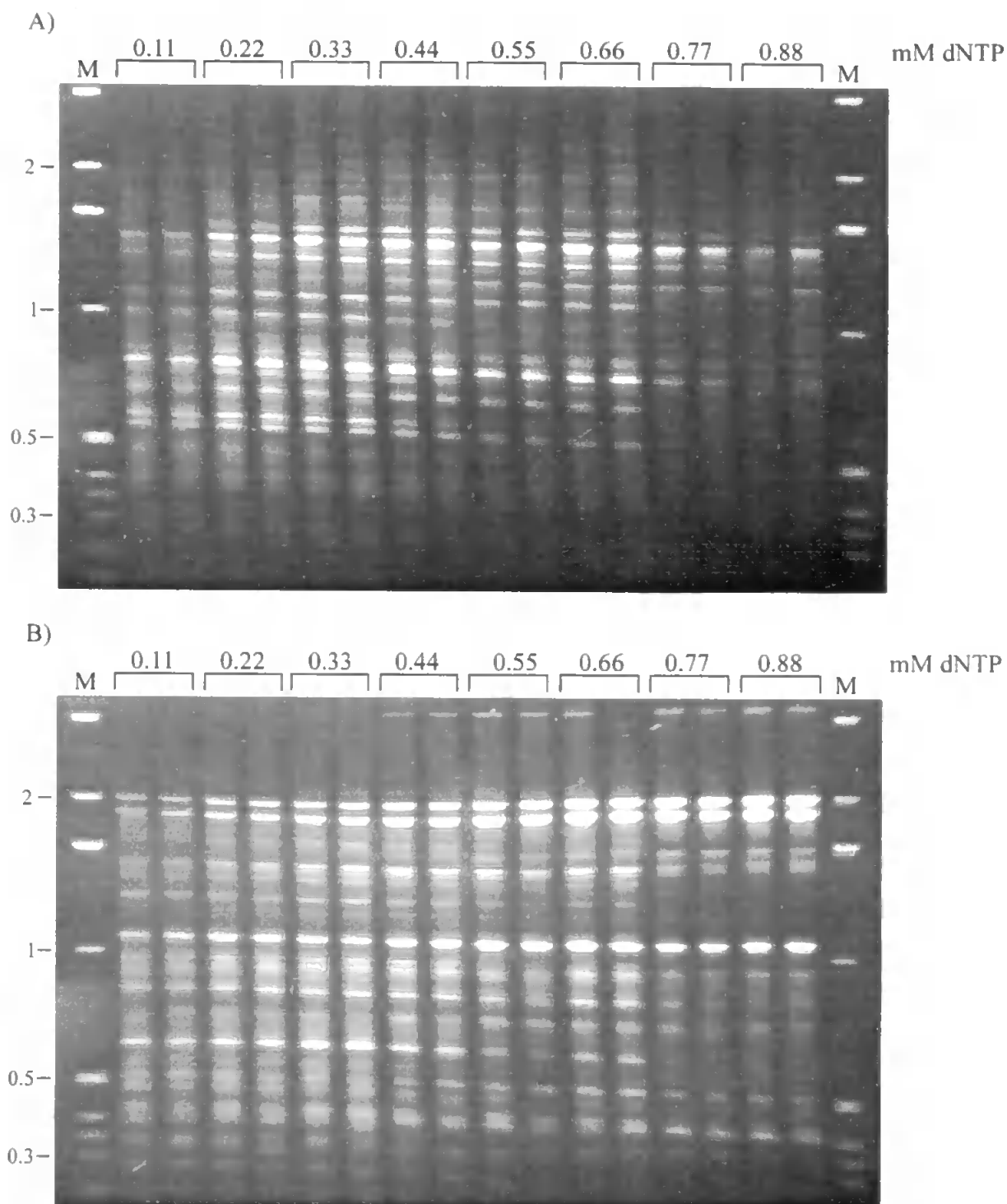


Figure 4.11 Effect of dNTP concentration on RAPD profiles. M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left of each gel. PCR reactions were performed with primer OPA9 (A) and OPB5 (B) using genomic DNA from *D. magna* and *E. intestinalis*, respectively under the optimised conditions (see section 3.4.1) except for the dNTP concentration (as indicated on the top of each gel).

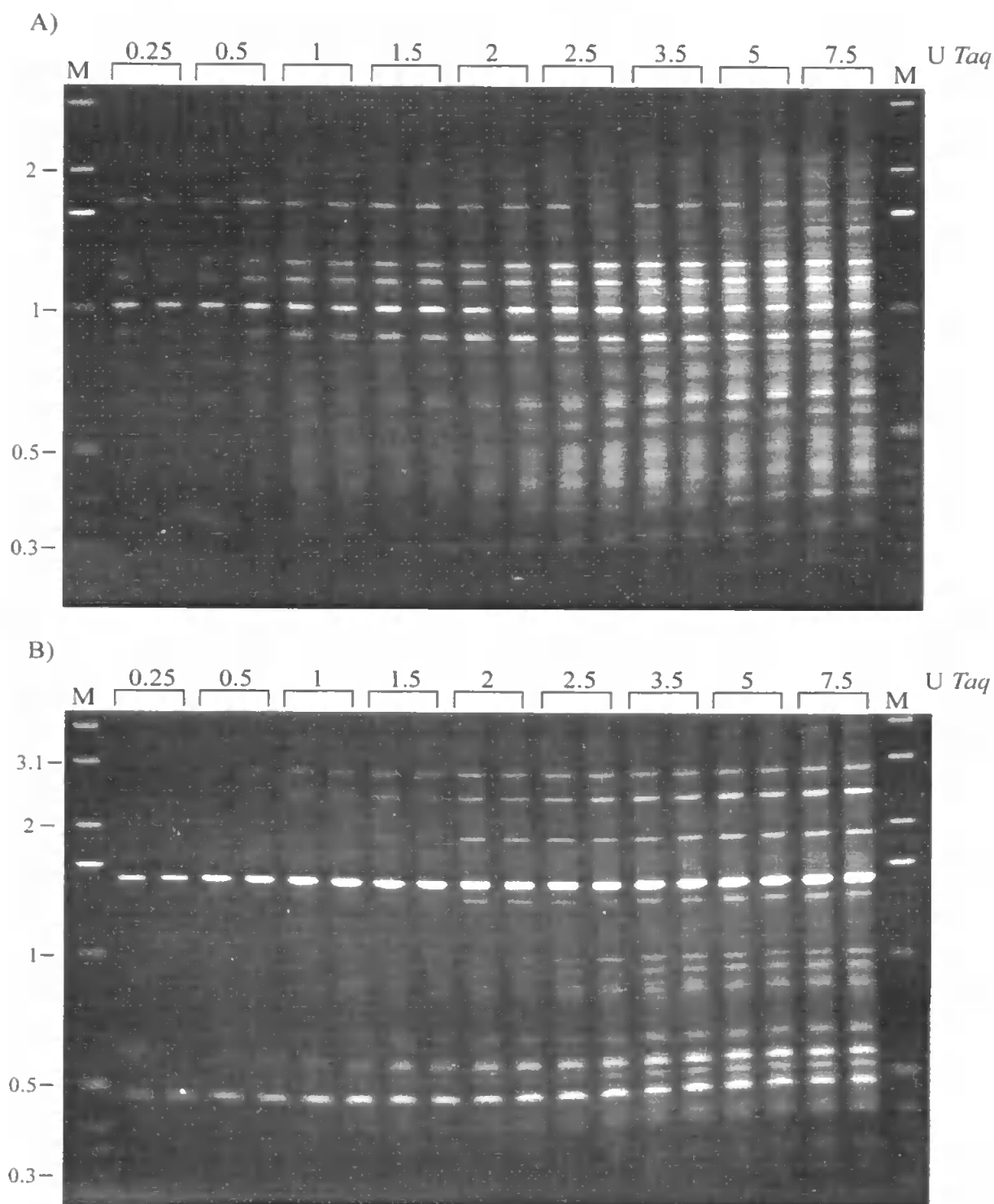


Figure 4.12 Effect of *Thermus aquaticus* DNA polymerase concentration on RAPD profiles. M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left of each gel. PCR reactions were performed with primer OPB5 using genomic DNA from *P. palmata* (A) and *E. coli* (B) under the optimised conditions (see section 3.4.1) except for the enzyme concentration (as indicated on the top of each gel). U: unit(s).

4.3.3.8 Effect of PCR buffer concentration on RAPD profiles

The buffer concentration was subjected to variation in the range 0.25-3 X using *D. magna* and *P. palmata* (Figure 4.13 and appendix III). As expected, the optimum concentration of buffer was 1 X. However, RAPD profiles were also generated at concentrations of 0.5 and 1.5 X. Outside this range, a decrease or increase in PCR buffer concentration had a significant effect on the profiles. No or very poor quality profiles were generated when the buffer concentration was either 0.25 X or 2-3 X.

4.3.4 Recommended RAPD reaction conditions

Table 4.1 gives an overview of some the results presented in this chapter. For each parameter (except for PCR buffer and bovin serum albumin) an optimised range to produce good quality RAPD profiles is given.

4.3.5 RAPD profiling obtained from different species

RAPD profilings of genomic templates isolated from *D. magna*, *E. coli*, calf thymus and human placenta using 11 different oligonucleotide primers are shown in Figure 4.14. Profiles generated using template DNA at both 20 ng and 5 ng were identical, demonstrating the high reproducibility of the methodology and the lack of template-dependent amplification artefacts.

4.3.6 Inter-individual reproducibility of RAPD profiling

The reproducibility of the RAPD method, using genomic DNA from individuals of the same species, is shown in Figure 4.15 and appendix III. The species tested were *P. palmata*, *P. dumerilii* and *M. edulis* (Figure 4.15) as well as *D. magna* (appendix III). Profiles obtained with 4 individuals of *P. palmata* and *P. dumerilii* (Figure 4.15) or 7 individuals of *D. magna* (appendix III) were reproducible. The best reproducibility was

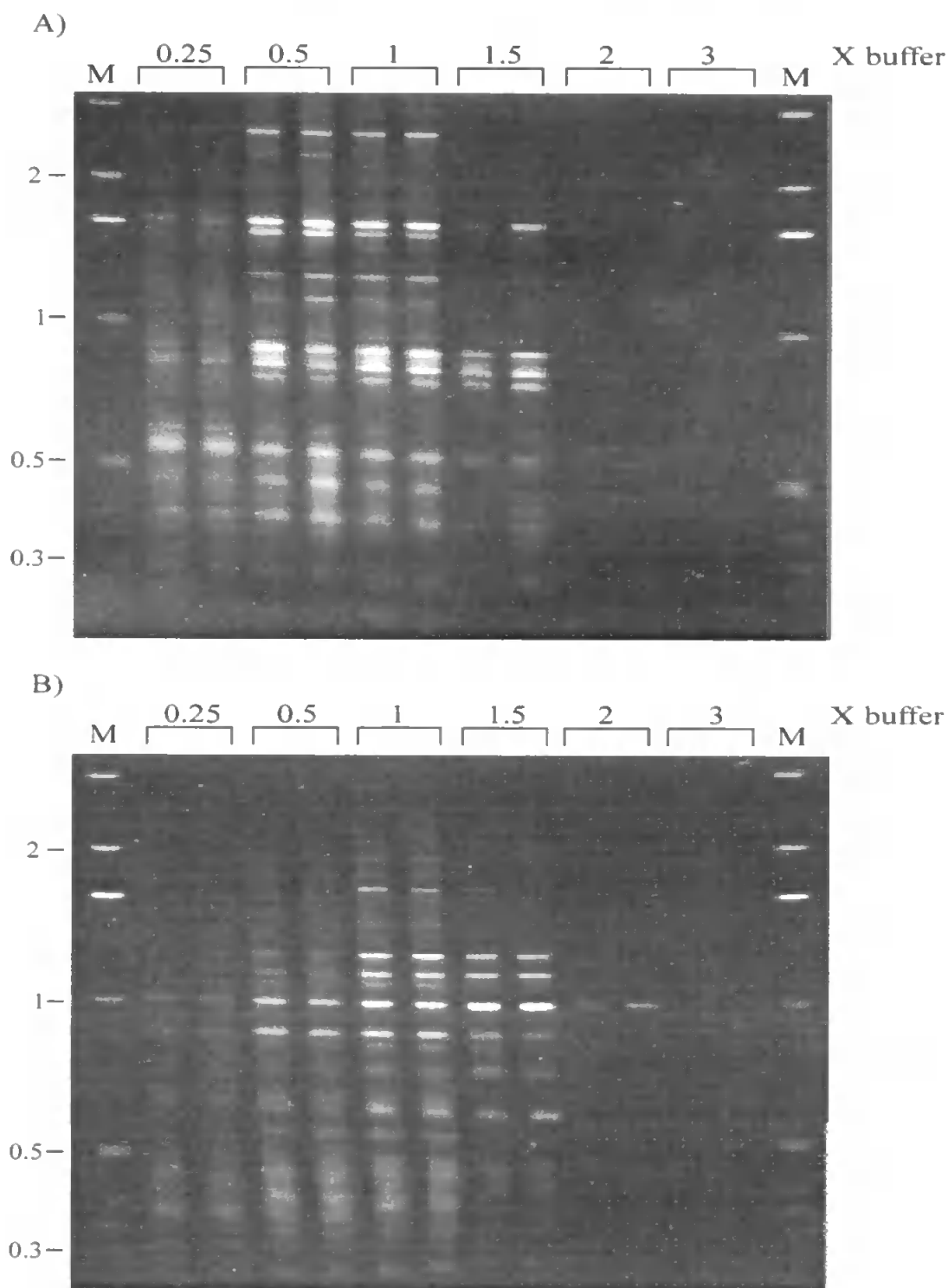


Figure 4.13 Effect of buffer concentration on RAPD profiles. M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left of each gel. PCR reactions were performed with primer OPB8 (A) and OPB5 (B) using genomic DNA from *D. magna* and *P. palmata*, respectively, under the optimised conditions (see section 3.4.1) except for the buffer concentration (as indicated on the top of each gel). X: concentration.

Table 4.1 Optimised conditions (range) to produce good quality RAPD profiles at high annealing temperature.

PCR parameters	Optimised conditions
Annealing temperature	50-54°C
Mg ⁺⁺ concentration	3-6 mM
dNTP concentration	0.33-0.44 µM
Primer concentration (10-mer)	1.0-4.0 µM
Number of native <i>Taq</i> DNA polymerase units	2.0-3.0 U
Amount of DNA	0.05-100 ng
PCR buffer	1 X (10 mM Tris-HCl pH 8.8 at 25°C, 50 mM KCl, 0.08% Nonidet P40)
Bovin serum albumin	2.5 µg
Thermal cycling conditions	<u>First cycle</u> : 95°C for 4 min, <u>39 cycles</u> : 95°C for 1 min, 50°C for 1 min, 74°C for 1 min, <u>Last cycle</u> : 95°C for 1 min, 50°C for 1 min, 74°C for 10 min.

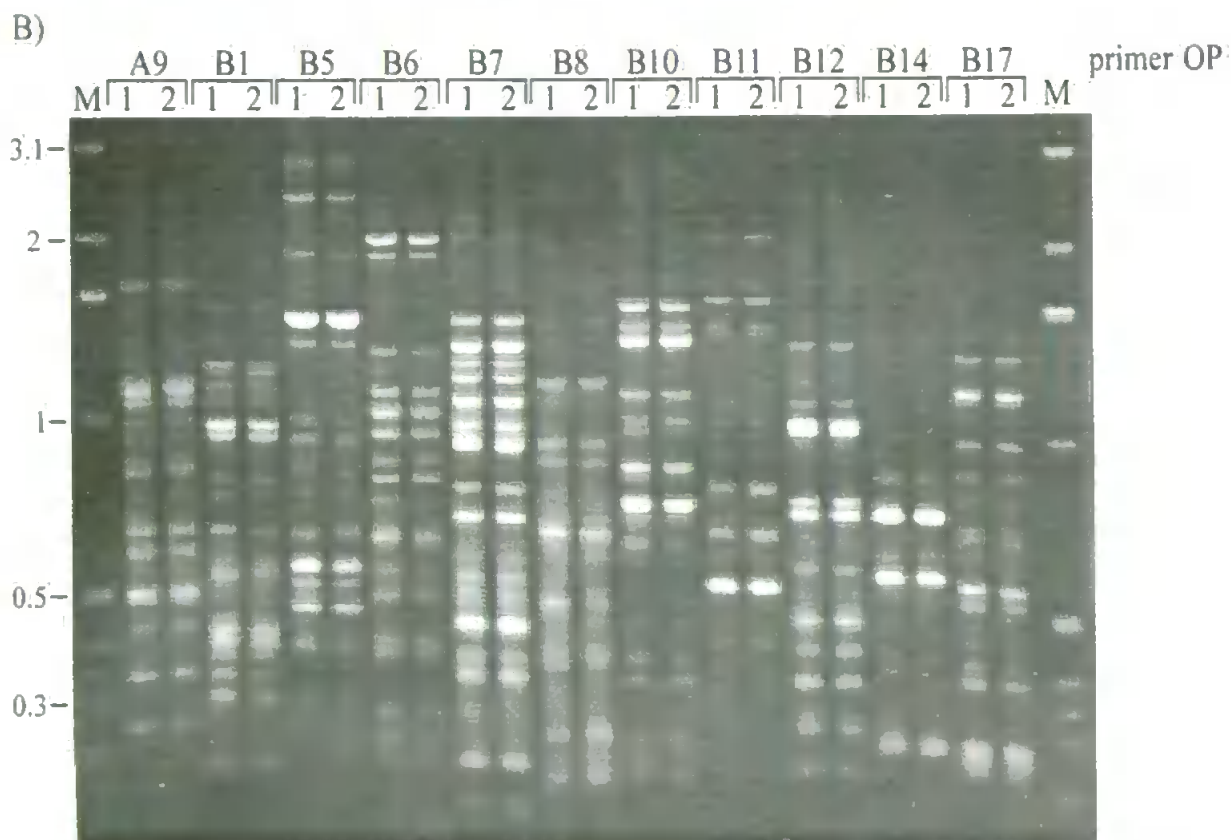
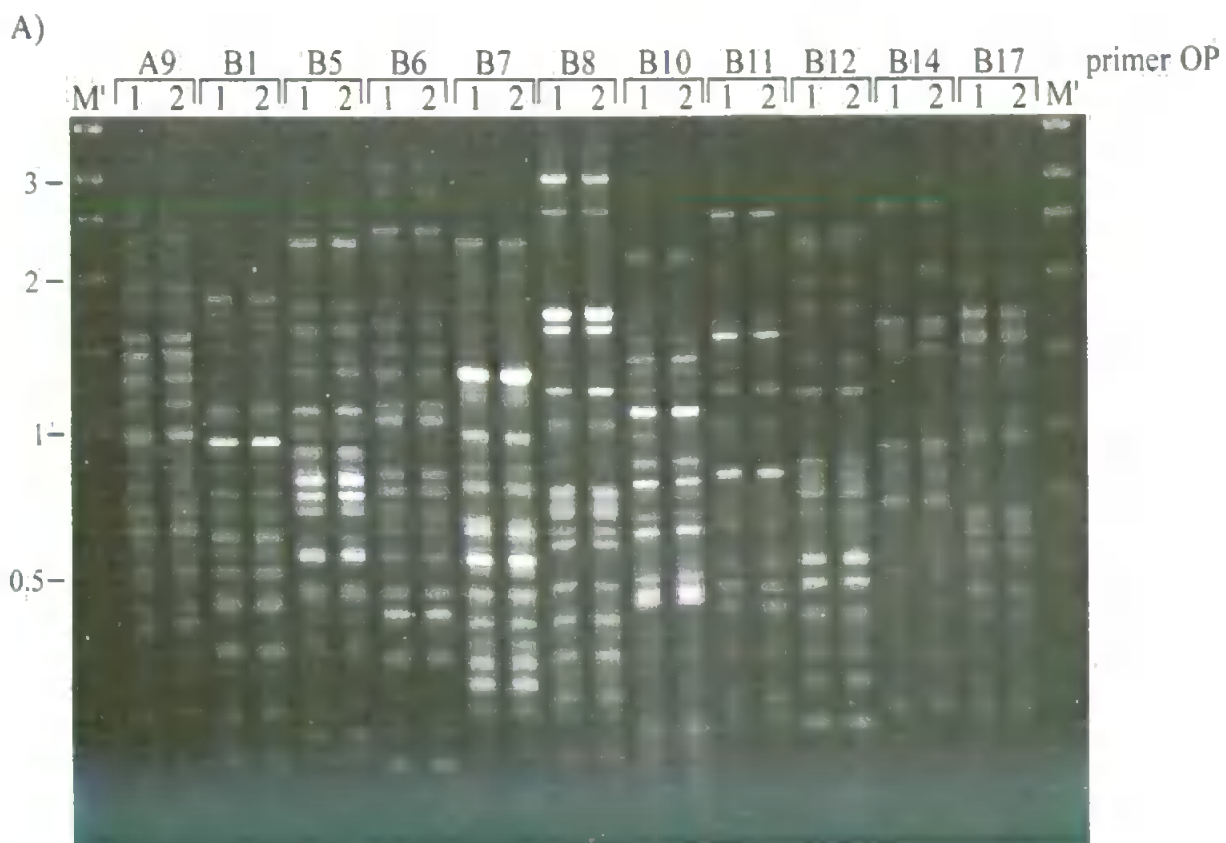
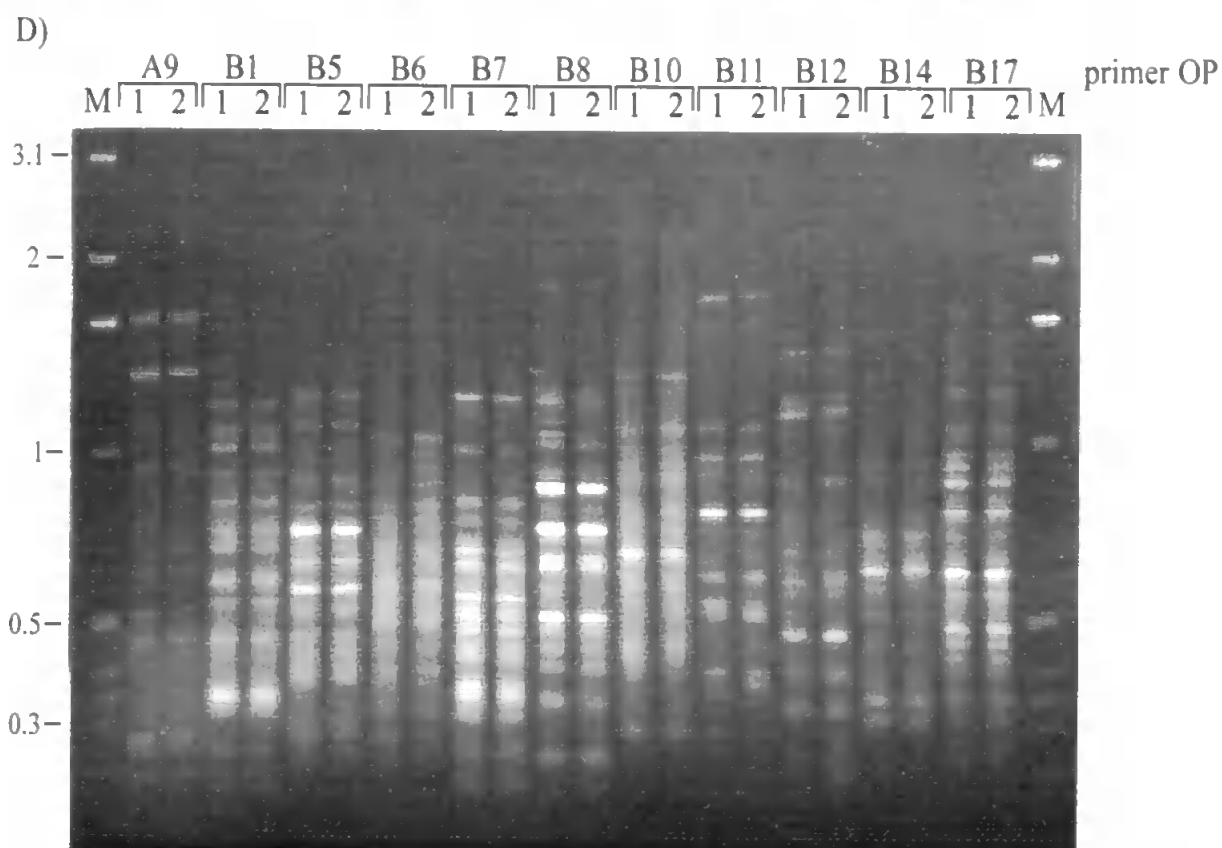
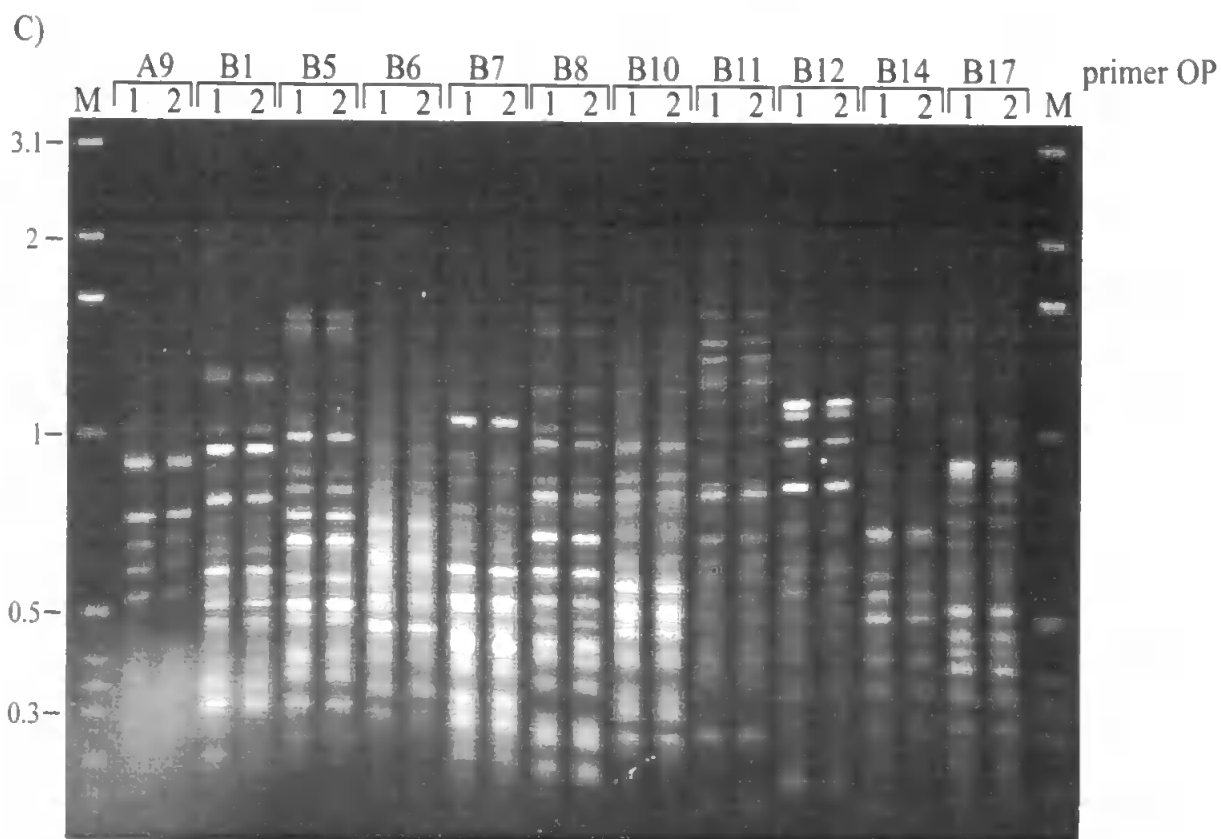


Figure 4.14 RAPD profiles of (A) *Daphnia magna*, (B) *Escherichia coli*, (C) calf thymus, and (D) human placenta. Primers as indicated at the top of each gel. Lanes 1 and



2: 20 and 5 ng DNA, respectively. M and M': 1 Kb DNA ladder from BRL and Igi, respectively. The molecular sizes (Kb) are indicated on the left of each gel.

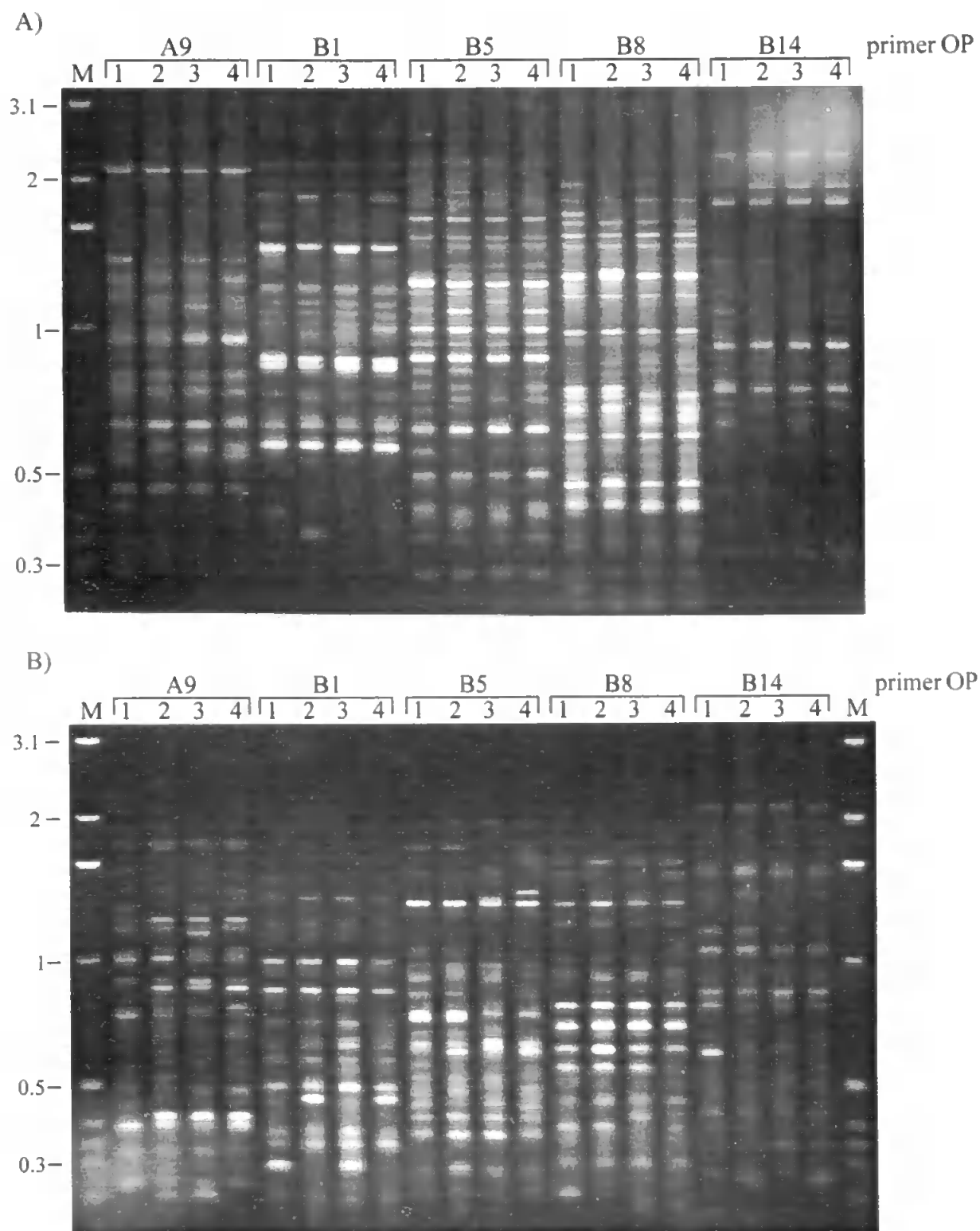
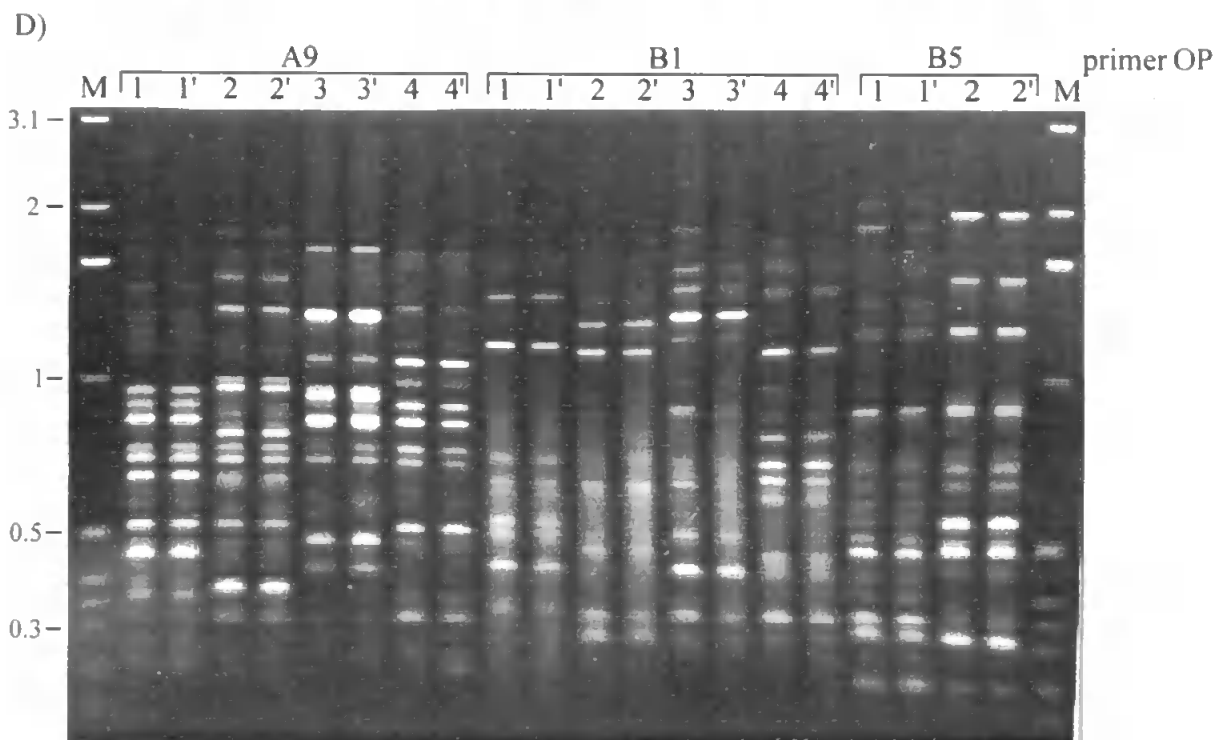
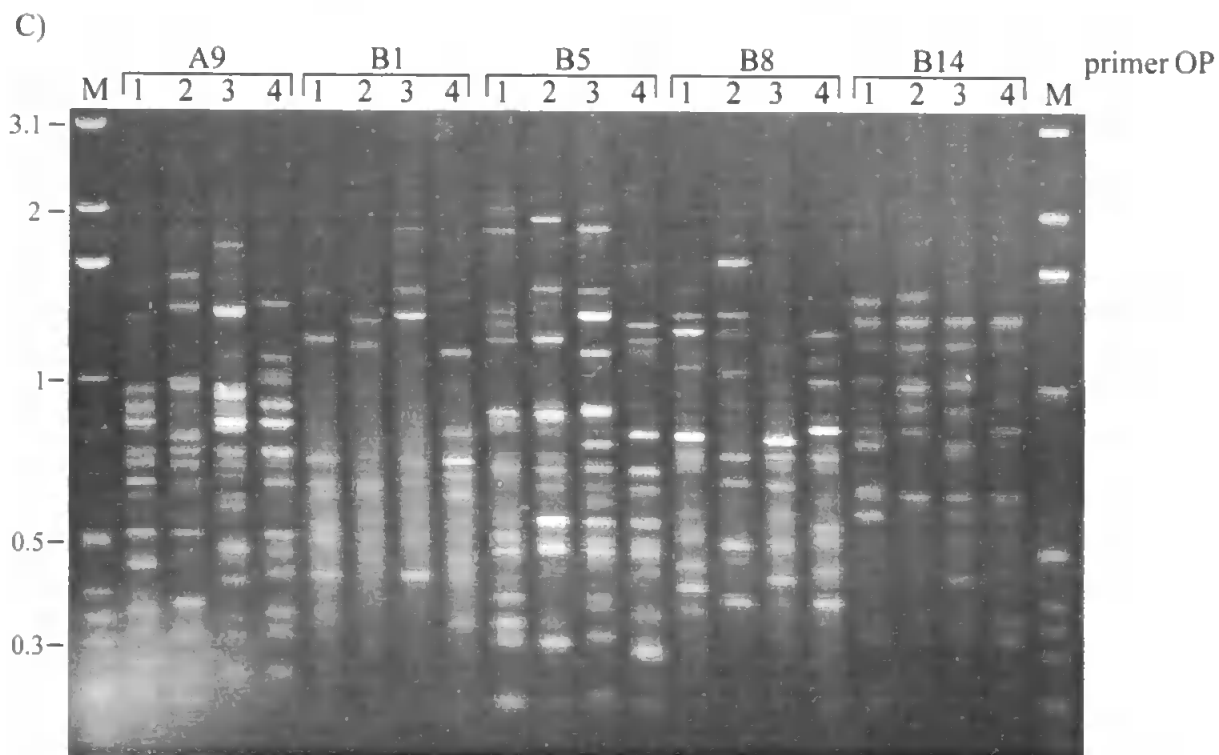


Figure 4.15 Reproducibility of RAPD profiles among 3 marine species. (A) *P. palmata*, (B) *P. dumerilii*, and (C) *M. edulis*. Primers as indicated at the top of each gel. M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left of each gel. Each number represents a single animal. (D) RAPD profiles reproducibility generated by the 4



individuals (*M. edulis*; used in Figure 4.15C) using 20 and 5 ng DNA. Lanes 1-4 and 1'-4': 20 and 5 ng genomic DNA, respectively. PCR reactions were performed under the optimised conditions (see section 3.4.1).

obtained with the red macroalgae *P. palmata* and the water flea *D. magna*. However, this was not the case when PCR reactions were performed using genomic DNA extracted from *M. edulis*. Indeed, a reproducible profile could not be obtained using the same oligonucleotide primers. Nevertheless, the fidelity of the PCR reaction conditions was confirmed by varying the template concentration, with comparable sample-specific profiles at DNA concentrations of both 5ng and 20 ng (Figure 4.15D). With regard to *P. dumerillii* (Figure 4.15B) the reproducibility was not perfect contrary to *P. palmata* (Figure 4.15A). However some of the differences in RAPD profiles obtained among the worms were shared whereas with *M. edulis* the RAPD profiles presented a high degree of variation.

4.3.7 Influence of time of DNA extraction and PCR on RAPD profiles

The results presented in Figure 4.16 and appendix III showed that RAPD profiles were identical whenever DNA extraction and PCR reactions were performed using the same sample. The only differences were due to a variation in the intensity of some bands.

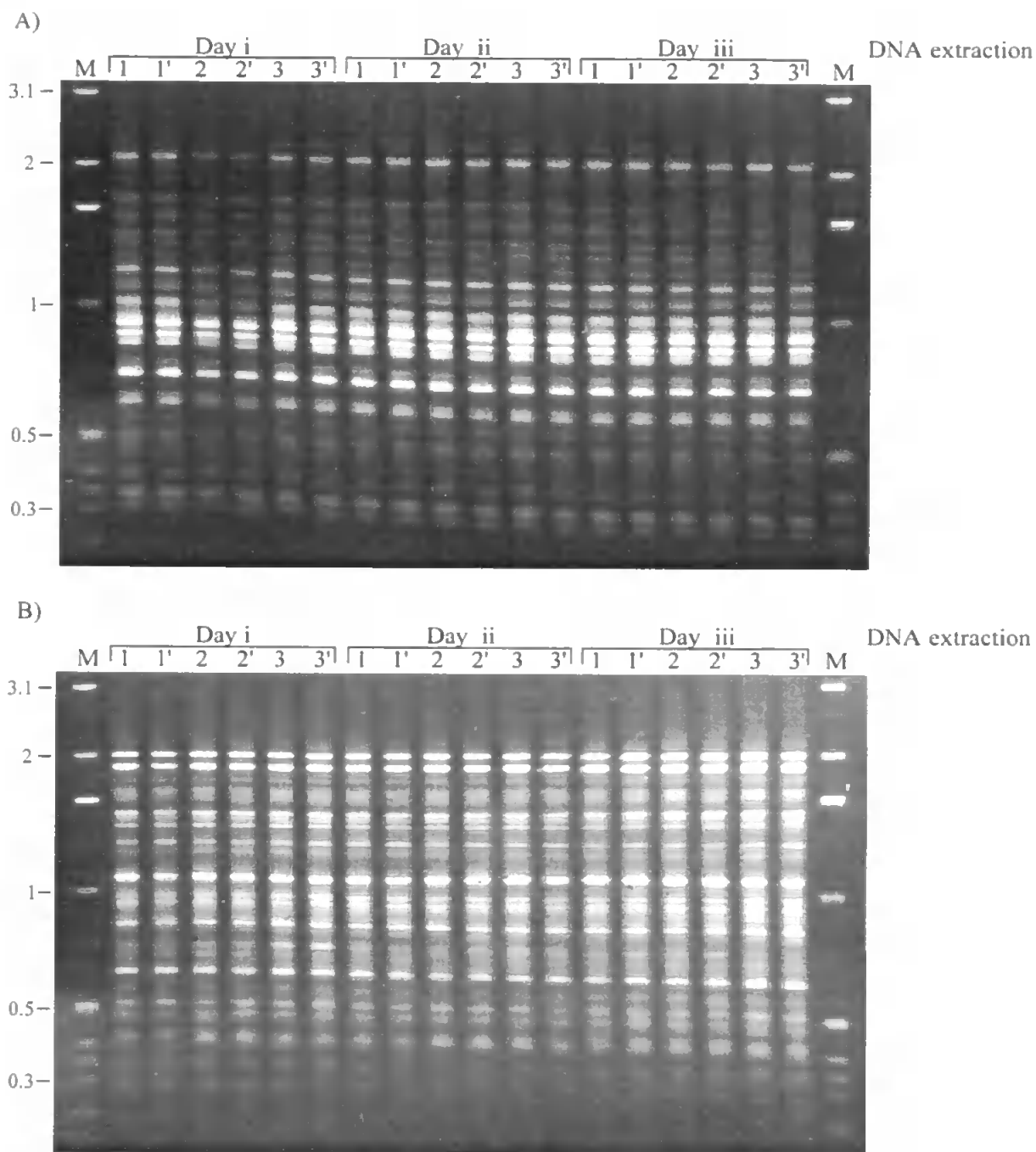


Figure 4.16 Effect of time of DNA extraction and PCR on RAPD profiles using *Daphnia magna* (A) and *Enteromorpha intestinalis* (B). DNA extraction was performed on day i, ii and iii as indicated on the top of each gel. Lanes 1, 2, 3 and 1', 2', 3' indicate that PCR reactions were performed on day iv and v, respectively. M: 1 Kb DNA ladder (BRL). The molecular sizes (kb) are indicated on the left of each gel. PCR reactions were performed under the optimised conditions (see section 3.4.1) using primer OPB5.

4.4 Discussion

The principal aim of this research project was to evaluate the potential of the RAPD technique to detect DNA damage and mutation. However before doing so, it was important to identify and overcome the potential problems associated with the technique. The arbitrary nature of randomly primed PCR methods has drawn criticism due to the formation of spurious amplifications (Ruano *et al.*, 1989; Mullis, 1991), affecting both the reproducibility (Khandka *et al.*, 1997; Ellsworth *et al.*, 1993) and the detection of Mendelian inheritance patterns (Ayliffe *et al.*, 1994; Riedy *et al.*, 1992; Scott *et al.*, 1992). Initially, when RAPD reactions were performed we encountered the problem of reproducibility in positive controls as well as the presence of amplicons in negative controls. It was initially thought that the pattern in the negative control was due to contamination of one of the reagents or the tubes, tips, pipettes, etc. Each of these were changed in turn but the problem still existed, suggesting that contamination was not the causative factor. This was confirmed by the fact that UV treated negative control displayed patterns whose complexity depended on the primer concentration. DNA exposed to high level of UV radiation cannot be amplified, due to the presence of DNA photoproducts such as thymine dimers which seriously hamper the processivity of DNA polymerases (Sarkar and Sommer, 1990 and Figure 4.3). In this way, contaminating DNA is prevented from acting as template for amplification. In addition, the major bands in negative controls displayed poor reproducibility in comparison to the profiles generated by positive controls. Thus, these results suggested that in the absence of DNA template spurious amplifications occurred as a consequence of non-specific reactions such as primer-mediated polymerisation.

The first objective of this study focused on determining the optimal conditions which are required for the generation of reproducible RAPD profiles from different

individuals of the same species. The parthenogenetically produced offspring of *D. magna* were chosen in order to eliminate the potential confusion caused by genomic changes due to sexual reproduction and also form a sound basis for the objective study of the key influences on assay reproducibility. The optimisation work was also performed using other species belonging to the bacterial, plant and animal kingdoms.

Despite having potential for many applications, the arbitrary nature of the randomly primed PCR methods has drawn criticism owing to the low stringency annealing conditions. Relatively low annealing temperatures (34-36°C) are used in RAPD to ensure a maximal number of primer binding events and consequent generation of a large number of amplified DNA fragments for analytical purposes. However, the low stringency of the accompanying DNA hybridisation can result in the formation of spurious amplifications (Ruano *et al.*, 1989; Mullis, 1991; Riedy *et al.*, 1992; Scott *et al.*, 1992; Ellsworth *et al.*, 1993; Ayliffe *et al.*, 1994; Khandka *et al.*, 1997). In this context, it was essential to significantly increase the stringency of the RAPD reactions by increasing step by step the annealing temperature and by using DMSO, a solvent which proved useful in this respect (Kidd and Ruano, 1995; Landre *et al.*, 1995). In the absence of DNA target, DMSO reduced the spurious amplifications. However, as it also decreased the performance of the PCR reactions in the presence of genomic template, DMSO could not be used in the reactions. The experiments presented in this chapter show that with selected primers, reproducible DNA profiles consisting of a large number of amplified fragments with a heterogeneous size range, can be produced at an annealing temperature of 50°C under optimised PCR conditions. This contrasts with the study of Williams *et al.* (1990) who reported that annealing temperatures above 40°C prevented amplification by 10-mer primers. It is also generally believed that the repeatability of the PCR assay is jeopardised by annealing temperatures higher than the denaturation temperature (T_d) of the oligonucleotide primer. T_d [known also as T_a (annealing temperature), or T_m (melting temperature)] may be calculated using the formula,

$T_d = (4 \times GC) + (2 \times AT) - 10^{\circ}\text{C}$, where GC and AT refer to the number of guanine plus cytosine and adenine plus thymine bases in the primer, respectively (Savva, 1996). Thus according to the formula, the highest applicable annealing temperature would be 30°C (i.e. $4 \times 10 + 2 \times 0 - 10$) for 10 mer-primers. The results of the present study do not support this statement. This formula which gives an estimation of the annealing temperature for longer primers (e.g. 20 bases) does not seem to be suitable for short primers. The purity and yield of the reaction products depend on several parameters, one of which is the annealing temperature. At both sub- and super-optimal annealing temperature, non-specific products may be formed, and the yield of products is reduced (Rychlik *et al.*, 1990). Other studies have also reported that RAPD profiles can be generated at high annealing temperature ($48-52^{\circ}\text{C}$) (Ellsworth *et al.*, 1993; Bentley and Bassam, 1996; Caetano-Anolles, 1998) for short primers. The main advantage of high stringency conditions is that nonspecific reactions are significantly reduced. Thus, protocols that use a high annealing temperature should always be preferred if the sensitivity of polymorphism detection is not jeopardised.

As clearly demonstrated by previous studies (Benter *et al.*, 1995; Newton and Graham, 1997), the influence of DNA purity on the success of PCR reactions was also evident in our experiments. DNA of good purity, free from other macromolecules and possible inhibitory compounds produced clear and discriminatory RAPD profiles. Indeed, the intensity of an amplified band depends on the efficiency of the interaction of the genomic sequence with the primer during the initial steps (Welsh *et al.*, 1995a). In this context, it is likely that when DNA is not properly purified, the proportion of cell components increase and interfere with the PCR events (e.g. annealing of the primers) and reagents (e.g. primers and *Taq* DNA polymerase). For the bulk of our experiments, genomic DNA prepared by a standard phenol/chloroform extraction purification was sufficient to produce RAPD profiles of good quality. Identical patterns were obtained whether DNA was highly purified (caesium chloride treated DNA) or not (phenol/chloroform extracted DNA).

When PCR is performed to amplify a single copy of genomic DNA, it is not always necessary to extract and purify the genomic DNA (Mercier *et al.*, 1990; McEwan and Wheeler, 1995). However as RAPD amplifies multiple loci, it is generally recommended to carefully extract DNA although RAPD profiles can also be obtained without purification of the genomic DNA (Mazurier *et al.*, 1992).

The concentration of DNA template is also crucial in the production of reproducible genomic profiles, not only to ensure the largest number of amplified bands and therefore maximum discrimination, but also to confirm the fidelity of the PCR reaction condition. If profiles from the same genomic template, or from different individuals of the same species vary, the reproducibility of the assay should be confirmed by repeating the PCR reaction using 2 different template concentrations, differing by at least two fold. If profiles still vary, then the results should always be treated with skepticism. This safeguard against concentration dependent spurious amplifications was highlighted by Welsh *et al.* (1995a), but unfortunately has not been adopted by many workers using the RAPD technique. In the present study, *M. edulis* genomic DNA extracted from different individuals and subjected to RAPD, produced non reproducible profiles although sample-specific patterns (i.e. without artefacts) were clearly generated. The non reproducibility between different *M. edulis* individuals could be attributed either to a true genetic diversity within this species or to a contamination with foreign DNA (e.g. the presence of different strains of bacteria in the gill tissue). Our optimal range for genomic DNA (0.05-100 ng) is generally in agreement with other studies (Benter *et al.*, 1995; Bentley and Bassam, 1996; Caetano-Anolles, 1998). However, it is important to recognise that variations in the estimation of the DNA concentration of the same sample by two experimenters may also be an important factor depending on the method used.

The priming oligonucleotide is obviously crucial to the success of any RAPD protocol, and our experiments support the judicious choice of primer for each application

and DNA template. Nevertheless, our set of 11 primers, have been successfully applied to RAPD profiling of each genomic DNA template we have studied, covering the bacterial, plant and animal kingdoms. In this context, this set of primers shows the potential to generate RAPD profiles with any genomic DNA of sufficient quality. Khandka *et al.* (1997) reported that the optimal concentration of every primer should be determined empirically for each application. This study does not support this statement as RAPD profiles of high quality were obtained using different primers and species, under the same conditions.

Experiments to assess the influence of each PCR reagent on RAPD profiles revealed that consistent profiles were generated when component concentrations were subjected to variation (within the predefined optimal conditions). In contrast, artefactual variation has often been described when RAPD reactions were performed at low annealing temperatures (Khandka *et al.*, 1997; Ellsworth *et al.*, 1993) although the experimenters did not use two different DNA concentrations to prove that the changes in banding patterns were indeed artefacts (Welsh *et al.*, 1995a). With regard to the magnesium concentration, it has been suggested that a relatively low magnesium ion concentration prevents undesirable annealing events within the PCR (Wilkie *et al.*, 1993). Our results however, showing satisfactory reproducibility with Mg^{++} concentrations between 3-6 mM, support other studies which recommend the use of higher concentrations to improve the stability of the results (Benter *et al.*, 1995; Bentley and Bassam, 1996). It was also reported that 2.5 units of *Taq* DNA polymerase gave more reproducible results compared to low concentrations (e.g. 1 unit) (Benter *et al.*, 1995). The results presented in this chapter led us to the same conclusion.

In addition, one of our major goals was to minimise the presence of spurious amplifications (primer polymerisation) generated in the absence of template DNA (Ruano *et al.*, 1989; Mullis, 1991). By varying the dNTP concentrations between otherwise

identical reaction mixtures, it was shown that 'dirty' negative controls could be greatly reduced. A dNTP concentration of 0.33 mM produced a large number of bands in reactions containing template DNA with a complete or partial reduction in spurious amplifications in those reactions without genomic DNA templates. Since the complexity of the RAPD profiles (in positive control) was affected when the dNTP concentration was increased, it is likely that dNTP interfere between the 10-mer primers like a physical barrier. When the RAPD methodology is used, negative controls are not always presented in the literature probably because of the presence of products in reactions containing no target DNA. The thermodynamic driving force for PCR is the molar excess of reagents with respect to template. This situation leads to the formation of primer dimers and other artefacts which consume the stock of primers and enzyme with a consequent reduction in the yield of the appropriate products. Although methods such as the Booster PCR (Ruano *et al.*, 1991) and the Hot-StartTM PCR (Erlich *et al.*, 1991) have been developed to tackle these problems, these procedures are not convenient when PCR reactions are performed with a large number of samples. For instance, since the ultimate manipulation is performed within the heating block of the thermocycler, there is a considerable risk of sample contamination.

In many studies researchers reported that some PCR parameters (e.g. magnesium concentration and annealing temperature) should be optimised for each species and primer combination separately (Wolff *et al.*, 1993). In the presented study, our results suggested that there was no need to have different PCR conditions for each species and primer combination as RAPD profiles of satisfactory quality were generated using different species and 10-mer primers. Furthermore, it was also clear that identical RAPD profiles were obtained whenever the DNA extraction or PCR were performed. In another study, it was reported that DNA extractions at different times and amplification of the same DNA on different days produced minor bands that varied in intensity (Benter *et al.*, 1995).

In conclusion, the RAPD protocol was found to generate high quality genomic DNA profiles from phylogenetically different groups of organisms (bacteria, plants and animals) using the same set of primers. Since RAPD reactions are performed at a high annealing temperature, spurious amplifications are kept to a minimum and consistent profiles are generated when component concentrations are within the predefined optimal range, contrary to PCR reactions performed at low annealing temperature. Thus after rigorous optimisation, the RAPD method appears to be a robust assay. In this context, the evaluation of the potential of the RAPD method to detect toxicant-induced DNA effects in aquatic organisms could begin.

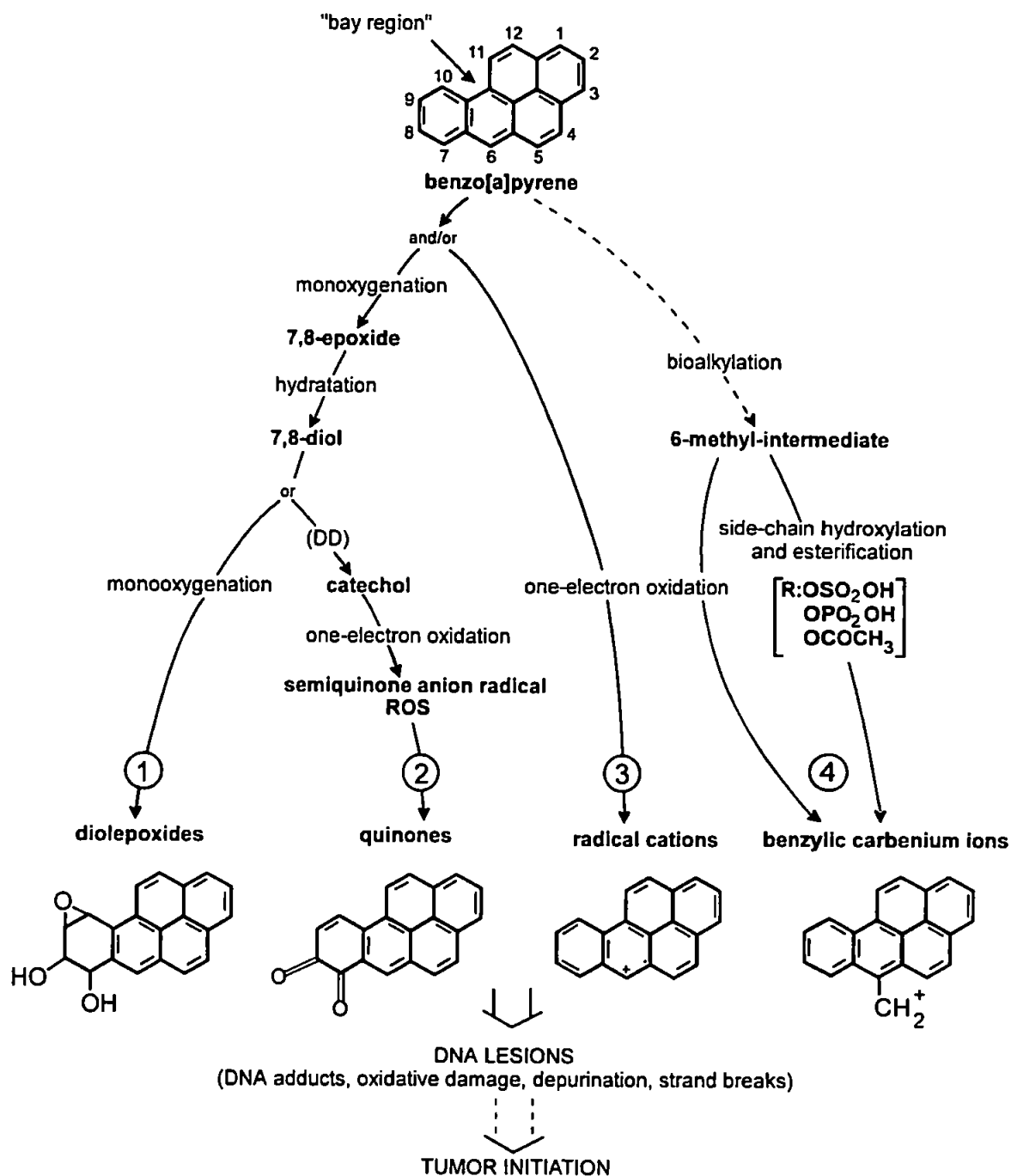
Chapter 5

QUALITATIVE ASSESSMENT OF DNA EFFECTS USING RAPD: COMPARISON OF GENOMIC TEMPLATE STABILITY WITH KEY FITNESS PARAMETERS IN *DAPHNIA MAGNA* EXPOSED TO BENZO(A)PYRENE

5.1 Introduction

Following the optimisation experiments (chapter 4), the potential of the RAPD method to detect DNA effects in aquatic organisms was evaluated. DNA effects include DNA damage (e.g. DNA adducts, DNA breaks) as well as mutations (point mutations and large rearrangements) and possibly other effects (e.g. structural effects) at the DNA level that can be induced by chemical or physical agents that directly and/or indirectly interact with genomic DNA. In this chapter, the effects of benzo(a)pyrene [B(a)P] were investigated because B(a)P is known to be carcinogenic to animals. In addition, the effects of B(a)P on DNA are well documented, and the presence of this chemical is ubiquitous in the environment which makes this study ecotoxicologically relevant.

Polycyclic aromatic hydrocarbons (PAHs) are formed by incomplete combustion of organic materials and are widespread in the atmosphere. PAHs are one of the most ubiquitous carcinogenic, mutagenic and toxic contaminants found in aquatic systems (Kennish, 1992). Exposure to PAHs is virtually unavoidable, and they are strongly suspected of being a causative factor in several human cancers (Phillips, 1983). Among PAHs, B(a)P is one of the most studied. B(a)P can be converted into toxic metabolites which have been shown to induce different kind of DNA lesions such as DNA adducts (Venier and Canova, 1996), micronuclei (Venier *et al.*, 1997), strand breaks (Mitchelmore *et al.*, 1998), chromosomal aberrations (Matsuoka *et al.*, 1998), oxidative damage (Canova *et al.*, 1998) and mutations (Rodriguez and Loechler, 1993). B(a)P is also known to create adducts on proteins such as hemoglobin (Shugart *et al.*, 1987). Four different pathways have been proposed for the metabolic activation of B(a)P (Figure 5.1). The most widely accepted activation mechanism (pathway 1) is based on ring oxidation to diol epoxides. In particular, the (+)-anti-B(a)P-7,8-diol-9,10 epoxide forms stable DNA adducts whose mutagenicity and carcinogenicity have been widely studied (Buening *et al.*, 1978; Wei *et*



DD: dihydrodiol dehydrogenase; ROS: reactive oxygen species

Figure 5.1. Possible pathways for activation of B(a)P leading to DNA lesions *in vivo*. Reprinted from *Mutation Research*, volume 399, Canova *et al.*, 1998, Tissue dose, DNA adducts, oxidative DNA damage and cyp 1a-immunopositive proteins in mussels exposed to waterborne benzo(a)pyrene, pages 17-30, with permission from Elsevier Science.

al., 1995). Pathway 1 also leads to the production of phenols, diols and tetrols (Sims *et al.*, 1974). Dehydrogenation of 7,8-diols mediated by the dihydrodiol dehydrogenase can divert diols from pathway 1 to yield transient catechols, quinones and quinone redox cycling (pathway 2). Free radicals which are produced through pathway 2 account for oxidative damage and DNA fragmentation (Devanesan *et al.*, 1996; Flowers *et al.*, 1996). Pathway 3 involves the formation of radical cation, possibly mediated by the cytochrome P450 or by peroxidative reactions (Cavalieri and Rogan, 1995). Finally, pathway 4 leads to the formation of the ultimate DNA-reactive metabolite, benzylic carbenium ion due to endogenous methylation of B(a)P (Stansbury *et al.*, 1994).

The objective of this study was to evaluate the potential of the RAPD technique to detect B(a)P-induced DNA effects in *D. magna*, and to compare any changes in RAPD profiles induced by exposure to B(a)P with fitness and Darwinian parameters.

5.2 Materials and methods

5.2.1 Culture of *Daphnia magna*

D. magna (clone 5) was cultured in M7 medium at $20 \pm 2^\circ\text{C}$ as described in section 3.1.1.

5.2.2 Preparation of test solutions and toxicity tests

Test solutions for chronic exposures were prepared from stock solutions of B(a)P at a concentration of 2 mg/ml in dimethylformamide (DMF). Once prepared, test solutions were stored at 4°C prior to use. Both B(a)P solutions, and the DMF solvent controls were added to the *D. magna* culture media in a volume of 100 μl of DMF in 1 L of medium ensuring a level below the 0.05 % maximum percentage of the solvent recommended by the American Society for Testing Materials (ASTM, 1975).

5.2.2.1 Acute toxicity

The acute toxicity of B(a)P was assessed by determining the LC50 of the chemical for *D. magna* over a period of 48 h. Freshly born neonates (less than 48 h) were exposed in replicate groups of 20, to concentrations of B(a)P equivalent to 0.1, 0.15, 0.2, 0.25 and 0.5 mgL⁻¹. Animals were fed (see section 3.1.1) during the test, and surviving animals were counted to determine the 48h-LC50.

5.2.2.2 Chronic toxicity

The chronic toxicity test of B(a)P to *D. magna* was performed under the same experimental conditions as for the acute toxicity tests, with a blank control, DMF solvent control and B(a)P concentrations of 0.0125, 0.025, 0.05, 0.1, and 0.2 mgL⁻¹. The experiment lasted 14 d. Surviving animals were counted at 0, 3, 5, 7, 10 and 14 d. Moribund, non-swimming (but alive) animals were removed from culture at regular intervals on and between counting d. These animals and the healthy *D. magna* following B(a)P exposure for 14 d were placed individually in 1.5 ml microcentrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C, prior to DNA extraction and RAPD profiling.

5.2.3 Growth and reproductive measurements

The length of *D. magna* (apex to base) surviving at day 0, 3, 5, 7, 10 and 14 was measured by video capture and image analysis using a Quantimet 570 image analyser (Cambridge Instruments, Cambridge, UK) and neonates were counted and recovered at daily intervals (see section 3.6.1).

5.2.4 Calculation of fitness parameters

The fitness parameters measured were the intrinsic rate of natural increase (r_m), the age-specific survival (l_x), the age-specific fecundity (m_x), and the net reproductive rate (R_0). Minimum generation time (T_{min}) and the inter-brood time (Bt) were also measured

(see section 3.6.2).

5.2.5 Generation of *Daphnia magna* DNA profiles using RAPD

Total DNA from *D. magna* was extracted and purified using either a conventional phenol/chloroform method (section 3.2.1.1) or a commercially available extraction kit (see section 3.2.1.2). Two methods were used because traces of organic compounds may inhibit PCR reactions, and one of the methods could have failed to produce DNA of sufficient quality to obtain robust RAPD profiles. Furthermore, both protocols of DNA extraction proved to generate similar RAPD patterns (see Figure 4.7). The extracted DNA was electrophoresed in 1.2 % agarose gels at 100 V for 2 h and the DNA concentration of unknown samples was determined by comparison with known amounts of Lambda phage DNA (see section 3.3.1). RAPD profiles were generated using 10-mer primers OPA9 and OPB7 (see section 3.4.2) under the optimised conditions as described in section 3.4.1. PCR products were then electrophoresed in 1.2 % agarose gel at 90 V for 6 h, stained with ethidium bromide and visualised under UV light. A photograph was also taken and the image of the gel was saved in the computer for analysis when necessary (see section 3.5).

5.2.6 Estimation of genomic template stability and transformation of the data

Genomic template stability (GTS) represents a qualitative estimation of the obvious changes occurring in RAPD profiles and a qualitative estimation of the DNA effects. For more details please refer to section 3.6.3 and appendix II. To compare the sensitivity of each parameter (GTS, l_x , m_x , r_m and R_o), changes in these values were calculated as a percentage of their control value (set to 100 %).

5.2.7 Statistical analyses

For more details see section 3.6.5.

5.3 Results

5.3.1 Acute toxicity tests

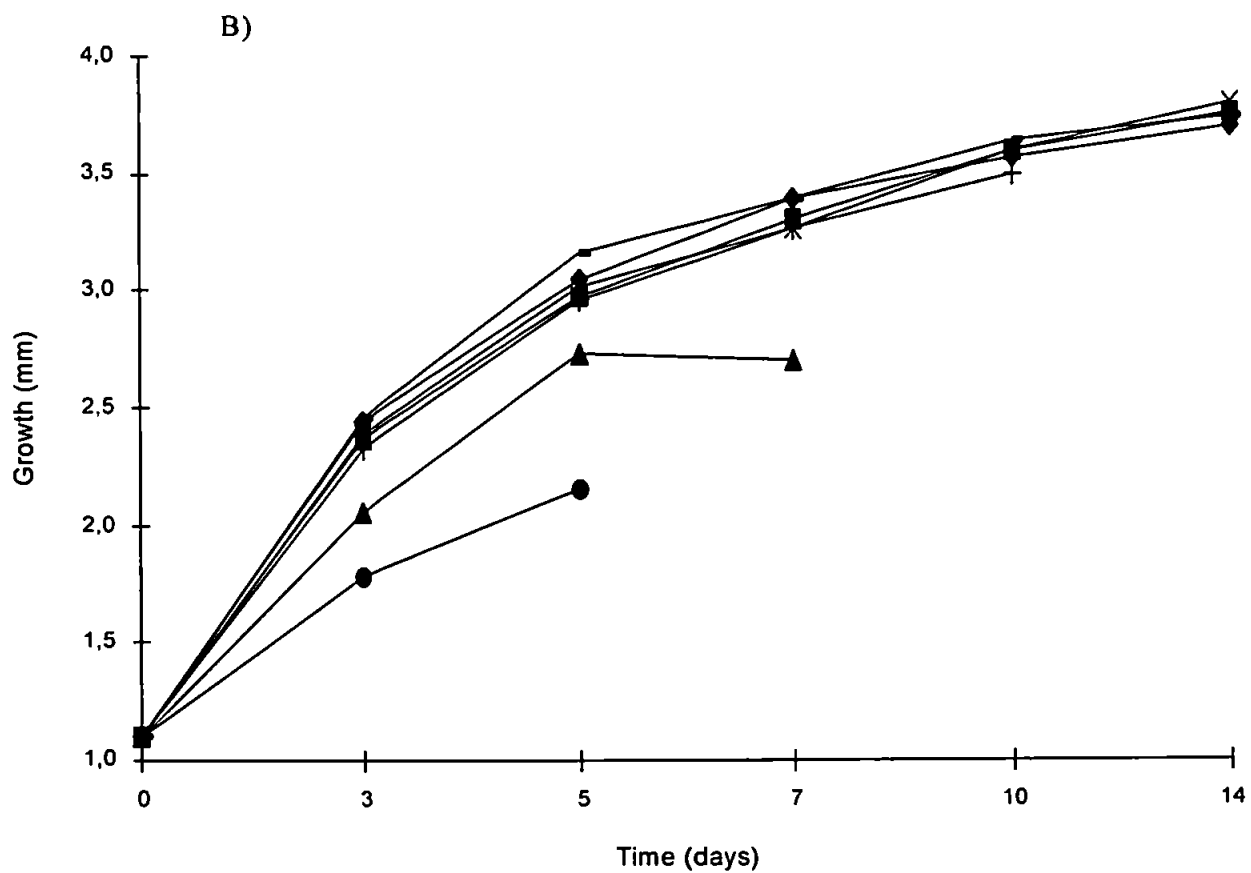
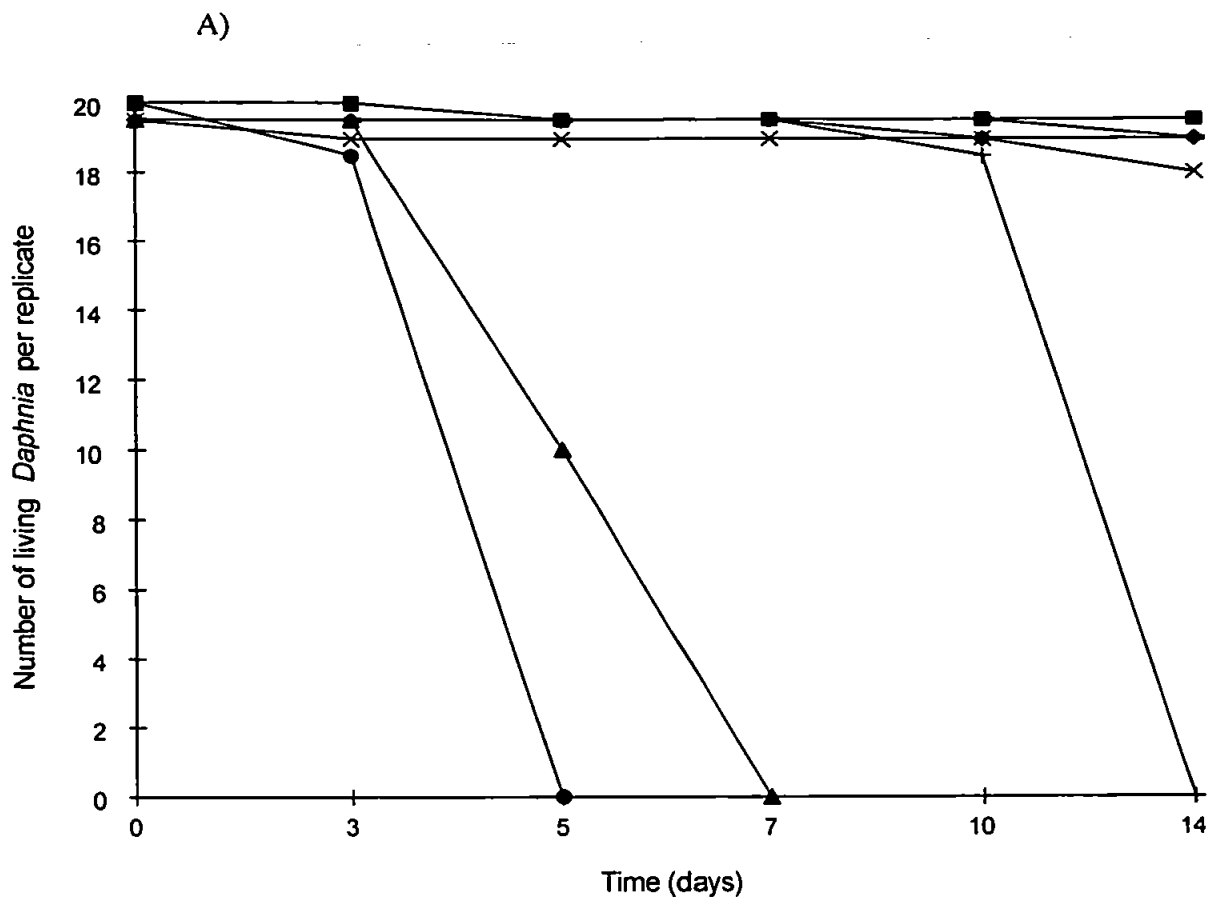
Using survivorship data, the 48h-LC50 of B(a)P for *D. magna* was 0.25 ± 0.04 mgL⁻¹ B(a)P ($\chi^2 = 63.6$, $p < 0.005$).

5.3.2 Effects at population levels

Demographic trends for the populations of *D. magna* exposed to differing concentrations of B(a)P are shown in Table 5.1. From these results, it can be seen that, with increases in toxicant concentration in the range 0.025-0.2 mgL⁻¹ B(a)P, consistent reductions occurred in the maximum number of eggs per female, maximum body size, and number of broods. However, the mean period for *D. magna* to become ovigerous was identical among groups. The data also indicate that *D. magna* exposed to gradual increases in B(a)P concentrations displayed a lengthened time between the first and second brood. The number of living organisms was significantly reduced at the highest B(a)P concentrations; for instance, organisms could not survive more than 5, 7 and 14 d at 0.2, 0.1 and 0.05 mgL⁻¹ B(a)P, respectively (Figure 5.2A). In contrast, *D. magna* from the other groups [i.e. control, control + DMF, 0.0125, and 0.0025 mgL⁻¹ B(a)P] survived throughout the exposure period. Although both populations growing at 0.1 and 0.2 mgL⁻¹ had a smaller body size than controls ($p < 0.001$), there were no significant differences between the control groups and the other B(a)P exposed groups (Figure 5.2B). In addition, the size of sub-24 h juveniles (first generation) was identical between the following groups control, solvent control, 0.0125, 0.025, and 0.05 mgL⁻¹ B(a)P ($p < 0.05$, appendix III). The length of the neonates generated by the population of *D. magna* exposed to 0.1 mgL⁻¹ B(a)P was not determined. Finally, the total number of offspring was significantly reduced at 0.025 mgL⁻¹ and higher B(a)P concentrations (Figure 5.2C and Table 5.1).

B(a)P concentrations (mgL ⁻¹)	Mean period to become ovigerous (days)	T _{min} (days)	Maximum body size (mm)	Maximum number of eggs/female	Number of broods	Inter brood period (days)	Total number of neonates	Age specific survival (I _x)	Age specific fecundity (m _x)	Net productive rate (R ₀)	Intrinsic rate of natural increase (r _m)
Blank Control	5±0.00	6	4.03	12	3±0.00	3	826.5±71.4	5.97±0.04	42.45±1.97	42.35±2.13	0.59±0.22
DMF Control	5±0.00	6	3.89	12	3±0.00	3	921±101.8	5.95±0.07	47.55±2.97	47.17±3.51	0.66±0.21
0.0125	5±0.00	6	3.9	12	3±0.00	3.5	861.6±133.6	5.90±0.14	43.78±5.69	43.07±6.68	0.78±0.16
0.025	5±0.00	7	4.03	11	2.5±0.49	4	499±22.6	5.82±0.11	26.48±2.39	25.63±2.09	0.45±0.15
0.05	5.1±0.01	6	3.67	11	2±0.00	4	227±45.3	4.95±0.07	11.66±1.97	11.61±1.90	0.33±0.04
0.1	5.31±0.00	7	3.16	9	1±0.00	-	40.5±6.4	2.50±0.63	2.08±0.40	2.08±0.40	0.06±0.01
0.2	-	-	2.44	5	0	-	0	1.92±0.03	0	0	0

Table 5.1 Demographic trends of *Daphnia magna* populations under various B(a)P treatments. Values which represent mean \pm SD were calculated using both replicates (i.e. 40 *D. magna* per concentration). All parameters were determined throughout the experiment until *D. magna* were unable to swim properly. Parameters such as minimum generation time (T_{min}), age-specific survival (l_x), age-specific fecundity (m_x), net reproductive rate (R_0) and intrinsic rate of natural increase (r_m) are described in section 3.6.2. Parameters referring to reproduction could not always be calculated for the 2 highest B(a)P concentrations (i.e. when number of broods < 2).



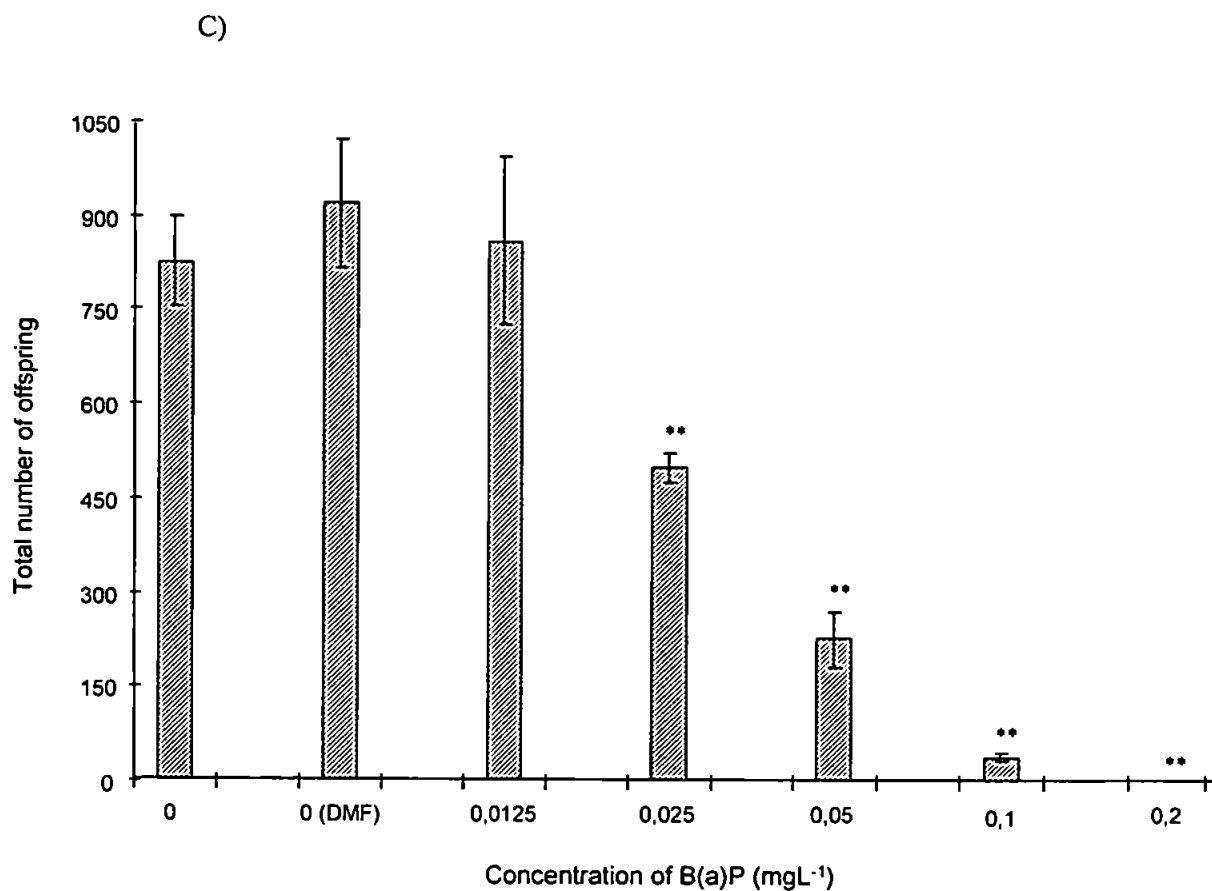


Figure 5.2 Variation in (A) number of living *Daphnia magna* per replicate, (B) growth, and (C) total number of offspring in populations of *Daphnia magna* exposed to B(a)P. —■— 0 (control), —◆— 0 (solvent control), —■— 0.0125, —×— 0.025, —+— 0.05, —▲— 0.1, and —●— 0.2 mgL⁻¹ B(a)P. ** indicate a significant difference from control ($p < 0.01$).

B(a)P induced some phenotypic changes in *D. magna*. Figure 5.3 shows that the possible malformation of eggs of one animal exposed to 0.05 mgL⁻¹ B(a)P for 10 days. In addition, the exposed *D. magna* seemed to be darker compared to the control animal.

5.3.3 Changes in fitness parameters

Alterations to the key fitness parameters; age-specific survival (l_x), age-specific fecundity (m_x), net reproductive rate (R_0) and intrinsic rate of population increase (r_m) are presented in Table 5.1 and Figure 5.6, before and after transformation respectively (see section 3.6.4). *D. magna* exposed to concentrations higher than 0.05 mgL⁻¹ exhibited reduced life-spans (l_x) compared to the controls. Age-specific fecundity (m_x) also gradually decreased when the population of *D. magna* was exposed to 0.05 mgL⁻¹ and higher B(a)P concentrations. The intrinsic rate of natural increase and the net productive rate were shown to be significantly reduced by increasing B(a)P concentrations above 0.0125 mgL⁻¹ ($p < 0.001$). R_0 and m_x appeared to be the most sensitive fitness parameters, as r_m was calculated to be only significantly different at 0.1 and 0.2 mgL⁻¹ B(a)P. None of the fitness parameters appeared to be altered at the lowest hydrocarbon concentration.

5.3.4 RAPD profiling

DNA amplified was extracted from moribund, non-swimming (but alive) organisms after 14, 7 and 5 days at 0.05, 0.1 and 0.2 mgL⁻¹ B(a)P, respectively. For the other groups [i.e. control, control + DMF, 0.0125, and 0.025 mgL⁻¹ B(a)P], healthy organisms were sacrificed after 14 days. RAPD profiles produced by 10-mer primers OPA9 and OPB7 are shown in Figure 5.4 and 5.5, respectively. The patterns show significant differences between unexposed and exposed individuals, with visible changes in the number and size of amplified DNA fragments, and both increases and decreases of DNA band intensities.

A)



B)

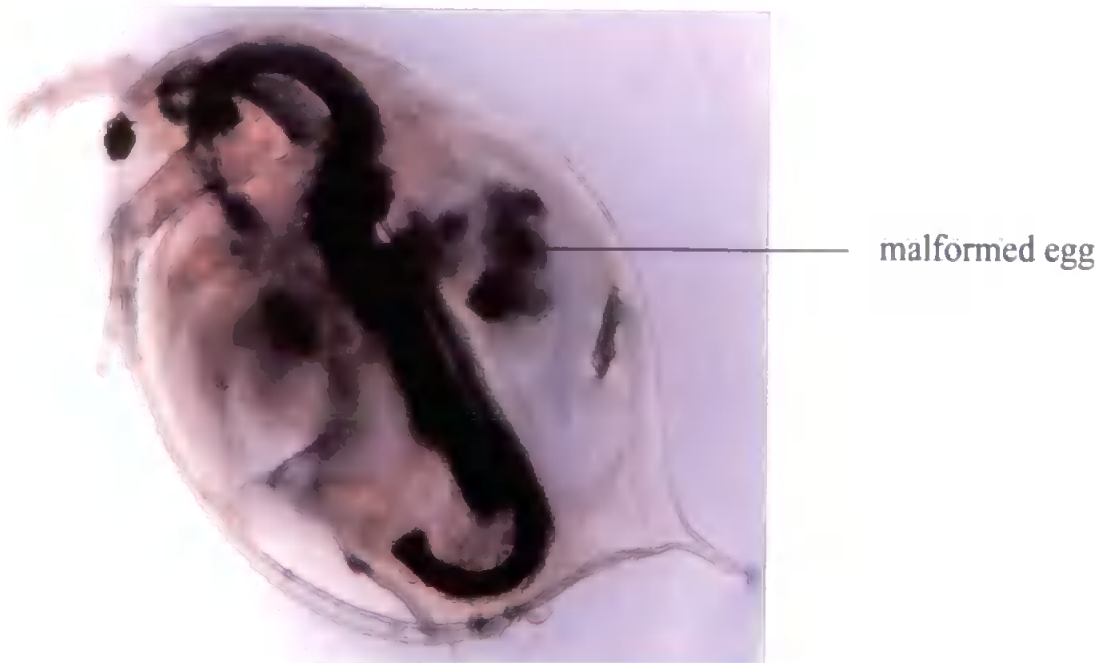


Figure 5.3 Phenotypic effects in *Daphnia magna* exposed to B(a)P. A) control *D. magna* after 10 days, B) *D. magna* exposed to 0.05 mgL⁻¹ for 10 days. Scale (A and B):

0.4 mm

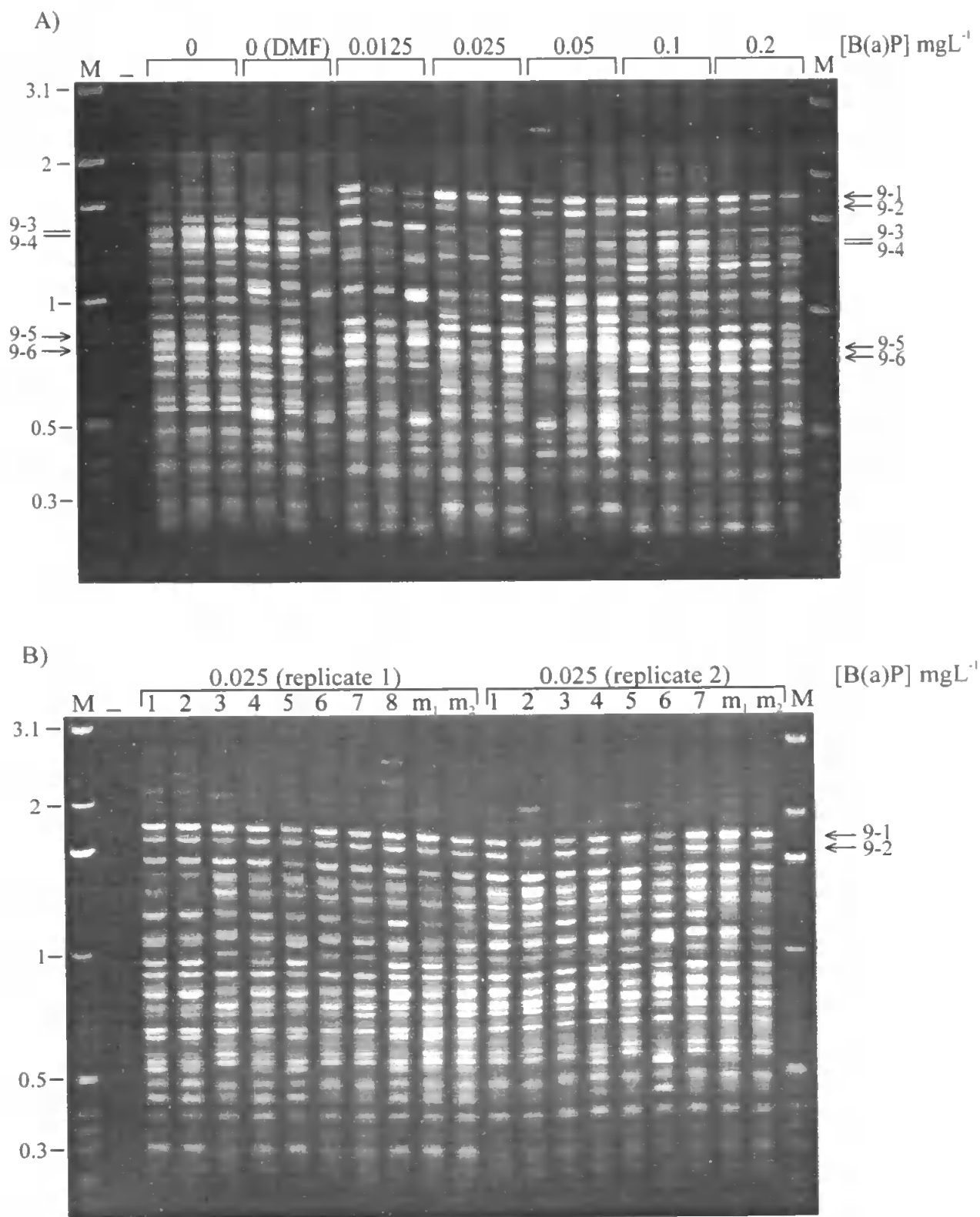
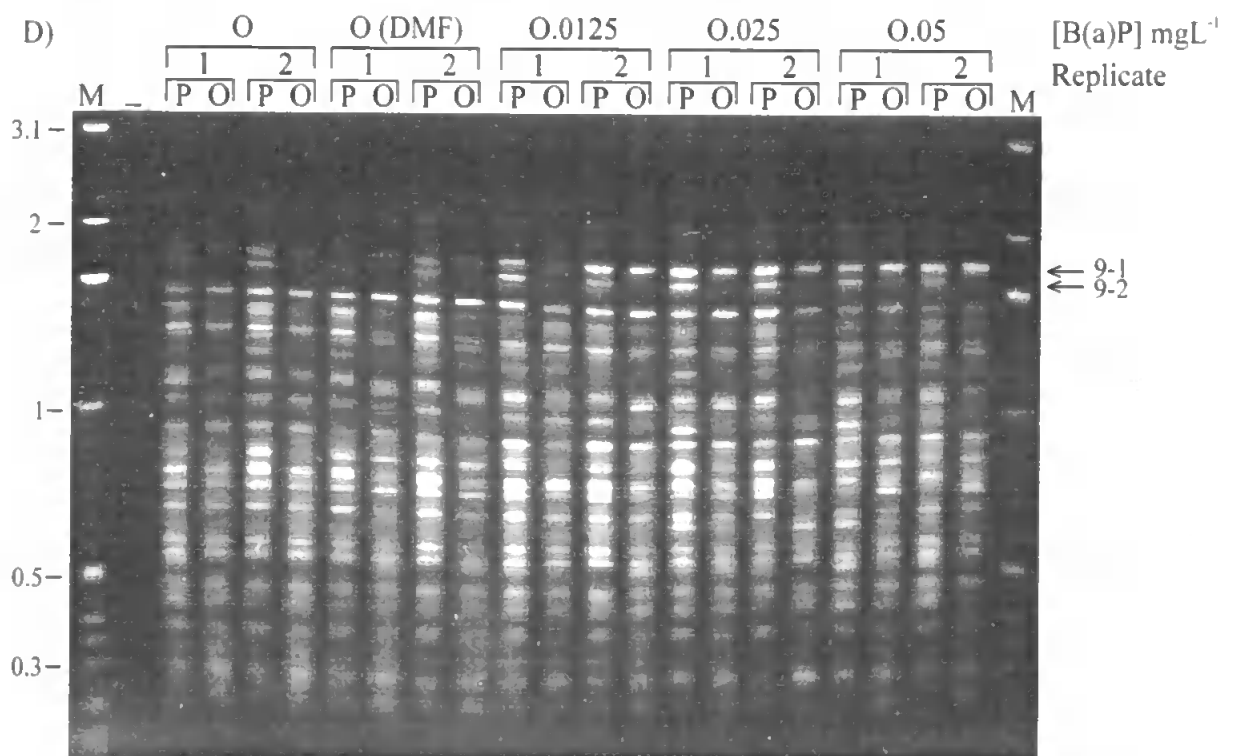
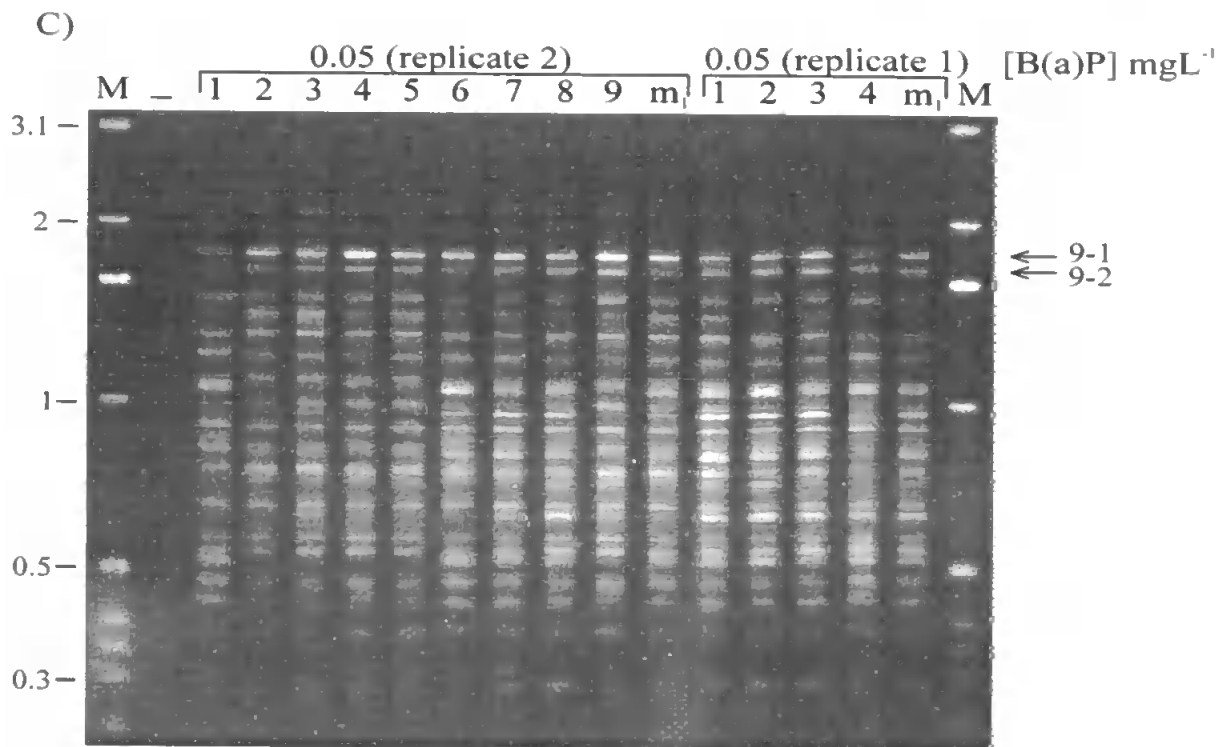


Figure 5.4 RAPD profiles of *Daphnia magna* exposed to B(a)P. A) Patterns generated by 3 individuals of each concentration (replicate 1). B) and C) Reproducibility of bands 9-1 and 9-2 from *D. magna* exposed to 0.025 and 0.05 mgL⁻¹, respectively. The numbers and 'm' represent individual animals and a mixture of 4 *D. magna*, respectively. D) RAPD profiles



generated from parent and offspring (last generation) in both replicates. P = parent (4 individuals), O = offspring (20-30 neonates). M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left. -: no DNA control. PCR reactions were performed using primer OPA9.

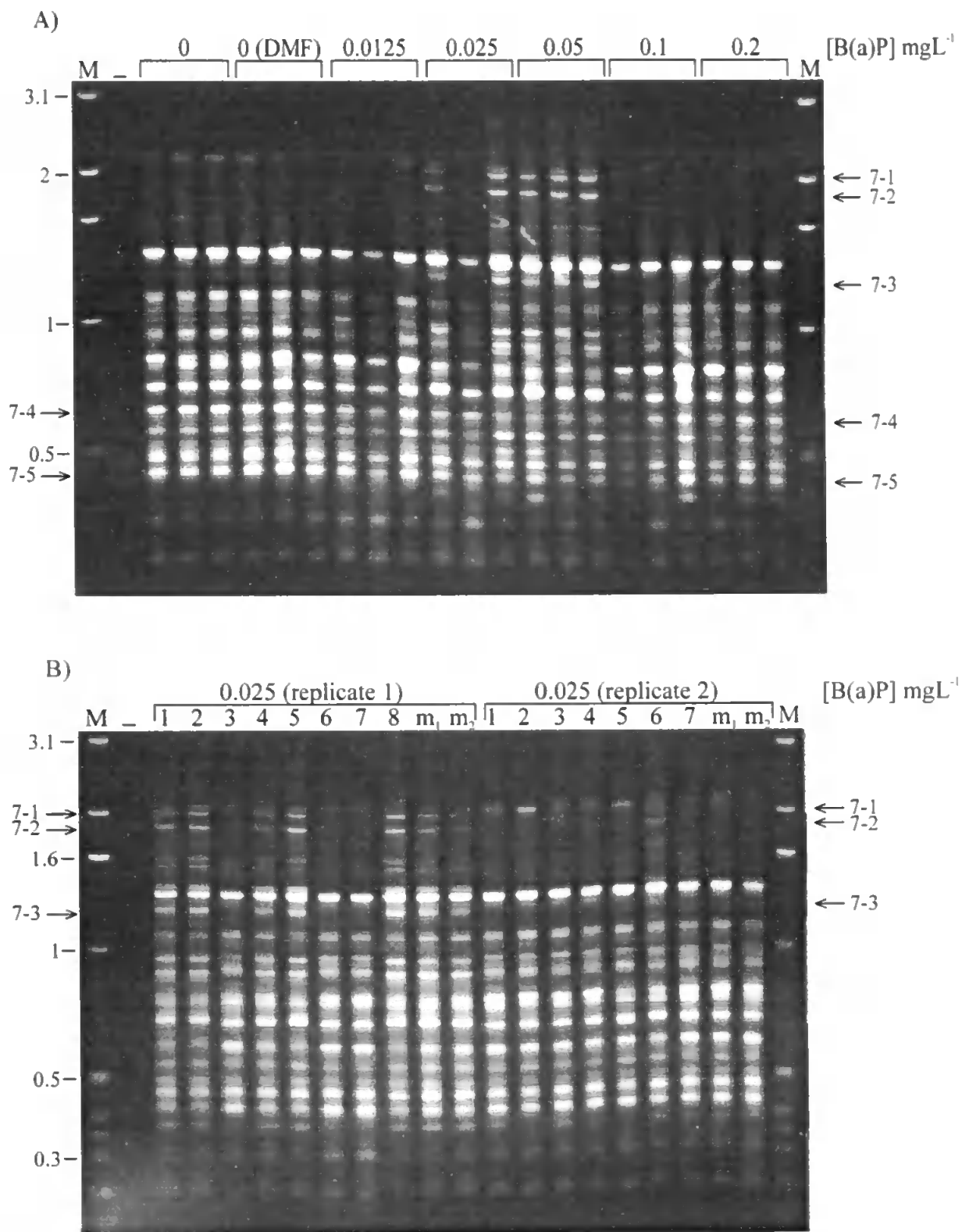
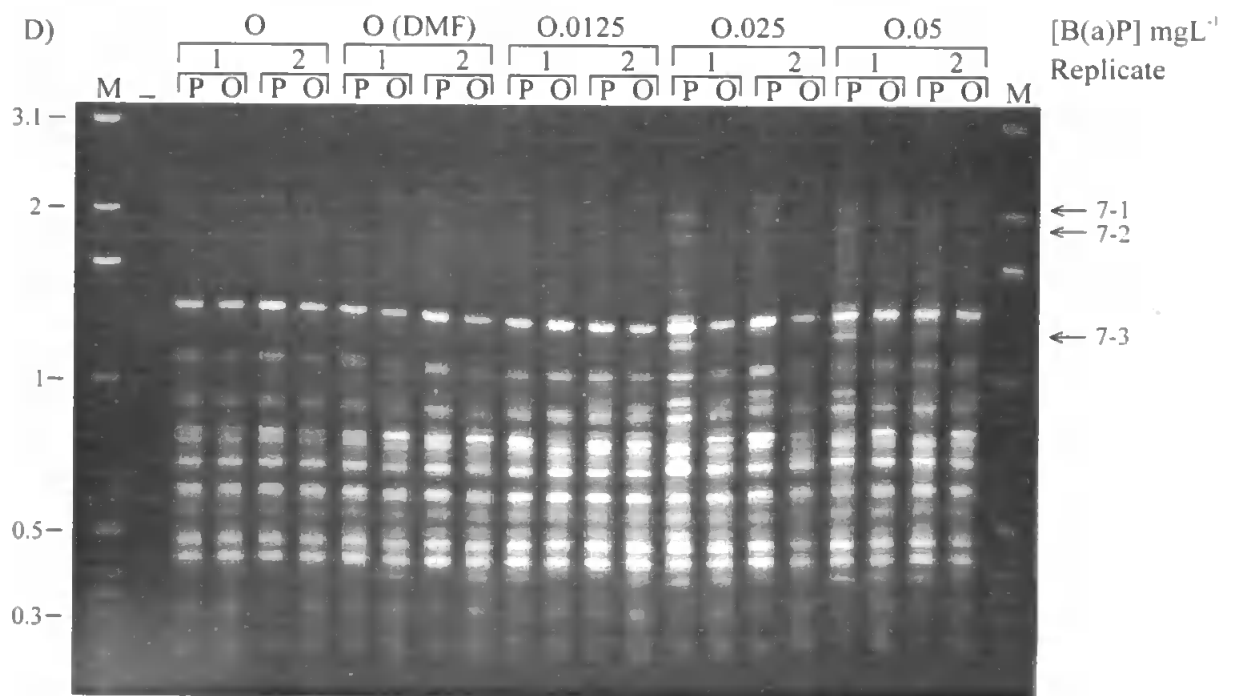
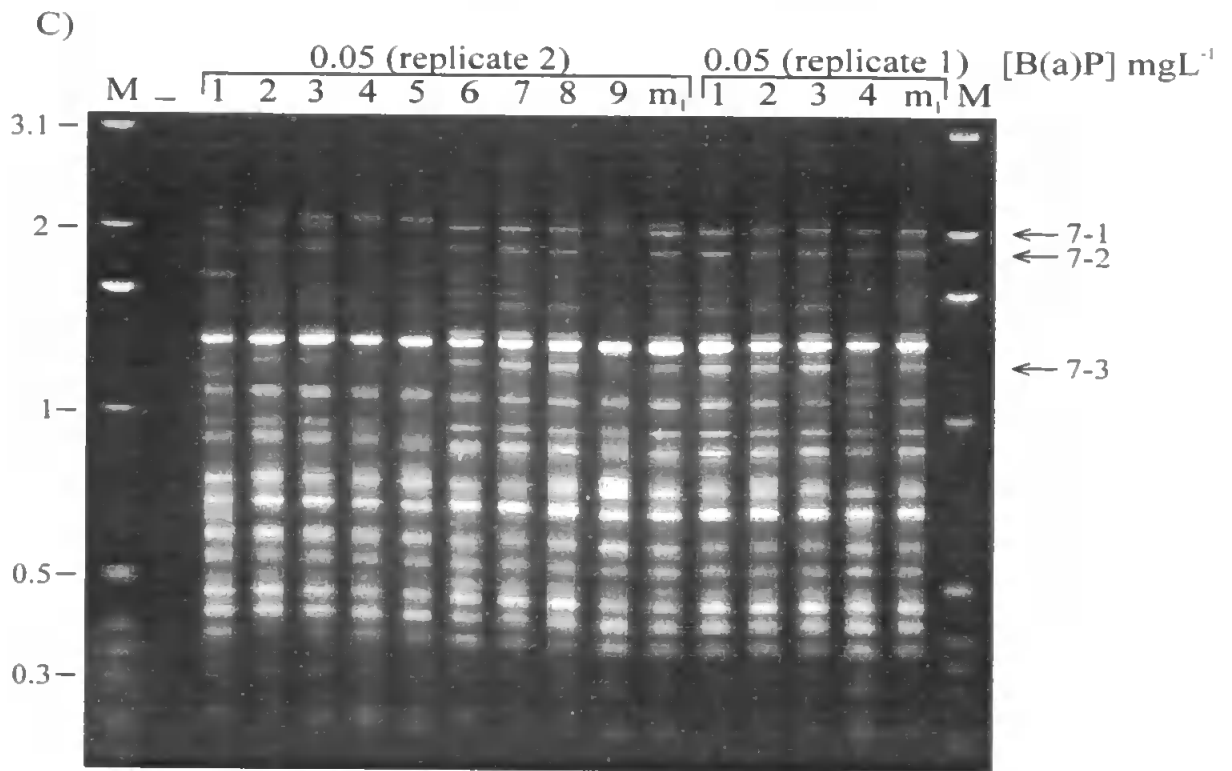


Figure 5.5 RAPD profiles of *Daphnia magna* exposed to B(a)P. A) Patterns generated by 3 individuals of each concentration (replicate 1). B) and C) Reproducibility of bands 7-1, 7-2 and 7-3 from *D. magna* exposed to 0.025 and 0.05 mgL⁻¹, respectively. The numbers and 'm' represent individual animals and a mixture of 4 *D. magna*, respectively.



D) RAPD profiles generated from parent and offspring (last generation) in both replicates. P = parent (4 individuals), O = offspring (20-30 neonates). M: 1 Kb DNA ladder (BRL). The molecular sizes (Kb) are indicated on the left. -: no DNA control. PCR reactions were performed using primer OPB7.

Changes in RAPD patterns were particularly evident with primer OPA9 (Figure 5.4A). For instance, extra bands 9-1 and 9-2 generally appeared in the patterns generated by exposed *D. magna*. Since these two bands may also appear in control *D. magna* (as very faint bands) they could be considered as variation in band intensity. To avoid any confusion, they will be always described as extra bands. PCR products produced by B(a)P exposed *D. magna* also disappeared; for example bands 9-3 and 9-4 disappeared and reappeared at low and high B(a)P concentrations, respectively. Finally band intensity was also subjected to variation. Bands 9-5 and 9-6 represent an example of an increase and a decrease in band intensity, respectively. Changes in RAPD profiles were also produced with primer OPB7 (Figure 5.5) but to a lesser extent in comparison to primer OPA9. For instance, extra bands (7-1, 7-2, and 7-3) only appeared in *D. magna* exposed to 0.025 and 0.05 mgL⁻¹ B(a)P. Variation in band intensity also occurred in RAPD profiles generated by the treated population (e.g. bands 7-4, 7-5). Further experiments confirmed that the variation in band intensities was not a consequence of either a variation in the concentration of template DNA (see appendix III) or a variation in PCR reagent concentration (e.g. *Taq* DNA polymerase) since a master mix was employed. The reproducibility of the RAPD profiling method in detecting B(a)P-induced DNA changes was also determined using both replicates (Figure 5.4B and 5.4C). This experiment was performed to confirm if extra bands 9-1 and 9-2 appeared in the patterns produced by the organisms exposed to 0.025 (Figure 5.4B) and 0.05 (Figure 5.4C) mgL⁻¹ B(a)P. The results showed that both bands were very reproducible between different individuals and mixtures of *D. magna*. The frequency of bands 9-1 and 9-2 was calculated to be at least of 95% at 0.025 and 0.05 mgL⁻¹ B(a)P. When the same samples were subjected to analysis with primer OPB7, bands 7-1, 7-2 and 7-3 appeared all together or not at all (Figure 5.5B and 5.5C). The frequency with which these bands appeared at 0.025 (Figure 5.5B) and 0.05 (Figure 5.5C) mgL⁻¹ B(a)P was calculated to be of 42 % and 87 %, respectively.

An accessory experiment designed to determine the effects of B(a)P exposure on subsequent generations of *D. magna* was performed by comparing profiles of pooled DNA from the offspring (last generation) of B(a)P exposed animals against the banding pattern obtained with maternal DNA with primer OPA9 (Figure 5.4D) and OPB7 (Figure 5.5D). Figure 5.4D revealed that band 9-1 clearly appeared in RAPD profiles generated by the last generation of offspring [except for replicate 1 at 0.0125 mgL⁻¹ B(a)P]. Results also indicate that extra bands (7-1, 7-2, 7-3) present in maternal DNA profiles were not visible in neonatal profiles at low concentrations of B(a)P (0.0125 and 0.025 mgL⁻¹) but may appear at higher concentrations (0.05 mgL⁻¹, replicate 1).

5.3.5 Comparison of fitness parameters with RAPD profiles

To compare the sensitivity of l_x , m_x , r_m , and R_0 with genomic template stability, changes in each parameter were calculated as a percentage of their control value (set to 100 %). All the parameters presented in Figure 6.6 were measured until *D. magna* were unable to swim but still alive after 14, 7 and 5 d at 0.05, 0.1 and 0.2 mgL⁻¹ B(a)P, respectively. For the other groups [i.e. control, control + DMF, 0.0125, and 0.025 mgL⁻¹ B(a)P], the measures were performed throughout the experiment. Changes in RAPD profiles were expressed as reduction in genomic template stability (a qualitative measure reflecting the obvious changes to the number and intensity of DNA bands in DNA patterns generated by toxicant-exposed daphnids) in relation to profiles obtained from control *D. magna*. Before transformation (i.e. control value set to 100 %), the genomic template stability was 96.5, 91.3, 63.4, 44.9, 28.6, 46.3, and 53.6% in control, control + DMF, 0.0125, 0.025, 0.05, 0.1, and 0.2 mgL⁻¹ B(a)P, respectively. These values were calculated with primer OPA9 and OPB7. Although m_x and R_0 are the most sensitive fitness parameters, changes in DNA profiles are more sensitive than any other population parameters. Interestingly, based on observation, fitness parameters were found to be negatively correlated to B(a)P

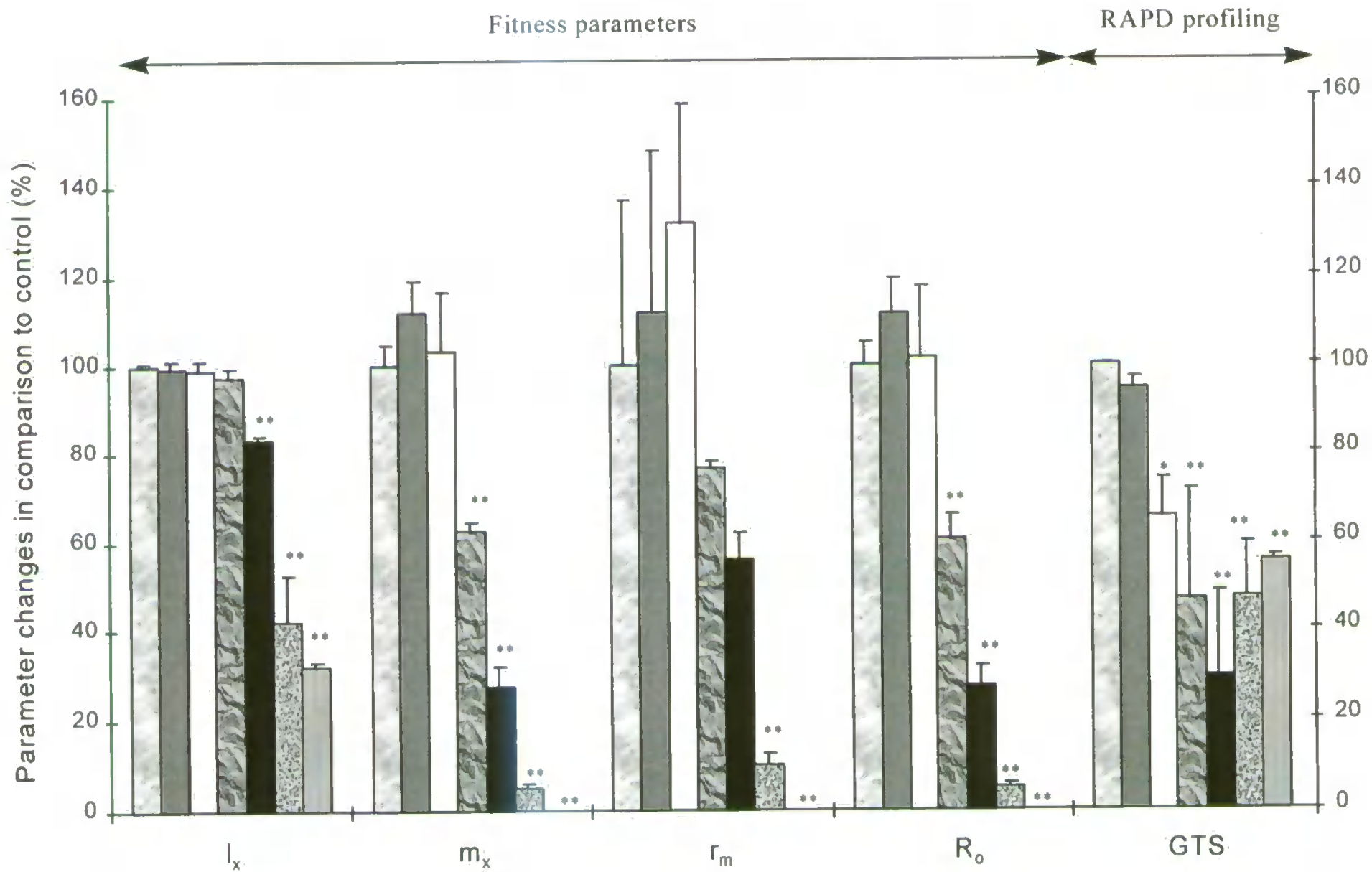
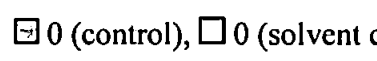








Figure 5.6 Comparison between key fitness parameters and genomic template stability (GTS) in populations of *Daphnia magna* exposed to B(a)P. l_x = age-specific survival; m_x = age-specific fecundity; R_0 = net reproductive rate, and r_m = intrinsic rate of natural increase.  0 (control),  0 (solvent control),  0.0125,  0.025,  0.05,  0.1, and  0.2 mgL⁻¹ B(a)P. * and ** indicate a significant difference from control ($p < 0.05$ and $p < 0.01$, respectively). Error bars represent standard deviation.

concentrations, whereas the genomic template stability followed a reversed Gauss curve (Figure 5.6). Genomic template stability was subjected to a gradual decrease in the range 0.0125-0.05 mgL⁻¹ B(a)P, but it increased at the 2 highest B(a)P concentrations in comparison to 0.05 mgL⁻¹ B(a)P.

5.4 Discussion

In ecotoxicology, the specific evaluation and environmental monitoring of potentially genotoxic agents would be improved with the development of sensitive and selective methods to detect toxicant-induced alterations in the genomes of a wide range of biota. Nevertheless, the value of such procedures would be further enhanced by linking molecular/cellular effects to higher order changes such as reductions in Darwinian fitness and decreases in species diversity (Wurgler and Kramers, 1992; Anderson *et al.*, 1994b; Depledge, 1994a). This study evaluates the suitability of a DNA-profiling/fingerprinting assay combined with the measurement of parameters at the population level with the objective to better understand the impact of B(a)P on *D. magna*.

RAPD profiles detect alterations to genomic DNA through the use of randomly primed PCR reactions. These effects include changes in oligonucleotide priming sites and variations in the activity of the *Taq* DNA polymerase. Such effects lead to visible changes in the electrophoretic profiles of RAPD reaction products. Changes include the appearance of extra amplified bands, the apparent disappearance of amplified bands, and the changes in amplified band fluorescence. However, although obvious changes occurred in RAPD profiles obtained from the exposed population, there were no obvious trends among patterns obtained at the different B(a)P concentrations. New PCR amplification products may reveal a change in the DNA sequence due to point mutations [resulting in (a) new annealing event(s)], and/or large deletions (bringing 2 pre-existing annealing sites closer),

and/or homologous recombination (juxtaposing 2 sequences that match the sequence of the primer) and/or structural effects (see below). Indeed, this method (in fact, the AP-PCR technique) has already been successfully used for the detection of mutations (Ionov *et al.*, 1993; Peinado *et al.*, 1992). Following exposure to mutagens, DNA replication (Christner *et al.*, 1994; Lawrence and Hinkle, 1996) and error-prone DNA repair (Sancar and Sancar, 1988) are generally implicated in generating mutations (Livneh *et al.*, 1993). Although the experiment did not exceed 14 days, DNA replication occurred at an intensive rate since *D. magna* have a rapid growth rate (Figure 5.2B). Thus, the frequency of mutations in the exposed populations may be elevated compared to control *D. magna*. Although new PCR products can be amplified if a mutation occurs within the annealing site, the probability that a mutation will arise within such small regions will be low in comparison to the rest of the genome. However, the RAPD assay can potentially detect mutations in a much larger region than that covered by the priming binding sites (Bowditch *et al.*, 1993). For instance, new annealing events could be attributed indirectly to mutations inducing genomic structural changes (please see below). Mutations can only be responsible for the appearance of new bands if they occur at the same locus in at least a minimum number of cells to be amplified by PCR. This is likely as numerous studies have reported 'hot spot' interactions between DNA and metabolised B(a)P products (Boles and Hogan; 1984; Ross *et al.*, 1994; Ross and Nesnow, 1999). It is now well established that genotoxins leave fingerprints because of specific binding to the target genomic DNA (Vogelstein and Kinzler, 1992).

Changes in RAPD profiles (e.g. appearance of new amplicons) may also result from structural changes induced by B(a)P adducts, mutations and/or by non genotoxic events such as transposition and DNA amplification. The presence of bulky adducts (Shugart *et al.*, 1987) which potentially block the PCR enzyme (Moore *et al.*, 1981; Huang *et al.*, 1993; Van Beerendonk *et al.*, 1994) are expected to have significant effects on RAPD profiles. The *Taq* DNA polymerase may also bypass a DNA adduct, a process which is

conditioned by the local sequence context, the conformation of the adduct, and the nature of the polymerase (Ide *et al.*, 1991). Furthermore, the disappearance of bands is not likely to be due to random mutations because the same event must happen in most of the cells if not all. Finally, the third observed change in RAPD patterns, the variation in band intensity, is likely to be due to the sum of all DNA alterations (e.g. B(a)P adducts, mutations, rearrangements, structural changes) induced by B(a)P. In an unrelated work, RAPD profiles generated from rats exposed to benzo(a)pyrene revealed appearing and disappearing bands in comparison to control RAPD patterns (Savva *et al.*, 1994). Savva (1996) hypothesised that DNA adduct formation would alter the DNA fingerprint of individuals due to the fact that the presence of an adduct in a priming site on the DNA would prevent the binding of the primer to that priming site. In addition, the DNA fingerprints will be altered when priming sites are lost or gained as a result of mutations and when certain PCR products are not synthesised as a result of DNA strand breaks (Savva, 1996).

Variation among RAPD profiles can be estimated by calculating indexes such as the band sharing index or the nucleon diversity index. The band sharing index involves the calculation of the fraction of bands which are shared between individuals (Lynch, 1990). The nucleon diversity index relies upon the band pattern frequencies of the population (Nei, 1987). Genetic distances between populations can then be calculated using Roger's genetic distance (Nei, 1987) and dendrograms can be drawn using the neighbour-joining algorithm (Saitou and Nei, 1987). One of our objectives was to compare the variation in RAPD profiles with the effects arising at the population level. However, the different indexes documented in the literature were not appropriate because of the relatively complex calculations involved. The band sharing index, despite being very simple to estimate, was discarded because it does not take into consideration the absence of bands in profiles. Therefore, a simple method of calculating the variation in RAPD profiles was developed. In this study, we have defined the genomic template stability, a qualitative

index taking into account the obvious variations occurring in RAPD profiles in comparison to control patterns. This index is directly related to the extent of DNA damage, mutations, the efficiency of DNA repair and replication, and possibly to other effects occurring at the DNA level. A high level of DNA damage does not necessarily decrease the genomic template stability (in comparison to a low level of DNA alterations) because DNA repair and replication may be inhibited due to excessive, lethal actions of the B(a)P induced adducts. If the survivorship of a population is affected, a toxic effect can completely inhibit a biological response (e.g. fitness parameters); in contrast, the genomic template stability cannot be completely affected because the induction of DNA damage may not increase linearly (plateau effect). Furthermore, since genomic template stability is likely to be related to all kinds of DNA effects, it would be difficult to anticipate a dose-response relationship. In this context, the genomic template stability could be misleading; for instance, it displayed the same value at 0.025 and 0.1 mgL⁻¹ B(a)P (Figure 5.6). However, to better understand the effect of B(a)P on *D. magna*, the changes in RAPD profiles must be interpreted carefully. Indeed, each change in RAPD profile may have specific significance(s) and the measure of some parameters at the population level can facilitate the interpretation of the data at the molecular level. For instance, a significant reduction in growth correlates with a significant inhibition in DNA replication, suggesting that the extent of DNA damage may be important in the majority of the cells. Unfortunately, since genomic DNA and proteins are affected by B(a)P, with consequent toxic and genotoxic effects, it may be difficult to discern clear relationships.

It is proposed that alterations to RAPD profiles can be regarded as alterations in genomic DNA template stability and that this qualitative measure of DNA effects can be directly compared with changes in key Darwinian fitness parameters. The results from this experiment clearly suggest that genomic DNA template stability, can be a more sensitive reflection than growth parameters and of at least equal or even greater sensitivity than other

measures of fitness such as age-specific survival, age-specific fecundity, net-reproductive rate and the intrinsic rate of population increase. It is also important to note from this series of experiments, that RAPD profiling can detect DNA changes earlier than other changes in conventional toxicity assays measuring fitness parameters. After exposing larval *Xenopus laevis* to B(a)P, Sadinski *et al.* (1995) suggested that DNA adducts and micronuclei were sensitive measures of sublethal DNA damage, as well as possible short-term indicators of indirect effects on fitness parameters. The present study using a novel and simple approach also supports this notion in a clonal freshwater invertebrate species.

Figures 5.4D and 5.5D shows that changes in RAPD profiles generated by the exposed populations can be transmitted to the neonates. However, since neonates were exposed to B(a)P for a short period (maximum 24 h) changes in RAPD profiles may be attributed to the direct action of B(a)P. However, if the length of time was not sufficient to induce changes in neonatal patterns some of the modifications in parental *D. magna* could be transmitted to the next generation. This could implicate the occurrence of mutation in non-somatic cells. This possibility was investigated in more details in chapter 10.

The 96h-LC50 for *D. pulex* to B(a)P has been reported in the literature as, for instance, 5 μgL^{-1} B(a)P (Trucco *et al.*, 1983), and 50 μgL^{-1} B(a)P (Govers *et al.*, 1984). In the present study, the 48h-LC50 of B(a)P for *D. magna* (clone 5) was 250 μgL^{-1} B(a)P. However, the great variability among different studies makes direct comparisons difficult because the toxicity of a compound depends on diverse parameters such as the quality of the medium, amount of food and the use of different clones of the same species (Meador, 1991; Soares *et al.*, 1992; Koivisto and Ketola, 1995) and obviously the duration of the experiment. The growth experiment suggested that *D. magna* exposed to 0.1 and 0.2 mgL^{-1} B(a)P had a size significantly reduced. By attaining reproductive maturity at a smaller body size, the *D. magna* are able to buffer the impact of lower body growth rate on the age at the first reproduction (Lynch, 1985). Generally, when food is limited, *D. magna* generate fewer

but larger neonates compared to organisms fed adequate amounts of food (Enserik *et al.*, 1993). Thus, the identical size of the neonates at first reproduction (see appendix III) plus the fact that growth rates were a good indicator of the intrinsic rate of population increase (r_m) (Lampert and Trubetskova, 1996) suggests that the decrease in size at maturity was more readily ascribed to a direct toxic effect of B(a)P. In other words, the effects on growth were not due to a limitation in food levels due to B(a)P. The data also indicate that *D. magna* exposed to gradual increases in B(a)P concentrations lengthened the time between the first and second brood. This response is generally known to lessen the impact of adverse effects on survival and the number of offspring at each breeding (Calow *et al.*, 1997). Finally, at least one of the *D. magna* exposed to 0.05 mgL^{-1} B(a)P revealed the presence of malformed eggs in its brood pouch. However, this result has to be considered as an illustration since it was not our intention to screen for the presence of abnormalities. Environmental pollutants including genotoxins have been shown to induce malformations in numerous studies (Kubota *et al.*, 1992; Sundelin and Eriksson, 1998; Brown *et al.*, 1999). In addition the exposed animal seemed to be darker than the control *D. magna*. This could be due to a high level of hemoglobin in the treated animal. Indeed it is well known that pale *Daphnia* (haemoglobin poor) lives in well-aerated water, while red *Daphnia* (haemoglobin rich) lives in low oxygen concentration (Kobayashi and Hoshi, 1982).

Results suggested that changes in RAPD profiles generated from the B(a)P exposed population were not always easy to interpret. For example, with primer OPA9 extra bands 9-1 and 9-2 appeared for all B(a)P concentrations (Figure 5.4A) whereas with primer OPB7 extra bands 7-1, 7-2, and 7-3 appeared only at 0.025 and 0.05 mgL^{-1} B(a)P (Figure 5.5A). The RAPD method allows a qualitative assessment of the DNA effects. Although quantitative data can be generated after scoring of the DNA fingerprints, the RAPD cannot be used to quantify the DNA effects. We hypothesise that the RAPD technique could give an overview of genotoxicity due to its potential to detect DNA damage (e.g. DNA adducts,

DNA breaks) and mutations. Whilst the RAPD technique clearly shows promise in the detection of pollutant-induced DNA effects, a great deal of further experimentation and validation is required. Firstly, it seems primordial to evaluate the potential of the RAPD assay to detect other genotoxin-induced DNA effects. Secondly, it is fundamental to investigate the relationship between each DNA lesion as well as mutation and their potential effects on RAPD profiles. Thirdly, as this study shows that the RAPD technology has the potential to detect B(a)P-induced DNA damage, future work should focus on the determination of sensitivity and selectivity of the RAPD technique by comparison with pre-existing DNA analysis methodologies [e.g. ^{32}P postlabelling (Gupta *et al.*, 1982), comet assay (Singh *et al.*, 1994)]. It is also important to assess the performance of this assay in the detection of chromosomal aberrations and mutations. Using this approach it should then be possible to determine the general applicability of this method to the detection of DNA effects under *in vitro* and *in vivo* conditions and the specific ability of RAPD to evaluate pollutant-induced genotoxicity in natural populations of invertebrate animals.

In conclusion, the RAPD technique shows potential as a reliable and reproducible assay for the detection of DNA effects induced by B(a)P in *D. magna*. Furthermore, the present study suggests that, when coupled with the measurement of pollutant induced effects at higher levels of biological organisation, this technique could prove to be a useful ecotoxicological tool. Nevertheless, it is clear that more studies are needed to assess the potential of the RAPD technique to detect DNA effects induced by other well known genotoxins using *D. magna* and possibly other species.

Chapter 6

COMPARISON OF ULTRAVIOLET RADIATION- INDUCED DNA EFFECTS DETECTED BY RAPD WITH CHLOROPHYLL FLUORESCENCE AND GROWTH IN A MARINE MACROALGAE, *PALMARIA PALMATA*

6.1 Introduction

To further examine the use of the RAPD for the detection of toxicant-induced DNA effects, this chapter investigated the effects of UV radiation, a physical genotoxin, as opposed to chapter 5 where a chemical genotoxin was utilised. UV radiation seemed a suitable 'candidate' not only because it is known to be mutagenic and carcinogenic (Anathaswamy and Pierceall, 1990) but also because the effect of UV radiation on DNA is well documented mainly in plants and mammals (e.g. Tachibana *et al.*, 1990; Stapleton, 1992; Liu and Smerdon, 1995; Akiyama *et al.*, 1996; Stapleton *et al.*, 1997). In addition, UV radiation has a timely relevance given the current debate about ozone depletion and the likely ecological effects.

There is great concern about ozone depletion in the earth's upper atmosphere and the associated higher levels of UV-B (280-320 nm) radiation which have the potential to adversely affect terrestrial and marine biota [World Health Organisation (WHO), 1994; Lumsden, 1997; Cordi *et al.*, 1999]. The role of the ozone layer is fundamental since it shields the earth from biologically damaging solar radiations. UV-B radiations which are absorbed by DNA, the most important cellular target, induce photochemical damage. Although DNA damage is usually accurately and efficiently repaired (Witkin, 1976; Szymkowski *et al.*, 1993; Todo *et al.*, 1993; Britt, 1995), in the case of a high level of DNA lesions, some of the alterations to the DNA may remain as permanent mutations (Hutchinson, 1987; Livneh *et al.*, 1993) and may initiate carcinogenesis (Brash, 1988; WHO, 1994). Numerous types of UV induced DNA damage include strand breaks (single and double), monomeric photoproducts (e.g. thymine glycols, pyrimidine hydrates, 8-hydroxyguanine), and dimeric photoproducts [e.g. cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone] (Taylor *et al.*, 1997). The major dimeric photoproducts are illustrated in Figure 6.1. Other lesions can be formed in DNA molecules by UV radiation

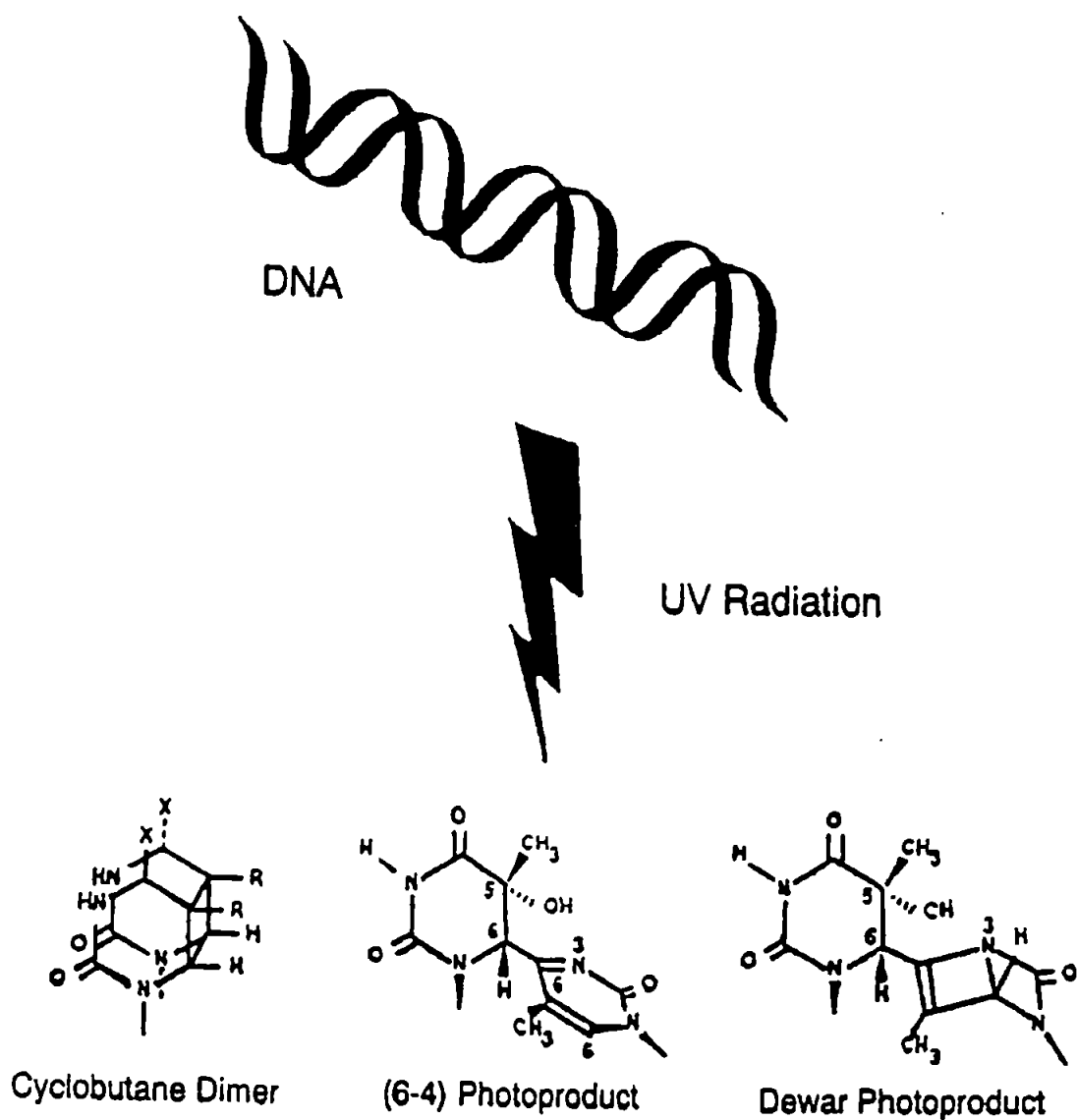


Figure 6.1 The major dimeric photoproducts formed in DNA as a result of UV radiation. Reprinted from 'The induction and repair of DNA photodamage in the environment', Mitchell and Karentz, 1993, in *Environmental UV photobiology*, Young (ed.), with permission from Plenum press.

and include DNA-protein crosslinks and also larger-scale genetic alterations such as chromosome breakage, sister chromatid exchanges and chromatid aberrations (McLennan, 1987). However, pyrimidine dimers which are toxic and mutagenic lesions are characteristic of the direct absorption that occurs at shorter UV wavelengths whereas strand breaks and 8-hydroxyguanine type lesions become increasingly important at longer wavelengths (WHO, 1994). Finally, UV radiation can also damage many other cellular targets such as RNA, proteins and lipids. In cellular and molecular studies, it has been found that the primary products of UV exposure are generally reactive species or free radicals which can rapidly form and may produce effects that last for hours, days or, in some cases, years (WHO, 1994).

The aims of this study were (a) to evaluate the suitability of the RAPD method to detect UV-induced DNA effects in the marine macroalga, *P. palmata*, and (b) to compare the sensitivity of genomic template stability with changes in chlorophyll fluorescence and growth after exposure to UV radiation.

6.2 Materials and methods

This study was performed in collaboration with Dr Britt Cordi, who conducted the exposure as well as the measurement of chlorophyll fluorescence and growth.

6.2.1 Collection of macroalgae

The macroalgae *P. palmata* (Rhodophyta) (see section 3.1.3) was collected at low tide from Wembury on the south-west coast of Devon, UK (50° latitude) between May and June 1997 (sea water temperature: 10°C). The thalli were harvested 24 h prior to the experiments and maintained in filtered sea water at constant temperature (15°C) and in low

light conditions ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$, fluorescent tubes Philips TLD 32W/83 HF, Holland) in growth cabinets.

6.2.2 UV treatments

Thalli were cut to a length of approximately 3 cm and covered with filtered sea water to a depth of 1.5 cm per Petri dish. Each dish was exposed to the various treatments for 3 h ($n = 4$ per Petri dish). Photosynthetically active radiation (PAR) and temperature conditions were identical to those in the acclimatisation period. PAR was measured with a PAR meter (Sky Instruments). UV-B was supplied by two UV-B tubes (Philips TL' 20W/12 RS, Holland) and UV-A by two UV-A tubes (Philips 1609 15 W, Holland). A constant UV-A irradiance of 1.3 W m^{-2} , which is approximately 4 times less than that calculated at 50° latitude in December (Driscoll *et al.*, 1992), was included in all UV-B treatments. The total irradiance of both UV-A and UV-B tubes was measured using UV-B (MP-229) and UV-A (MP-236) cosine sensors (MicroPulse Technology Ltd., UK). These sensors were calibrated in the experimental light field against a double monochromator spectroradiometer (model SR 9910, Macam Photometrics Ltd., UK). UV light was filtered with $35 \mu\text{m}$ cellulose diacetate foil which showed 0 % transmission below 286 nm (UV-C). For the control thalli, UV tubes were covered with Mylar 125 D which showed 0 % transmission below 320 nm (i.e. filtering out UV-B).

Irradiances and doses used in the UV-B treatments are listed in Table 6.1. The weighted UV-B doses were calculated according to the generalised plant response action spectrum normalised at 300 nm (Caldwell, 1971). The corresponding percentage ozone depletion was calculated for 15.07.97 at Plymouth (UK) during clear sky conditions using the computer model of Björn and Murphy (1985). The relatively high UV-B irradiance values were chosen to investigate the entire range of the exposure-response curves.

Table 6.1 Exposure irradiances and doses used in the experiment.

UV-B irradiance (W m^{-2})	0	1.4	2.6	9.0	12.7
Unweighted UV-B dose (kJ m^{-2})	0	15.1	28.1	97.2	137.2
Weighted UV-B dose (kJ m^{-2})	0	4.3	7.9	27.4	38.7
Calculated ozone depletion (%)	0	Ambient August	17	41	100

6.2.3 Measurements of chlorophyll fluorescence

In vivo chlorophyll fluorescence was measured with a Plant Efficiency Analyser (Hansatech instruments). The ratio F_v/F_m was determined before, immediately after, 21 h and 45 h after the UV exposure of 3 h. F_m is the maximal fluorescence, F_o the initial fluorescence and F_v is $F_m - F_o$. Before the measurements the thalli were dark-adapted for 10 min to ensure an oxidised electron transport chain. Prolonged dark adaptation (25 min) showed no change in F_o and F_m . The fluorescence was initiated by 1 second of red light pulses with a peak wavelength of 650 nm and an intensity of 3000 $\mu\text{mol}/\text{m}^2/\text{s}$. Illumination was provided by an array of 6 high intensity light emitting diodes (Hansatech instruments) which were focused onto the thallus surface to provide even illumination.

6.2.4 Growth measurements

The physiological variables were related to differences in thallus growth by measuring the increase in thallus area using a Quantimet 570 Automatic Image Analyser (Cambridge Instruments, Cambridge, UK) during a two week period. The process of image capture, image processing and measurements was programmed using QBASIC the command language of the Quantimet. Distance and light intensity were calibrated prior to measurements.

6.2.5 Generation of *Palmaria palmata* DNA profiles using RAPD

Total DNA from *P. palmata* was extracted and purified using a conventional phenol/chloroform method as described in section 3.2.2.1. The extracted DNA was electrophoresed in 1.2 % agarose gels at 100 V for 2 h and the DNA concentration of unknown samples was determined by comparison with known amounts of Lambda phage DNA (see section 3.3.1). DNA profiles of *P. palmata* were generated using the decamer oligonucleotides, OPA9, OPB1, OPB5, OPB14, and OPB17 (see section 3.4.2) under the

optimised conditions as described in section 3.4.1. PCR products were then electrophoresed in 1.2 % agarose gel at 90 V for 6 h, stained with ethidium bromide and visualised under UV light. A photograph was also taken and the image of the gel was saved electronically for further analysis when necessary (see section 3.5). Genomic template stability was calculated as described in section 3.6.3 and appendix II. The intensity of selected bands was determined using the Kodak Digital ScienceTM 1 D (see section 3.5).

6.2.6 Transformation of the data

To compare the sensitivity of each parameter [genomic template stability, chlorophyll fluorescence (F_v/F_m), and growth rate], changes in these values were calculated as a percentage of their control value (set to 100 %).

6.2.7 Statistical analysis of chlorophyll fluorescence, thallus growth and genomic template stability

Statistical analyses were conducted using software package Statgraphics (Statgraphics plus for Windows version 3.1, Statistical Graphics, USA). For F_v/F_m data, the spectrophotometric measurements and growth data, sequential sampling of each thallus at all time periods was performed so each thallus could act as its own control. Inter-individual variability was thus taken into account in the statistical analysis. A multivariate analysis of variance (ANOVA) for repeated measurements was applied. If the above tests showed significant differences between groups and overall significant time-treatment interaction, Bonferoni tests were conducted to find where significant differences occurred (Milliken and Johnson, 1984). Growth data was analysed using multiple regression analysis. Slopes of the regression lines were determined on log transformed data. This part was performed by Dr Britt Cordi (University of Plymouth, UK). For more details please see Cordi *et al.*, 1997.

GTS was statistically tested by performing one-way analysis of variance. The LSD (least significance difference) test was used to reveal statistical differences. Statistical analyses were performed using the software package STATGRAPHICS (Statgraphics plus for Windows version 3.1, Statistical Graphics, USA).

6.2.8 Effect of DNA concentration on band intensity

In order to investigate the effect of DNA concentration (*P. palmata*) on band intensity, a number of bands were selected across the width of the gels and the intensities were determined using the image analysis software. This extra experiment was performed on control *P. palmata*. The band which displayed the highest intensity was given the arbitrary score of 100 % and intensities of other bands were calculated as a percentage of their control value. Finally, data were pooled to calculate average and standard deviation.

6.3 Results

6.3.1 Measurements of chlorophyll fluorescence and growth rate

The results concerning the chlorophyll fluorescence and growth of the present study have previously been reported in Cordi *et al.* (1997). The effects of UV exposure on F_v/F_m ratio, and growth rates of *P. palmata* are recorded in Table 6.2. Briefly, reduced levels of chlorophyll fluorescence were measured with increasing UV-B irradiance. After a recovery period of 45 h, only thalli exposed to UV-A or the lowest UV-B irradiance (1.4 W m^{-2}) exhibited increases in chlorophyll fluorescence. Thalli exposed to 1.3 W m^{-2} UV-A and 1.4 W m^{-2} UV-B suffered 25 and 58 % reductions in growth rate, respectively compared to controls. Thalli exposed to higher UV-B levels were unable to grow.

Table 6.2 Chlorophyll fluorescence and growth rate measured after exposure of *Palmaria palmata* to UV radiation. † indicate that the algae was irradiated with UV-A only (in W m⁻²). For more details, please see Cordi *et al.* 1997.

Time after UV exposure (h)	Chlorophyll fluorescence and growth rate	UV-B irradiance (W m ⁻²)					
		0	1.3 [†]	1.4	2.6	9.0	12.7
0	Chlorophyll fluorescence, F _v /F _m ratio (%)	100	80	42	47	0	0
21	Recovery experiment Chlorophyll fluorescence, F _v /F _m ratio (%)	100	88	54	32	0	0
45	Recovery experiment Chlorophyll fluorescence, F _v /F _m ratio (%)	100	91	58	5	0	0
-	Growth rate (%) until day 11	100	75	42	0	0	0

6.3.2 RAPD DNA profiling

In total five 10-mer priming oligonucleotides were used to analyse the results. In all cases, RAPD patterns generated by UV-exposed algae were different from those obtained using control DNA (Figure 6.2 and appendix III). DNA patterns generated by each treatment group were reproducible, although each RAPD profile was obtained from individual alga. The principal events observed following the UV exposure were a variation in the band intensity, as well as the disappearance and appearance of new bands (Figure 6.2). Selected changes in comparison to control profiles are indicated in Figure 6.2. Table 6.3 presents a summary of all RAPD profile modifications while Figure 6.3 displays the analysis of band intensity. The decrease in band intensity was particularly obvious for algae exposed to the two highest UV-B irradiances (9.0 and 12.7 W m⁻²). In contrast, an increase in band intensity occurred for the three lowest UV irradiances (UV-A and UV-B irradiances of 1.4 and 2.6 W m⁻²). The number of disappearing RAPD bands which occurred for all UV irradiances tested, was greater at higher UV-B irradiances. Only bands of molecular size greater than 1Kb were shown to disappear (Figure 6.2). Finally, extra bands appeared for the three lowest UV irradiances; this event occurred, however, very rarely for 9.0 W m⁻² and never for 12.4 W m⁻².

In order to investigate the effect of DNA concentration on band intensity, 8 bands have been selected from Figure 6.4A and 6.4B (all bands except B1-2). The band which displayed the highest intensity was given the arbitrary score of 100 % and the intensities of the other bands (of same molecular weight) were calculated as a percentage of their control value. The results clearly showed that there was very little variation in band intensities within the range 1-120 ng of genomic DNA (Figure 6.5). Thus, the changes in band intensities observed from the UV-exposed algae were unlikely to be due to a variation in DNA concentration (Figures 6.4 and 6.5) as PCR reactions were performed using approximately 10 ng DNA. However, although most of the bands followed the type of

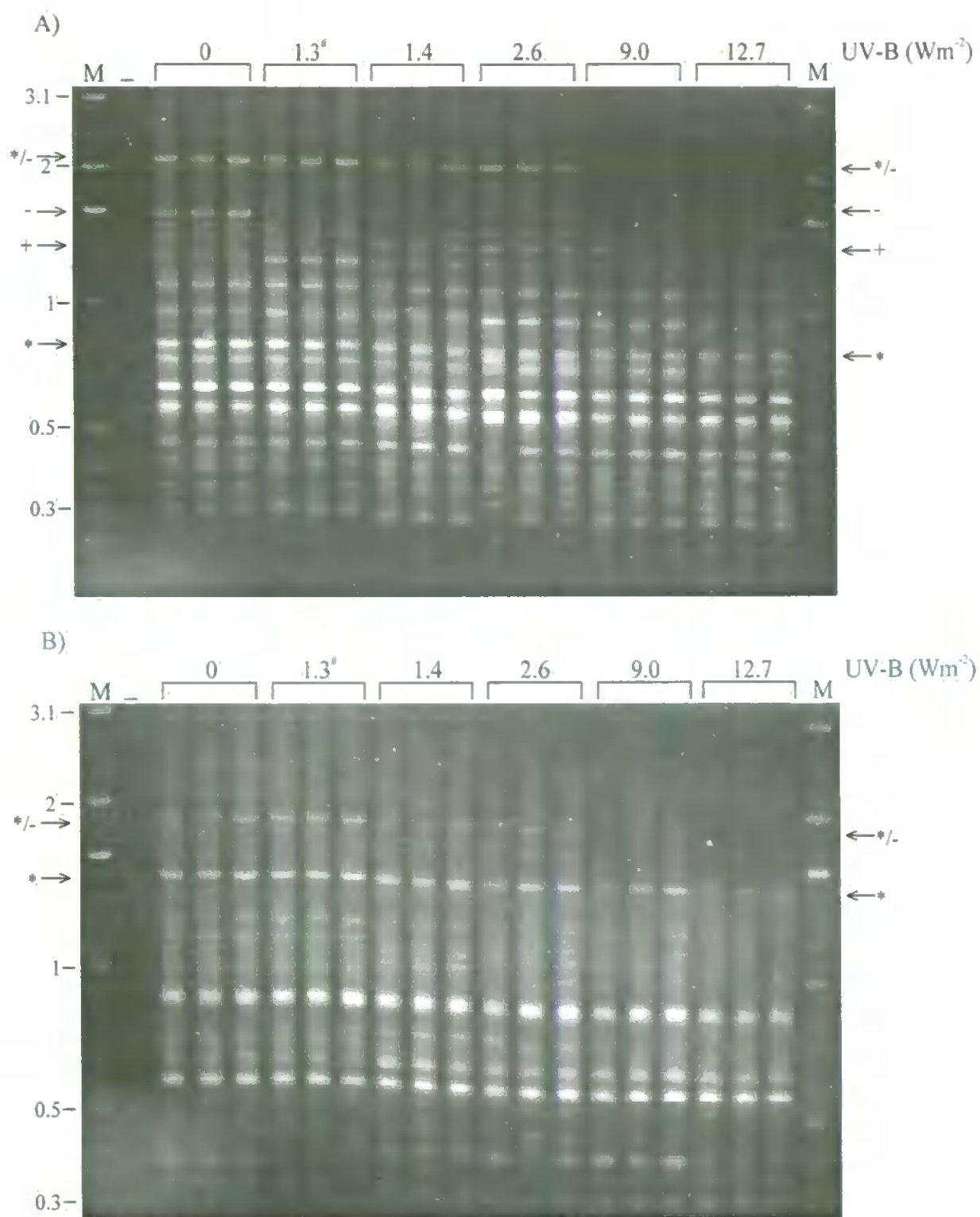
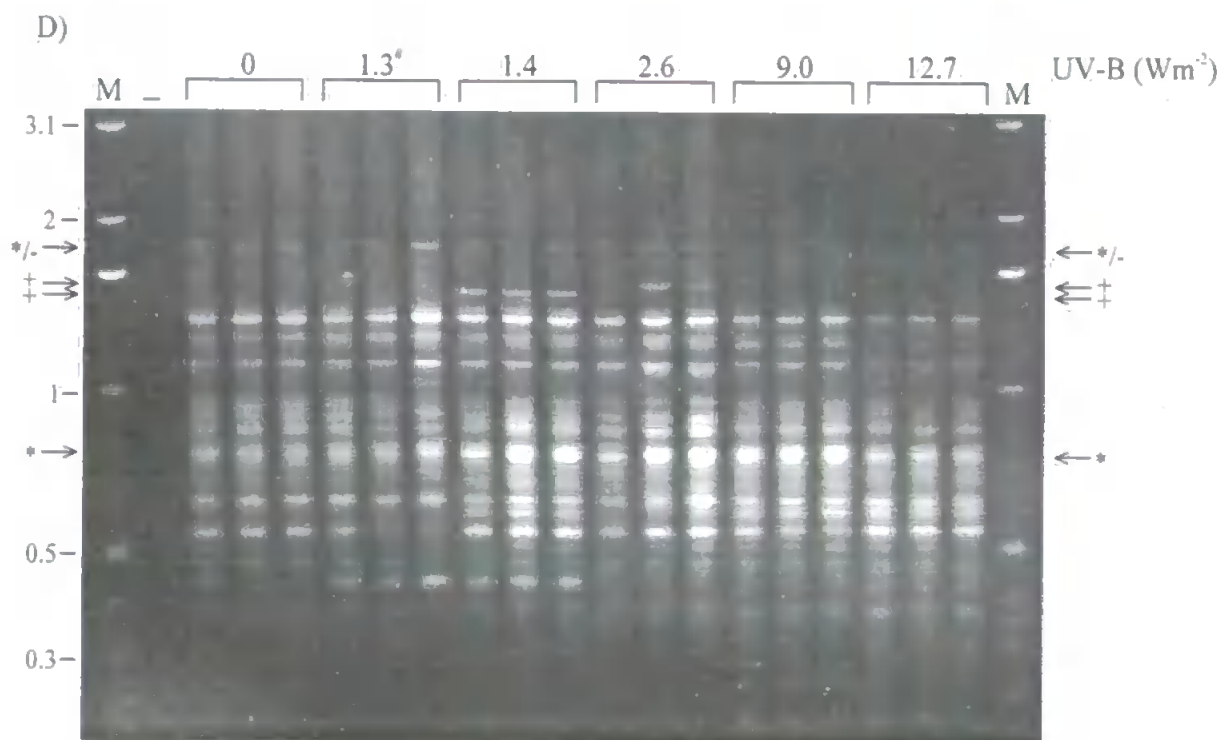
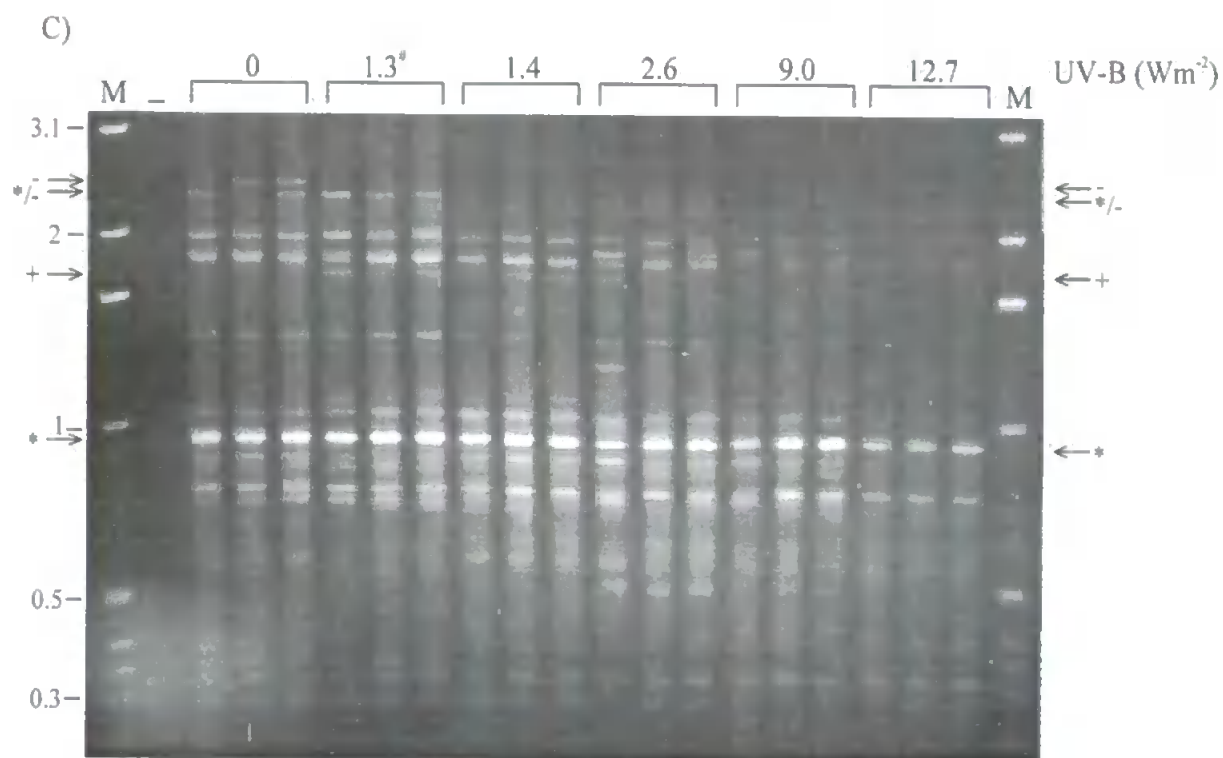


Figure 6.2 RAPD profiles of *Palmaria palmata* exposed to UV radiation. RAPD reactions were performed using oligonucleotide primers OPA9 (A), OPB1 (B), OPB14 (C), and OPB17 (D). UV-B irradiances as indicated at the top of each gel. [#] indicates that the alga were exposed to UV-A only. Each line represents an individual algae. M: 1 Kb DNA



ladder (BRL). The molecular sizes (Kb) are indicated on the left of each gel. -: no DNA control. Selected changes are indicated by arrows in comparison to control patterns; *: variation in band intensities, +: appearance of a new band, and -: disappearance of a band.

Table 6.3 RAPD profile changes after exposure of *Palmaria palmata* to UV radiation. Numbers indicate the frequency in % of each event described in the table. Results were generated using approximately 18 bands for each UV irradiance. † indicate that the algae was irradiated with UV-A only (in W m⁻²).

Changes in RAPD profiles compared with the control	UV-B irradiance (W m ⁻²)				
	1.3 [†]	1.4	2.6	9.0	12.7
Appearance of bands	5	10	5	5	0
Disappearance of bands	10	10	15	30	40
Decrease in band intensities	5	15	15	50	60
Increase in band intensities	80	65	65	15	0

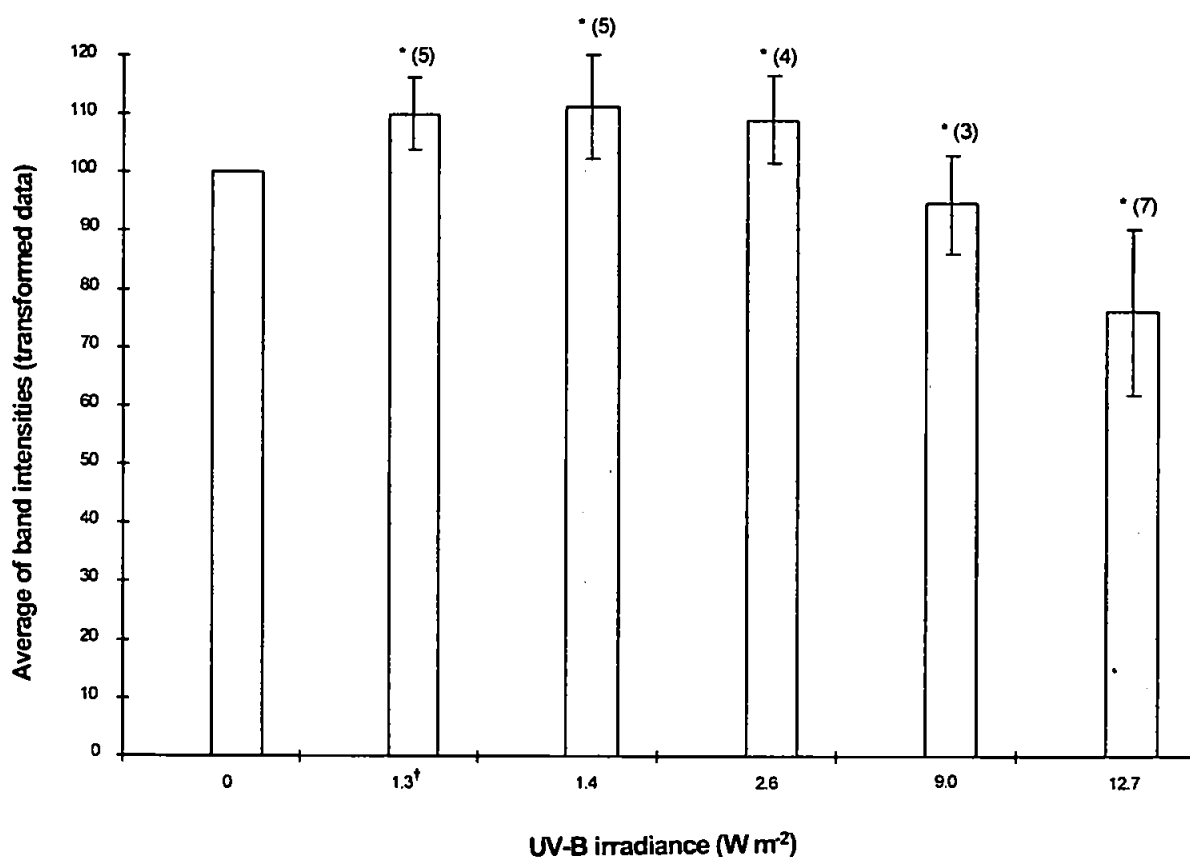


Figure 6.3 Variation in band intensities selected from RAPD profiles generated by UV-exposed *Palmaria palmata*. A total of 10 relatively intense bands appearing across the width of the gels were arbitrarily selected. The band intensities were calculated as a percentage of its own control value (set to 100 %). Average and standard deviation were calculated with the 10 selected bands. The figures in parentheses above the standard deviation indicate the number of bands (out of 10) being significantly different from control (*: $p < 0.05$). † indicate that the algae was irradiated with UV-A only (in W m^{-2}).

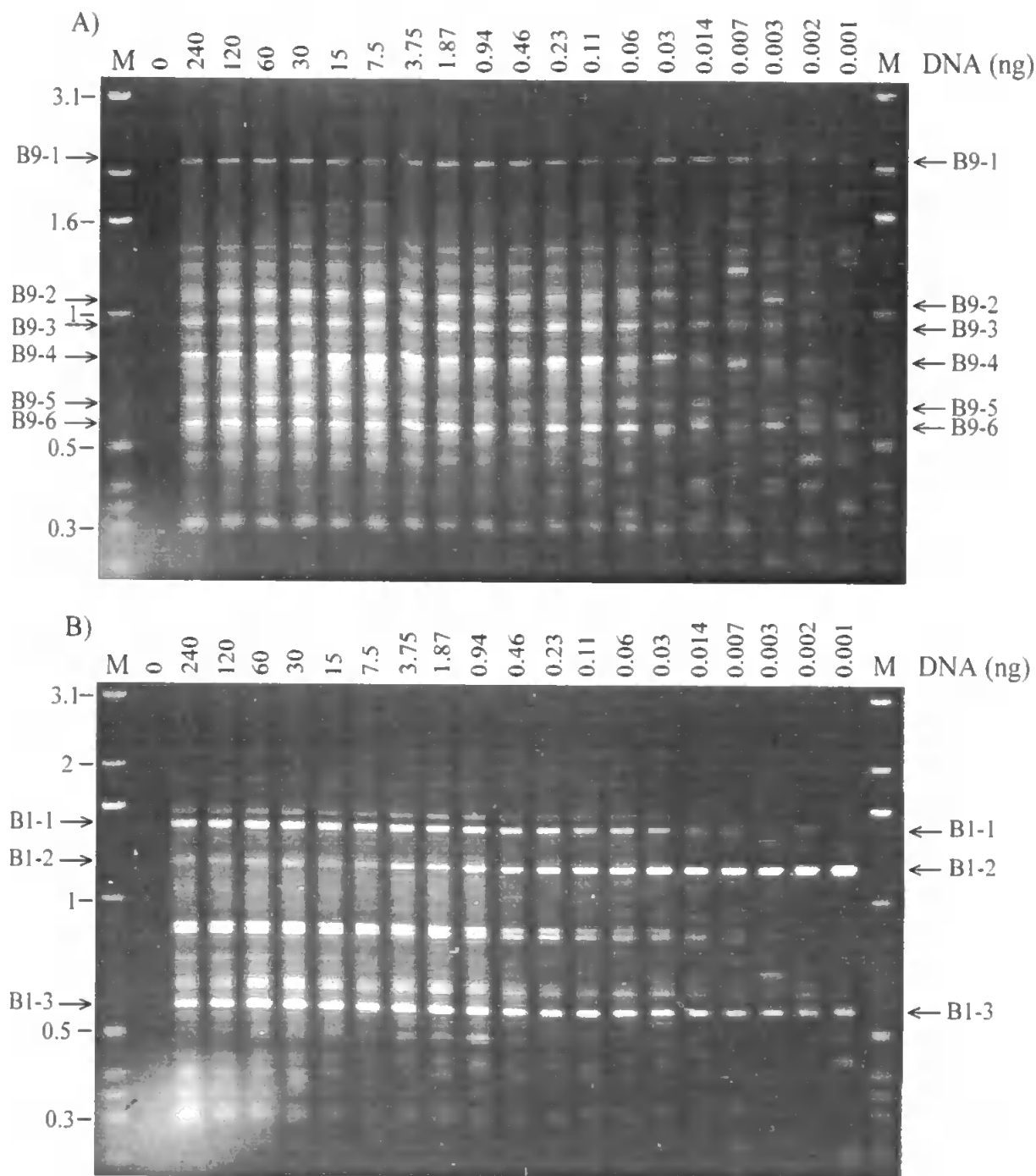


Figure 6.4 Effect of DNA concentration (*Palmaria palmata*) on band intensity. RAPD reactions were performed using oligonucleotide primers OPA9 (A) and OPB1 (B). DNA concentration as indicated on the top of each gel. Six bands were selected from Figure 6.4A (B9-1 to B9-6), and three from figure 6.4B (B1-1, B1-2, B1-3). M: 1 Kb DNA ladder (BRL). The molecular sizes (kb) are indicated on the left of each gel. -: no DNA control. PCR reactions were performed under optimised conditions except for DNA concentrations.

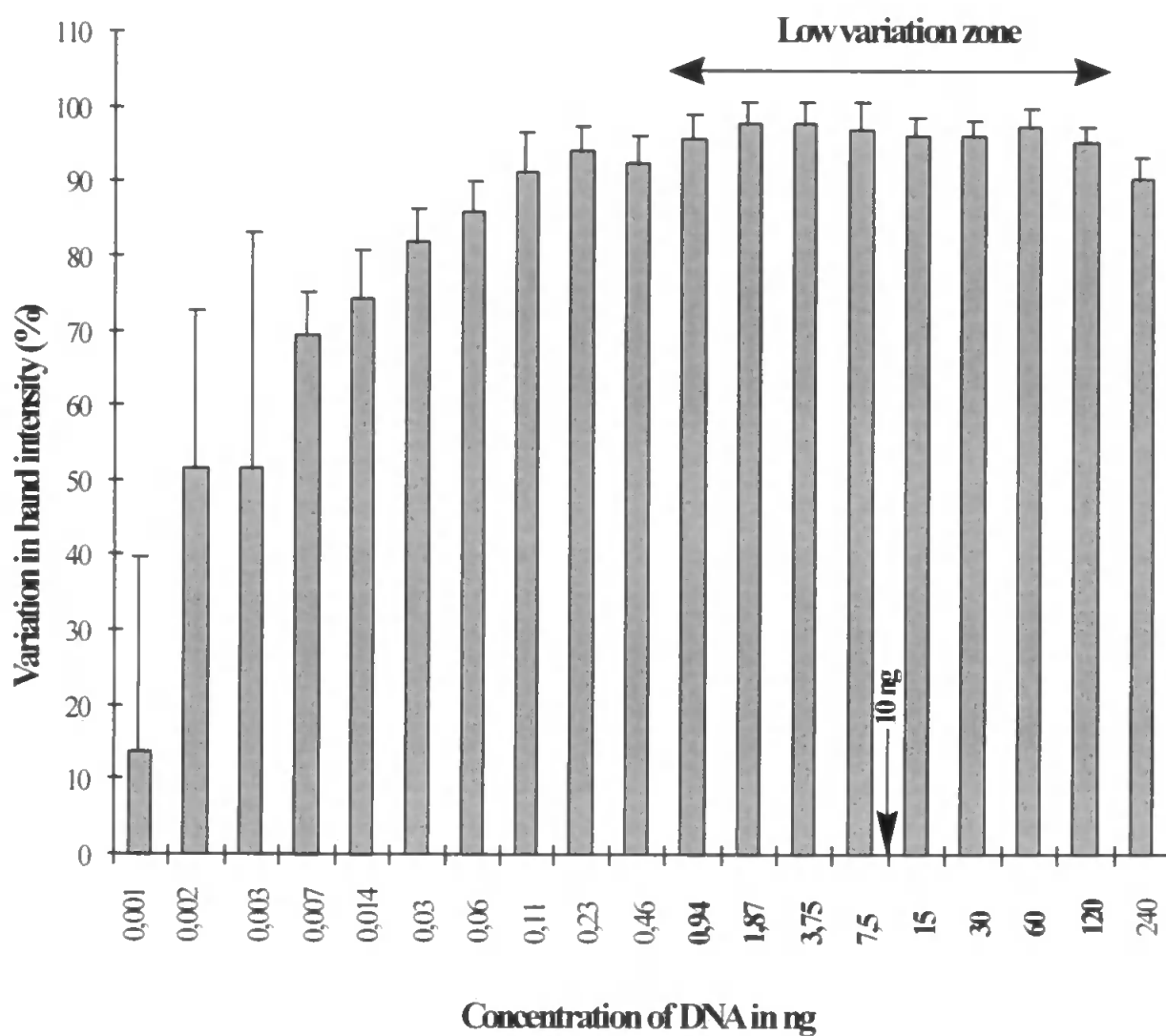


Figure 6.5 Effect of DNA concentration (*Palmaria palmata*) on band intensity. Eight bands were selected from Figure 6.4 (all bands except B1-2) and the average and standard deviation were calculated.

curve displayed in Figure 6.5, band intensity (e.g. B1-2 in Figure 6.4B) followed another type of curve. The intensity of band B1-2 increased with decreasing DNA concentration (appendix III).

6.3.3 Comparison of chlorophyll fluorescence, thallus growth and RAPD profiles

In Figure 6.6, the genomic template stability, a qualitative measure reflecting changes in RAPD patterns, was used to compare the modifications in RAPD profiles with reductions in chlorophyll fluorescence (F_v/F_m ratio) and growth rates. Following exposure to increasing UV-B irradiance, chlorophyll fluorescence and growth rates decreased gradually to zero. In contrast, the genomic template stability decreased after exposure to UV-A, but stabilised after UV-B exposure, varying from 52-60%.

6.4 Discussion

This study evaluates the suitability of a DNA-profiling/fingerprinting assay combined with the measurement of parameters at population level with the objective to better understand the impact of UV radiation on *P. palmata*. Although the effects of UV irradiance on the genetic material (Stapleton *et al.*, 1997), physiological parameters (e.g. photosynthesis, chlorophyll fluorescence, reviewed by Franklin and Forster, 1997) and decreased growth rates (Grobe and Murphy, 1994) have been studied separately, to our knowledge, there has been no attempt to elucidate the potential consequences of induced DNA damage on the physiology and Darwinian fitness parameters of exposed populations.

The effect of UV-B on marine biota is of particular interest as it may lead to a reduction in survival and productivity (Hader *et al.*, 1995). Investigations into the effects of enhanced UV-B radiation on phytoplankton and macroalgae are of great importance, as

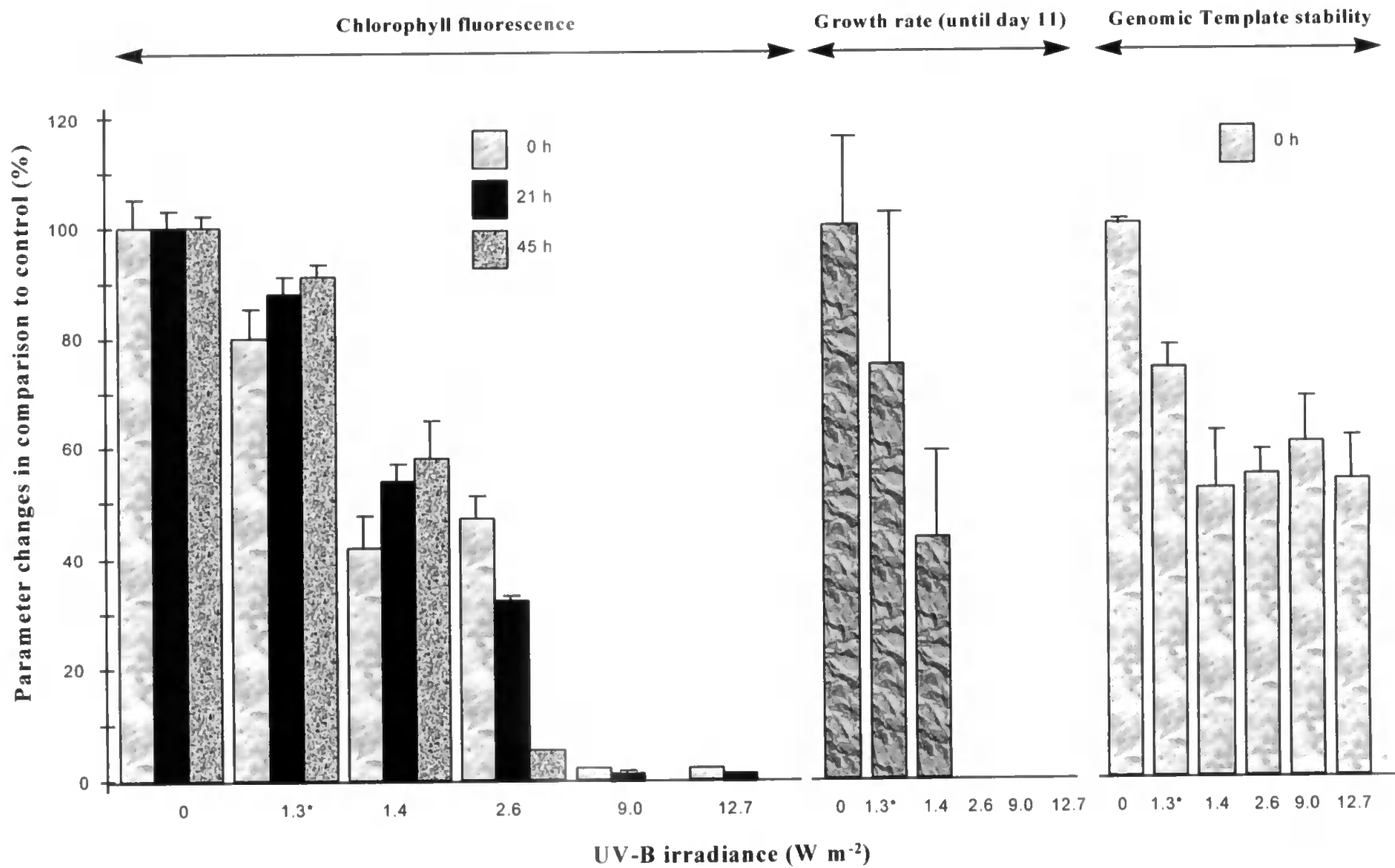


Figure 6.6 Comparison among chlorophyll fluorescence, thallus growth, and genomic template stability in populations of *Palmaria palmata* exposed to UV radiation. The different times represent the length of time (h) following the 3 h of UV exposure. * indicate that the algae was irradiated with UV-A only (in W m^{-2}). All the measured parameters were significantly different from control values ($p < 0.05$) apart from growth at 1.3 W m^{-2} . Error bars represent standard deviation.

these vital primary producers form the basis of aquatic food webs (Houghton and Woodwell, 1989). In addition, since UV-B radiation can penetrate to ecologically significant depths in water, aquatic life in the upper layers of the hydrosphere may be at risk (Hader *et al.*, 1995).

Previous studies have shown that changes in band patterns observed in DNA fingerprint analyses reflect DNA alterations from single base changes to complex chromosomal rearrangements (White *et al.*, 1990; Welsh *et al.*, 1991). Similarly, in the present study, DNA effects induced by UV-radiation reflected changes in RAPD profiles. The present study clearly demonstrated that the variation in band intensity could not be attributed to a variation in DNA concentration, neither to variation in one of the PCR component as a master mix was employed. The variation in band intensities and the disappearance of bands may be attributed to the presence of DNA photoproducts (e.g., thymine dimers, 6-4 photoproducts), which can act to block or reduce (bypass event) the polymerisation of DNA in the PCR reaction (Donahue *et al.*, 1994; Nelson *et al.*, 1996a). The number of disappearing bands increased with increasing UV-B irradiation; this suggested that the frequency of DNA photoproducts increased with increasing UV-B irradiance.

As well as the disappearance of some bands, extra bands were also detected in RAPD profiles (Figure 6.2). New PCR amplification products may occur because mutations including point mutations and large rearrangements arose in the genome of UV-irradiated macroalgae. Please refer to the discussion in chapter 5 for more details. Unlike mammals, plant cells enter meiosis only after significant vegetative growth. In this context, the induction of mutations, which accumulate over time in the somatic tissue, may therefore be passed to the gametophytes (Walbot and Cullis, 1985). This could have implications for the long-term survival of the species (Wurgler and Krammer, 1992). Mutations can only be responsible for the appearance of new bands if they occur at the

same locus in a sufficient number of cells to be amplified by PCR. Pyrimidine photoproducts are produced by UV radiation (Varghese and Wang, 1968), and thus following DNA replication of unrepaired bases, 'hot spot' mutations can be generated (Kamiya *et al.*, 1993; Nelson *et al.*, 1996b). Appearing bands may also be the result of structural changes induced by DNA photoproducts. The trans-syn-thymine dimer bends and unwinds DNA by approximately 22 and 15 degrees, respectively (Wang and Taylor, 1991). In another study, Hanawalt (1998) reported that cyclobutane pyrimidine dimers are formed abundantly compared to the 6-4 pyrimidine-pyrimidione photoproduct, but that the latter is the more distorting lesion, bending the DNA about 44 degrees, while the cyclobutane pyrimidine dimers bend the DNA only 9 degrees. Both of these lesions are subject to nucleotide excision repair but the 6-4 photoproduct is repaired more rapidly than the pyrimidine dimers (Lindahl, 1993). In the present study, the appearance of extra bands occurs principally in the three lowest UV irradiances possibly because extremely high irradiances of UV inhibit DNA repair (Howland, 1975) and replication (Vornarx *et al.*, 1998) in plant tissues.

In an earlier study, a non-mammalian test system for germ-cell mutagenesis was developed for detecting DNA alterations in F₁ progeny descended from the γ -irradiated male medaka fish using the AP-PCR technique. DNA alterations were detected as changes in patterns i.e band loss and/or band gain (Kubota *et al.*, 1995) and the frequency of band loss was shown to increase with increasing radiation doses (Kubota *et al.*, 1992). In the present study, the results of UV exposure of an algal species are consistent with this experiment performed on vertebrates. Govan *et al.* (1990) have also demonstrated the use of PCR to detect UV induced DNA damage in a small region of specific genes (147-440 bp). In the present study, only bands of molecular size greater than 1Kb were shown to disappear (Figure 6.2). The sensitivity for detection of DNA effects by the PCR method may depend upon the size of the target sequence; for instance, there is a greater probability

that an adduct lies within a 2 Kb region rather than within a 150 bp sequence. Therefore, as a general rule, amplification of the longer fragments is expected to be inhibited more than that of shorter fragments (e.g. when DNA adducts block the polymerisation of the DNA). However, some bands whose molecular weight are lower than others can be more affected. This implies that some regions of the DNA are more accessible to genotoxins due to the DNA conformation.

In this study, the two highest UV irradiances selected (i.e. 12.7 and 9.0 W m⁻²) were not environmentally realistic. They were chosen to investigate the entire range of the exposure-response curves. Chlorophyll fluorescence and growth rates decreased gradually to zero following exposure to increasing UV-B irradiance (Figure 6.6). Indeed, a toxic effect can completely inhibit a biological response (e.g. when the survival of a population is affected). In contrast, the genomic template stability exhibited the same degree of inhibition at all UV-B irradiances tested. Genomic template stability is influenced by different parameters as described in section 5.4. The plateau effect is ascribed to multiple changes in RAPD profiles (appearance, disappearance of bands, etc.) which tend to counterbalance each other (Table 6.3). In other words, for the highest UV irradiances, the high frequency of disappearing bands was compensated by the low frequency of newly appearing bands. Therefore, genomic template stability can be misleading. However, to better understand the effect of UV on *P. palmata*, the changes in RAPD profiles must be interpreted carefully. Indeed, the frequency of appearance and disappearance of bands, which may have specific significance, may allow a better understanding of the results if parameters at higher levels of biological organisation are measured. For instance, the absence of extra bands and the high frequency of disappearance of bands revealed that the survival of the individuals was greatly affected. The chlorophyll fluorescence and growth experiments support this observation.

The simultaneous use of more than one biomarker enhances the detection of toxic effects since different biomarker responses are induced at different stages of the plant's

health status curve (Depledge, 1994b). Chlorophyll fluorescence, an important indicator of reversible and irreversible changes in photosystem II (within the photosynthetic process) in plants and algae is known to respond to a wide variety of stressors, both natural and xenobiotic (Bolhar-Nordenkampf *et al.*, 1989; Cordi *et al.*, 1997; 1999). Chlorophyll fluorescence has been characterised as a sensitive general biomarker to UV exposure (Larkum and Wood, 1993; Cordi *et al.*, 1997; Cordi *et al.*, 1999). The results presented in this chapter suggest that chlorophyll fluorescence is a sensitive parameter. This is not surprising because chlorophyll fluorescence can reveal impairments at the molecular level. In this study, changes in RAPD patterns, chlorophyll fluorescence and growth rates indicate that UV-B radiation equivalent to a reduction of 17 % in upper atmospheric ozone caused irreversible damage in *P. palmata*. However, these results may be influenced by the sampling time, due to seasonal variations in the sensitivity of algae to UV exposure.

In conclusion, the RAPD method has been successfully used as a sensitive means of detecting UV-induced DNA effects. However, as with the B(a)P study (chapter 5), RAPD only allowed a qualitative assessment of the DNA effects. Although it seems obvious that the number of DNA photoproducts probably increased with increasing UV radiation, definitive evidence was not obtained. The present study suggests that the RAPD assay applied in conjunction with other biomarkers from higher levels of biological organisation would prove to be particularly relevant in the field of genetic ecotoxicology. Nevertheless, at this stage, further evaluation of the RAPD method for the detection of DNA effects was needed. In particular, it would be challenging to examine whether the RAPD assay could detect DNA effects induced by non-xenobiotic compounds.

Chapter 7

THE USE OF THE RAPD METHOD TO DETECT COPPER-INDUCED DNA EFFECTS IN *DAPHNIA MAGNA* AND COMPARISON WITH KEY FITNESS PARAMETERS

7.1 Introduction

After evaluating the effectiveness of the RAPD assay for the detection of DNA effects induced by chemical (chapter 5) and physical (chapter 6) xenobiotics in aquatic species, the assay was further assessed using *D. magna* which were exposed to a non-xenobiotic compound. Copper was chosen not only because it has been implicated in toxicity and genotoxicity but also because as much as 5 mgL⁻¹ copper has been reported to be discharged into sewers (James, 1990a).

Copper has long been known as essential to living organisms, in part through its fundamental role in electron transport, respiration, growth and development (Linder, 1991). This element has been associated in numerous processes in all kind of organisms. Figure 7.1 gives an overview of copper intake, absorption, distribution, content, function, and excretion in the average, normal human adult. In vertebrates cells, copper has been implicated in the stabilisation of chromosomes and it may be involved in transcription and/or transcriptional regulation effected by certain hormones (Linder, 1991). Copper which is associated with key enzymes or proteins such as the superoxide dismutase (Fridovich, 1995; Atienzar *et al.*, 1998b), metallothioneins (Hamer, 1986), cytochrome c oxidase (Steffens *et al.*, 1987), is well represented in invertebrates. For example, molluscs and arthropods probably contain many or even most of the common copper-dependent proteins that are present in animal species, and they also use copper in a unique way, by producing hemocyanins to carry oxygen to their tissues (Linder, 1991).

On the other hand, the binding of copper to DNA bases unwinds the double-helix (Eichhorn and Shin, 1968) and certain types of DNA damage can be generated. For instance, copper was reported to induce diverse kind of DNA damage such as single- and double-strand breaks, modified bases, abasic sites, DNA-protein crosslinks, and even bulky adducts representing intrastrand dimerisation of adjacent purine bases (Eichhorn and Shin,

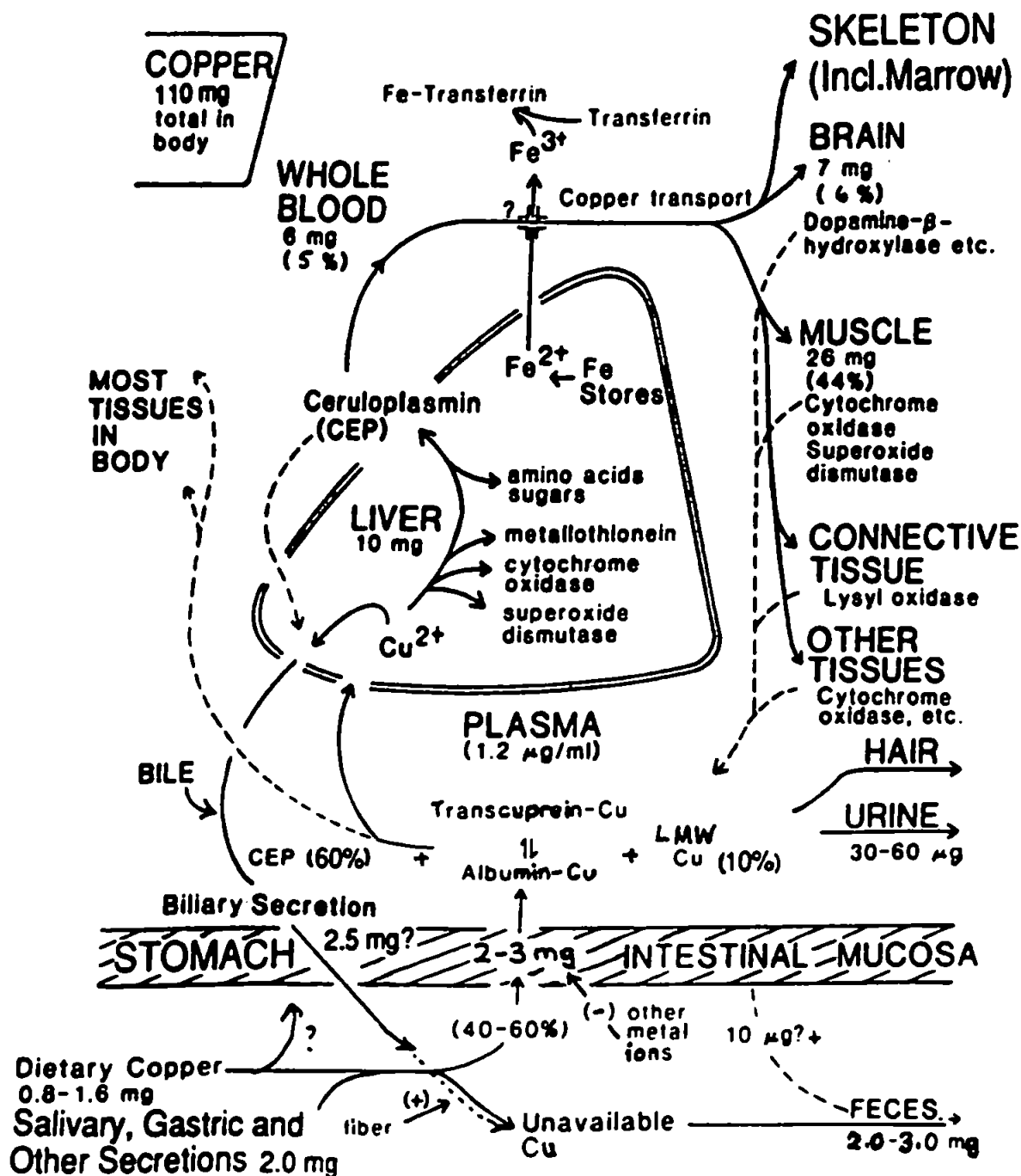


Figure 7.1 Overview of copper intake, absorption, distribution, content, function, and excretion in the average, normal human adult. Quantities are copper concentrations or total contents in tissues or average daily amounts eaten, secreted, absorbed, and excreted.

Reprinted from Linder, 1991, *Biochemistry of copper*, with permission from Plenum Press.

1968; Carmichael *et al.*, 1995; Rodriguez *et al.*, 1995; Toyokuni and Sagripanti, 1996; Drouin *et al.*, 1996b; Lloyd and Phillips, 1999). Copper, and other transition metals, catalyses the Fenton type reduction of hydrogen peroxide to form the hydroxyl radical, one of the most reactive radical oxygen species (Drouin *et al.*, 1996b). Figure 7.2 illustrates the reactions leading to DNA damage in aerobic solutions. Copper ion binding is a necessary but insufficient requirement for the induction of DNA damage; for instance the formation of the DNA-Cu(I)-H₂O₂ complex is also necessary (Yamamoto and Kawanishi, 1989). Local factors influencing the efficiency of formation of the DNA-Cu(I)-H₂O₂ complex or the efficiency of base oxidation by this complex are also important determinants of the damage distribution (Rodriguez *et al.*, 1995). There has been a debate about the mechanism of formation of DNA breakage due to Cu²⁺. Several authors have suggested that Cu²⁺ ions react with H₂O₂ to produce $\cdot\text{OH}$, which mediates the DNA strand breakage (Eberhardt *et al.*, 1989). However, other researchers have disputed the formation of $\cdot\text{OH}$ in reactions involving Cu²⁺ ions and H₂O₂ because $\cdot\text{OH}$ scavengers did not have any effect on the extent of DNA damage (Sutton and Winterbourn, 1989). The suspected intermediate was the oxo-copper complex. However, the production of $\cdot\text{OH}$ by DNA-Cu(I)-H₂O₂ is very rapid and $\cdot\text{OH}$ scavengers have no effect on such systems (Samuni *et al.*, 1981). In this context, the production of $\cdot\text{OH}$ by the complex DNA-Cu(I)-H₂O₂ is very likely. In addition, it has also been proposed that nonbound Cu(I) mediates frank strand break production (Drouin *et al.*, 1996b).

The effect of copper on invertebrates has often drawn the attention of the ecotoxicologists (Dave, 1984; Leblanc, 1985; Bjerregaard and Vislie, 1986; Koivisto and Ketola, 1995; Conradi and Depledge, 1998; Lepp and Salmon, 1999). Many studies have reported the effect on mortality, growth and reproduction after relatively short exposure. In a long term study, the amphipod *Corophium volutator* was exposed to copper concentrations in the range 0.2-1 mgL⁻¹ for 100 days (Conradi and Depledge, 1998). It was

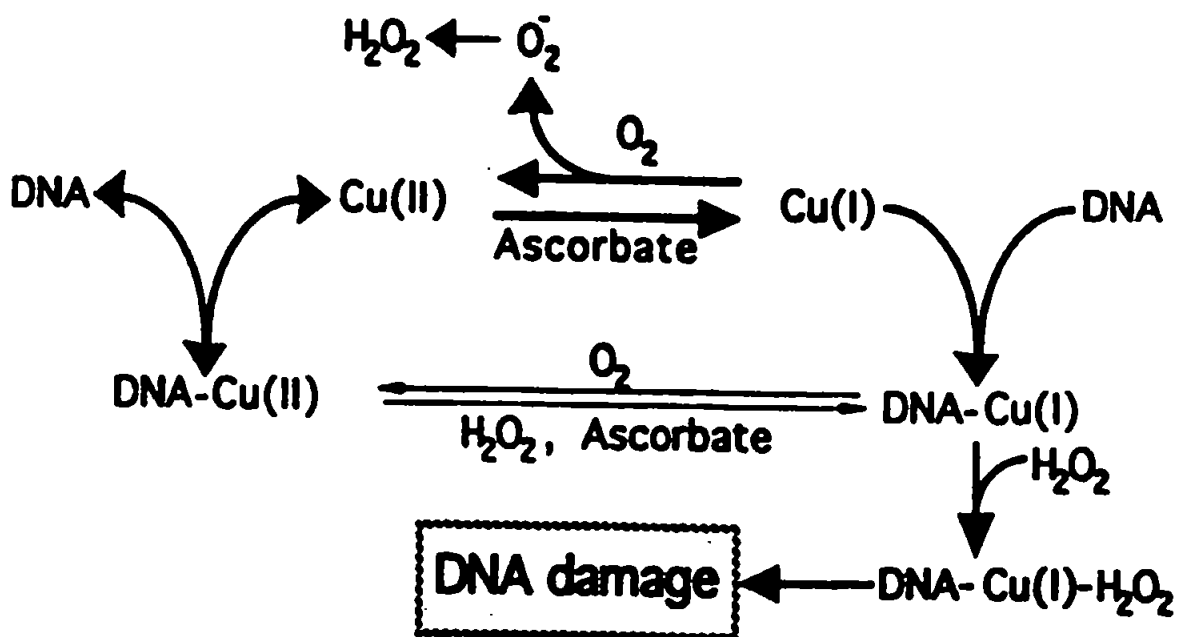


Figure 7.2 Reactions leading to DNA damage in aerobic solutions. Cu(II) distributes almost equally between DNA bound and free solution forms. In the presence of an excess of ascorbate, soluble Cu(II) is reduced and tightly bound to DNA; bound Cu(II) may also be reduced. The DNA-Cu(I) then forms a complex with H_2O_2 . Reprinted from *The Journal of Biological Chemistry*, volume 270, Rodriguez *et al.*, 1995, Mapping of copper/hydrogen peroxide-induced DNA damage at nucleotide resolution in human genomic DNA by ligation-mediated polymerase chain reaction, pages 17633-17640, with permission from The American Society for Biochemistry and Molecular Biology.

concluded that copper could lead to severe local population density decline because of the reduction of juvenile survivorship and fertility. Conradi and Depledge (1998) emphasised the fact that the chronic exposure of *C. volutator* to higher concentrations of copper could have more serious consequences such as the local extinction of this species since juveniles experienced such severe reductions in growth that they were unable to mature.

The aims of this study were to evaluate the potential of the RAPD technique to detect copper-induced DNA effects, and to compare changes in RAPD profiles induced by copper (i.e. genomic template stability) with fitness parameters measured in *D. magna*.

7.2 Materials and methods

7.2.1 Culture of *Daphnia magna*

D. magna (clone 5) was cultured in M7 medium at $20 \pm 2^\circ\text{C}$ as described in section 3.1.1.

7.2.2 Preparation of test solutions and toxicity tests

Test solutions for acute and chronic exposures were prepared from stock solutions of copper (CuCl_2) at a concentration of 3.90 and 0.24 mg/ml in distilled water, respectively, and were subjected to the same dilution (100 μl of the different solutions in 1 L of M7 medium).

7.2.2.1 Acute toxicity

The acute toxicity of copper (Cu) was assessed by determining the LC50 of the chemical for *D. magna* over a period of 48 h. Freshly born neonates (less than 48 h post-hatch) were exposed in replicate groups of 20, to concentrations of Cu equivalent to 10, 25, 62.5, 156.25 and 390.62 $\mu\text{g L}^{-1}$. Animals were fed (see section 3.1.1) during the test, and surviving animals were counted to determine the 48h-LC50.

7.2.2.2 Chronic toxicity

The chronic toxicity test of Cu to *D. magna* was performed under the same experimental conditions as for the acute toxicity tests. *D. magna* were exposed to the following Cu concentrations 0, 15, 30, 60, 90, and 120 μgL^{-1} for 15 d. Surviving animals were counted at day 1, 3, 5, 8, 10, 12 and 15. Moribund, non-swimming animals were removed from culture at regular intervals on and between counting days. Healthy animals after 14 d exposure and non healthy *D. magna*, which were significantly affected before the end of the experiment, were harvested and were stored at -80°C , prior to DNA extraction and RAPD DNA profiling.

As M7 medium contains 2 μgL^{-1} Cu, all concentrations previously mentioned in acute and chronic toxicity tests can be corrected by adding 2 to each of the values. However, as 2 μgL^{-1} was thought to be negligible, non-rectified values were used throughout the manuscript.

7.2.3 Growth and reproductive measurements

The length of every *D. magna* (apex to base) [except at the start of the experiment (day 1) where 20 *D. magna* were measured] surviving at day 1, 3, 5, 8, 10, 12 and 15 was measured by video capture and image analysis using a Quantimet 570 image analyser (Cambridge Instruments, Cambridge, UK). Neonates were counted and recovered at daily intervals. The length of new-born neonates (generally 10 animals per replicate) released at day 8, 10, and 15 was also determined.

7.2.4 Calculation of fitness parameters

The fitness parameters measured were the intrinsic rate of natural increase (r_m), the age-specific survival (l_x), the age-specific fecundity (m_x), and the net reproductive rate (R_0). Minimum generation time (T_{min}) and the inter-brood time (Bt) were also measured. For more details please refer to section 3.6.2.

7.2.5 Generation of *Daphnia magna* DNA profiles using RAPD

Total DNA from *D. magna* was extracted and purified using a conventional phenol/chloroform method (see section 3.2.1.1). The extracted DNA was electrophoresed in 1.2 % agarose gels at 100 V for 2 h and the DNA concentration of unknown samples was determined by comparison with known amounts of Lambda phage DNA (see section 3.3.1). DNA profiles of *D. magna* were generated using 10-mer primers, OPA9, OPB1, OPB5, OPB6, OPB7, OPB8, OPB10, OPB11, OPB12, OPB14 and OPB17 (see section 3.4.2) under the optimised conditions as described in section 3.4.1. PCR products were then electrophoresed in 1.2 % agarose gel at 90 V for 6 h, stained with ethidium bromide and visualised under UV light. A photograph was also taken and the image of the gel was saved electronically for further analysis when necessary (see section 3.5).

7.2.6 Estimation of genomic template stability and transformation of the data

Genomic template stability (GTS) was determined as described in section 3.6.3 and appendix II. To compare the sensitivity of each parameter (GTS, l_x , m_x , r_m and R_0), changes in these values were calculated as a percentage of their control value (set to 100 %).

7.2.7 Statistical analyses

For more details please refer to section 3.6.5.

7.3 Results

7.3.1 Acute toxicity

The 48h-LC50 of Cu for *D. magna* (clone 5) was $165.1 \mu\text{gL}^{-1}$ Cu. The upper and lower values of the 95% confidence limit were 147.7 and $192.2 \mu\text{gL}^{-1}$ Cu, respectively ($p < 0.05$).

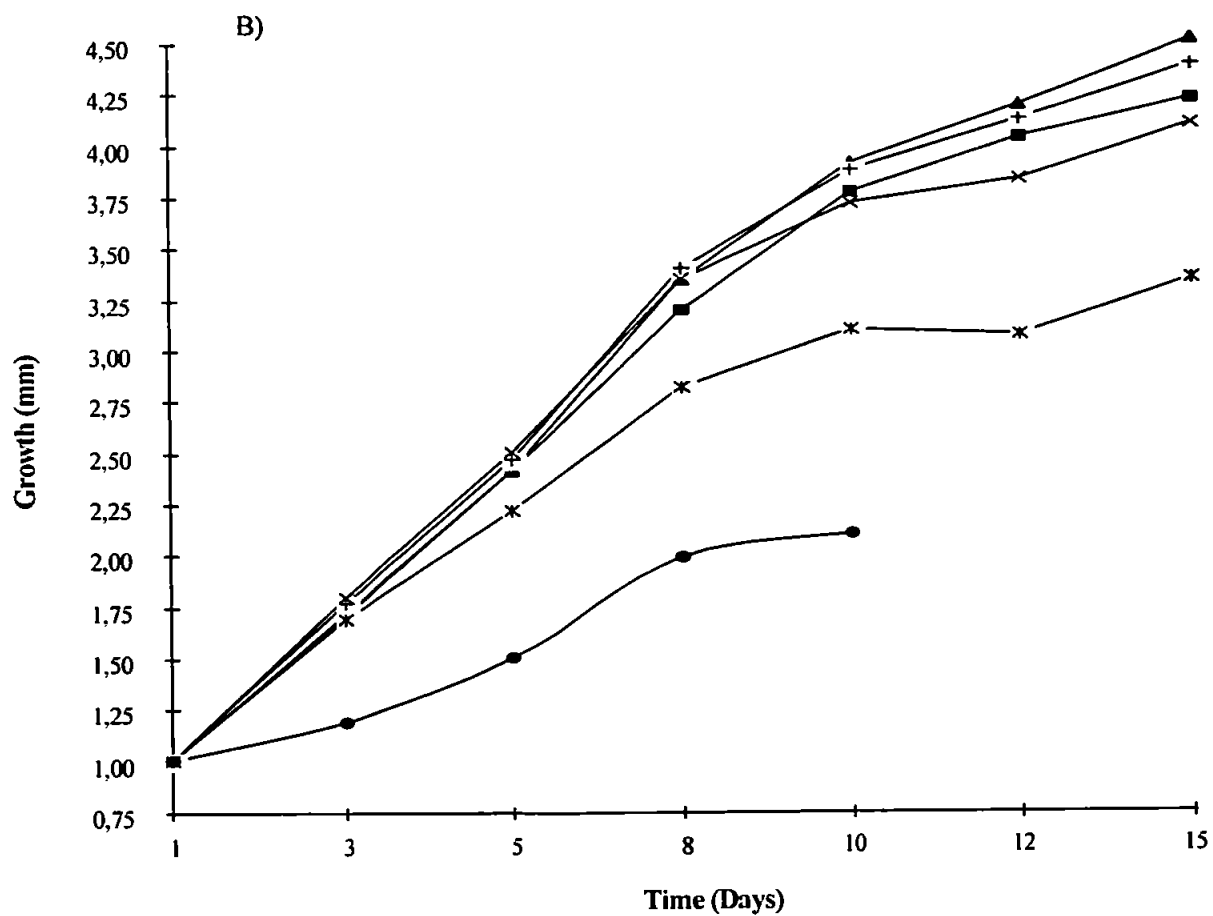
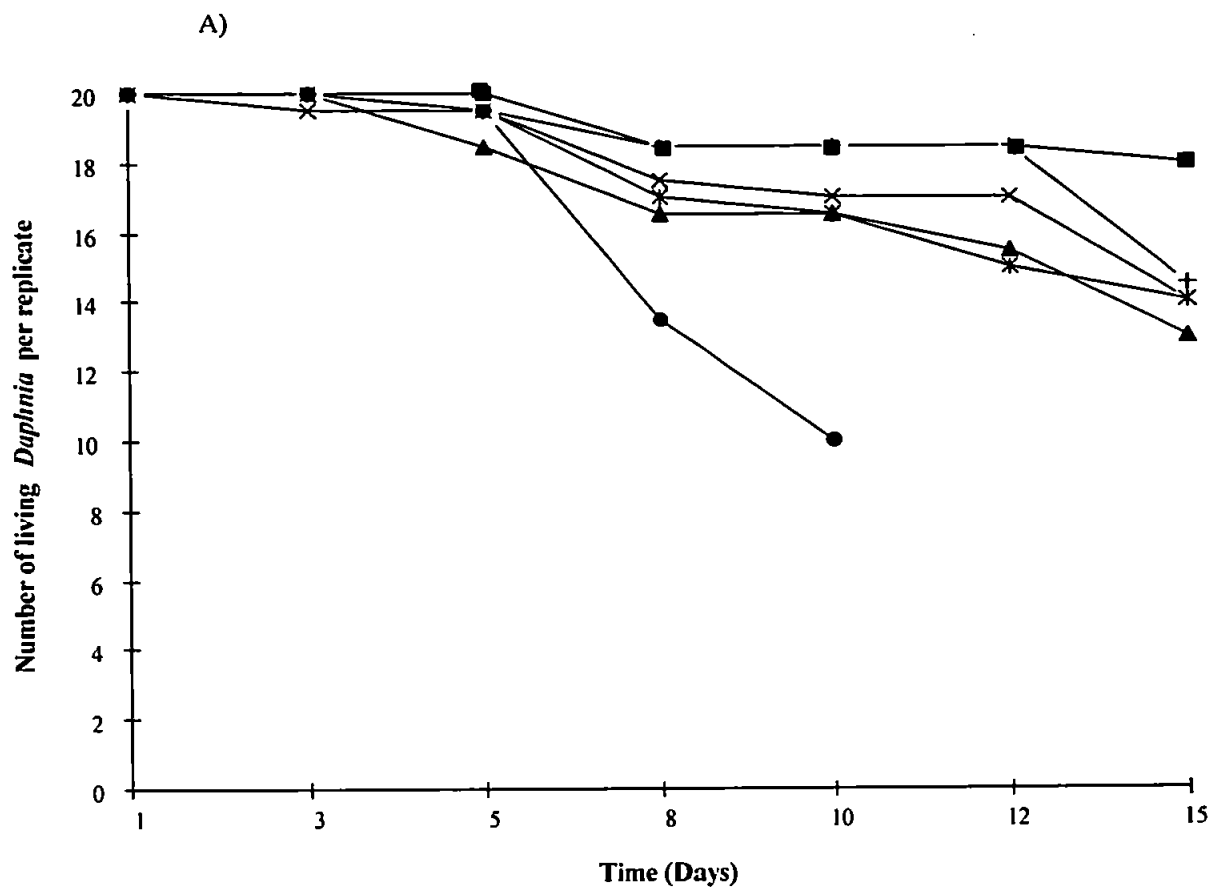
7.3.2 Changes at population levels

Demographic trends for the populations of *D. magna* exposed to differing concentrations of Cu are shown in Table 7.1. In the range 30 - $120 \mu\text{gL}^{-1}$ Cu, consistent reductions occurred in maximum number of eggs per female and maximum body size. In contrast, the longevity and the number of broods were only affected at $120 \mu\text{gL}^{-1}$ Cu. *D. magna* exposed to the highest Cu concentration could not survive for more than 10 days whereas animals from the other groups survived throughout the experiment (Table 7.1 and Figure 7.3A). The mean period for *D. magna* to become ovigerous was identical among groups and animals exposed to gradual increases in Cu concentrations displayed the same time between broods except for *D. magna* exposed to $120 \mu\text{gL}^{-1}$ Cu (Table 7.1). Both populations growing at 120 and $90 \mu\text{gL}^{-1}$ Cu had a smaller body size than controls ($p < 0.001$), and there were no significant differences between control and other Cu exposed groups (Figure 7.3B). In addition, the size of sub-24 h juveniles at day 8, and 10 was identical between all groups ($p < 0.01$; appendix III). At day 15 the length of the offspring was different from control at 15 , 60 , and $90 \mu\text{gL}^{-1}$ Cu ($p < 0.01$; appendix III). Finally, the total number of offspring was significantly reduced at 90 and $120 \mu\text{gL}^{-1}$ Cu ($p < 0.01$; Figure 7.3C and Table 7.1) compared to the control.

Copper concentration ($\mu\text{g/L}$)	Longevity (days)	Number of days to become ovigerous	Maximum body size (mm)	Maximum number of eggs/female	Number of broods	Inter- brood period (days)	Total number of neonates	Age specific survival (l_x)	Age specific fecundity (m_x)	Net productive rate (R_0)	Intrinsic rate of natural increase (r_m)
0	15	5	4.6	38	3	3	1471.0 ± 20.4	6.67 ± 0.46	80.48 ± 0.38	55.50 ± 4.38	0.21 ± 0.02
15	15	5	4.88	40	3	3	1424.5 ± 33.2	6.00 ± 0.07	97.22 ± 1.93	53.80 ± 0.14	0.18 ± 0.06
30	15	5	4.77	30	3	3	1409.0 ± 19.8	6.47 ± 0.32	83.50 ± 12.71	49.13 ± 3.78	0.22 ± 0.01
60	15	5	4.46	27	3	3	1269.5 ± 61.5	6.22 ± 0.39	80.17 ± 6.88	46.13 ± 0.18	0.12 ± 0.07
90	15	5	3.72	24	3	3	618.0 ± 141.4	6.10 ± 0.28	40.27 ± 6.00	19.95 ± 7.21	0.08 ± 0.07
120	10	5	2.58	6	1	-	2.5 ± 3.5	4.15 ± 0.21	0.25 ± 0.35	0.13 ± 0.18	-0.06

Σ

Table 7.1. Demographic trends of *Daphnia magna* populations under various copper treatments. Values which represent mean \pm SD were calculated using both replicates (i.e. 40 *daphnia* per concentration). All parameters were determined throughout the experiment until *Daphnia* were unable to swim properly.



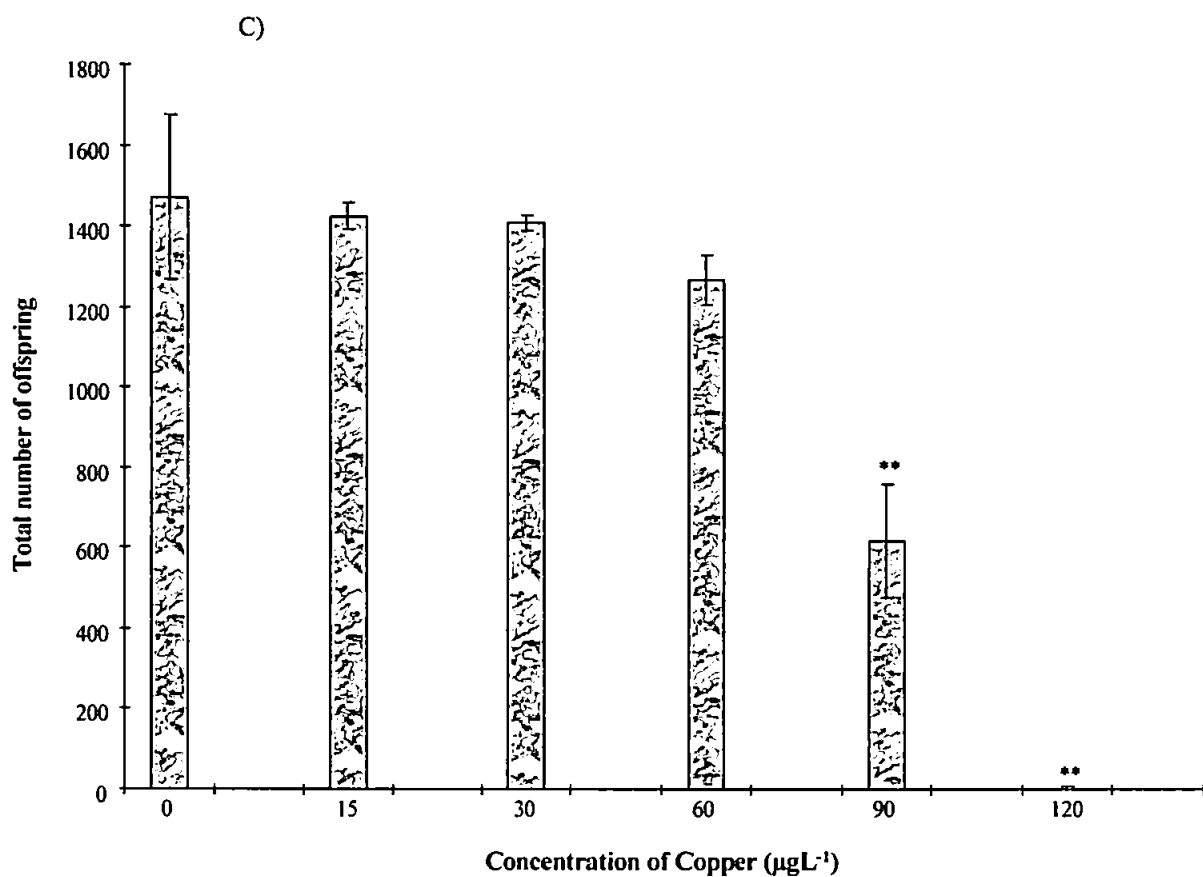


Figure 7.3 Variation in (A) number of living *Daphnia magna* per replicate, (B) growth, and (C) total number of offspring in populations of *Daphnia magna* exposed to copper. —■— 0, —▲— 15, —+— 30, —×— 60, —*— 90, and —●— 120 $\mu\text{g/L}$ Cu. ** indicates a significant difference from control ($p < 0.01$).

7.3.3 Changes in fitness parameters

Alterations to the key fitness parameters of age-specific survival (l_x), age-specific fecundity (m_x), net reproductive rate (R_0) and intrinsic rate of population increase (r_m) are presented in Table 7.1 and Figure 7.7, before and after transformation, respectively. *D. magna* exposed to $120 \mu\text{gL}^{-1}$ Cu exhibited a significant reduction in l_x compared to the controls ($p < 0.05$). In contrast, the remaining fitness parameters (age-specific fecundity, net reproductive rate and intrinsic rate of population increase) were more sensitive than age-specific survival as they were at least significantly different for the 2 highest Cu concentrations (90 and $120 \mu\text{gL}^{-1}$ Cu) in comparison to control ($p < 0.05$). R_0 and m_x which appeared to be the most sensitive fitness parameters for the detection of toxic effects, were also significantly different from control at 60 and $15 \mu\text{gL}^{-1}$ Cu, respectively.

7.3.4 RAPD profiling

The DNA template for RAPD profiling was extracted from healthy organisms aged 15 d from all groups except for *D. magna* exposed to $120 \mu\text{gL}^{-1}$ Cu. Genetic material obtained from those latter animals was carried out on weak but alive *D. magna* aged 10 d. In total, 11 oligonucleotide primers were used in the analysis and the presence of changes in the RAPD profiles obtained from the exposed population depended on the primer used. DNA profiles presented in Figure 7.4 were generated using 4 primers (primer OPA9, OPB7, OPB10, and OPB14) and a mixture of 4 individuals from each replicate. RAPD profiles generated by individuals and a mixture of individuals from both replicates with all primers are presented in appendix III apart from primer OPB14 (Figure 7.6). Profiles generated by the primers presented in the main body of the thesis (Figure 7.4) revealed significant differences between control and exposed individuals, with visible changes in the number and size of amplified DNA fragments, and both increases and decreases of DNA band intensities. Arrows on the right of each gel (Figure 7.4) show some of the obvious

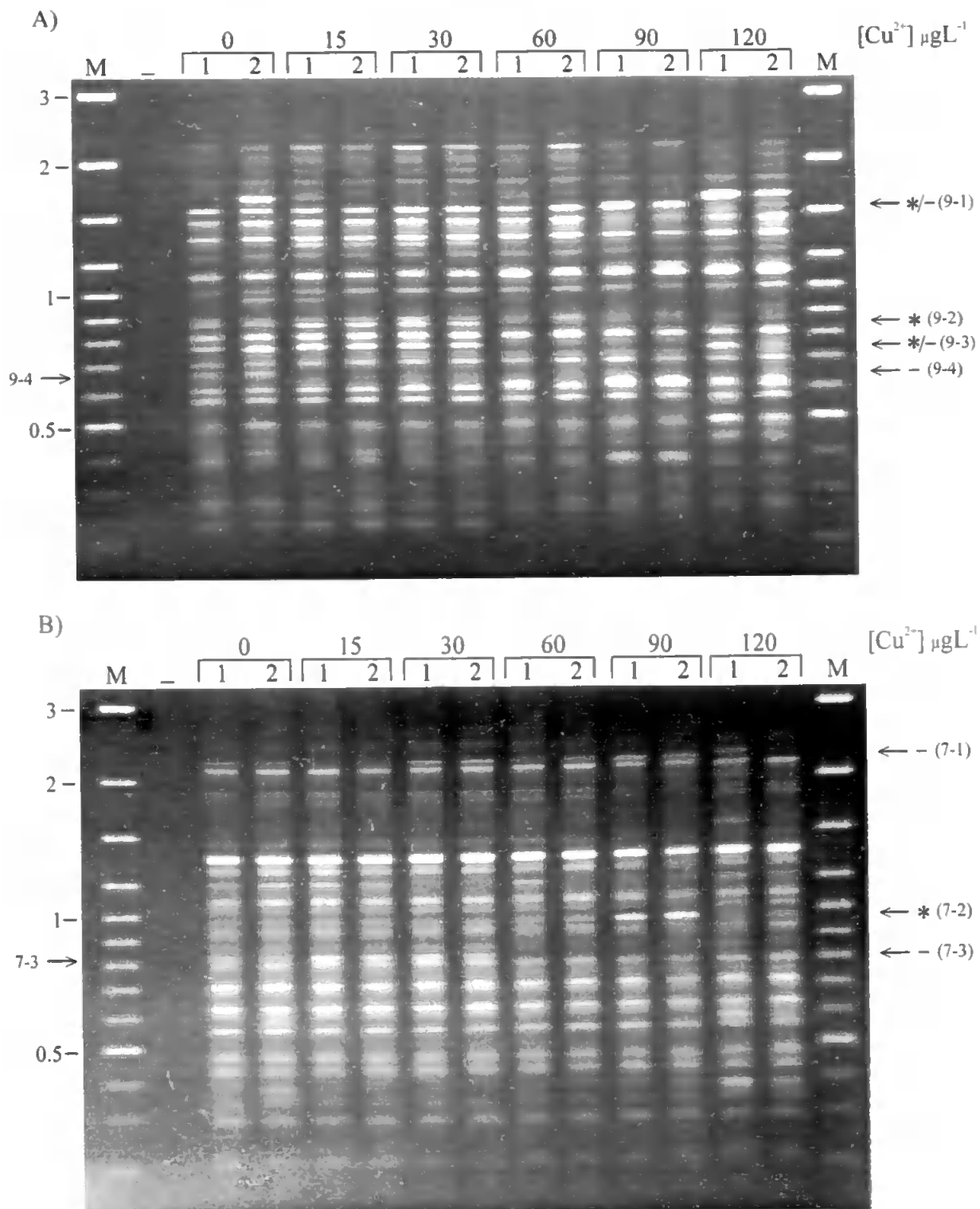
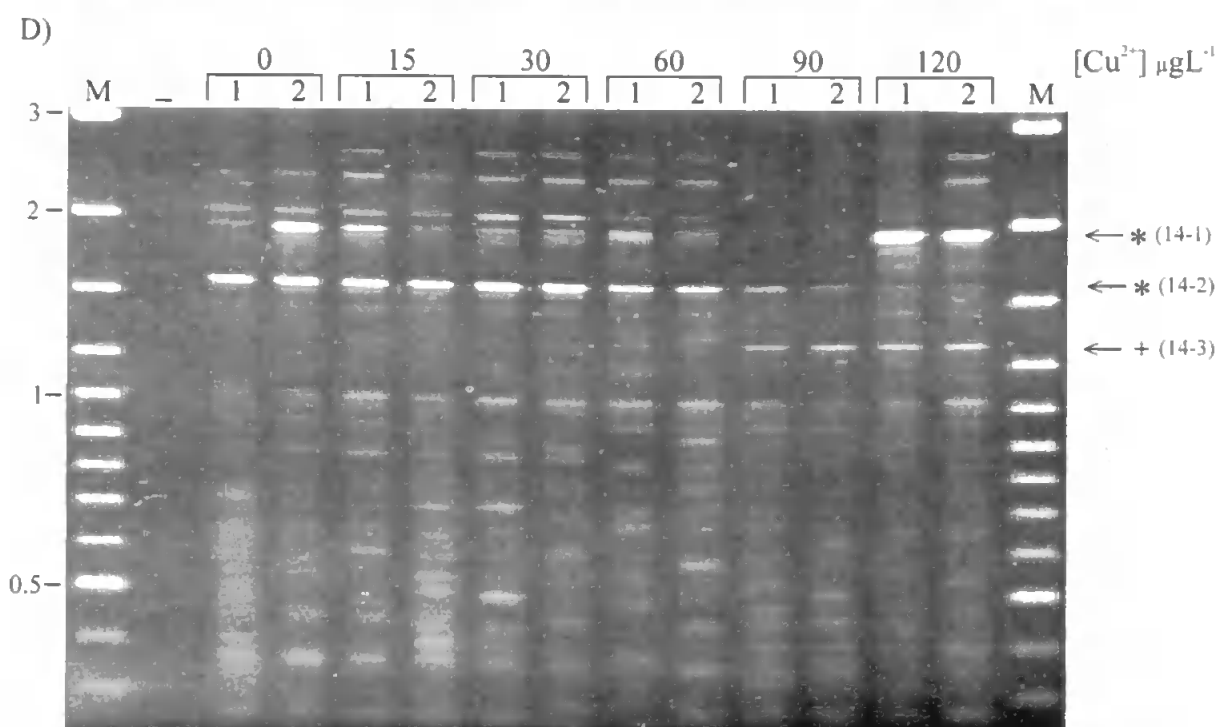
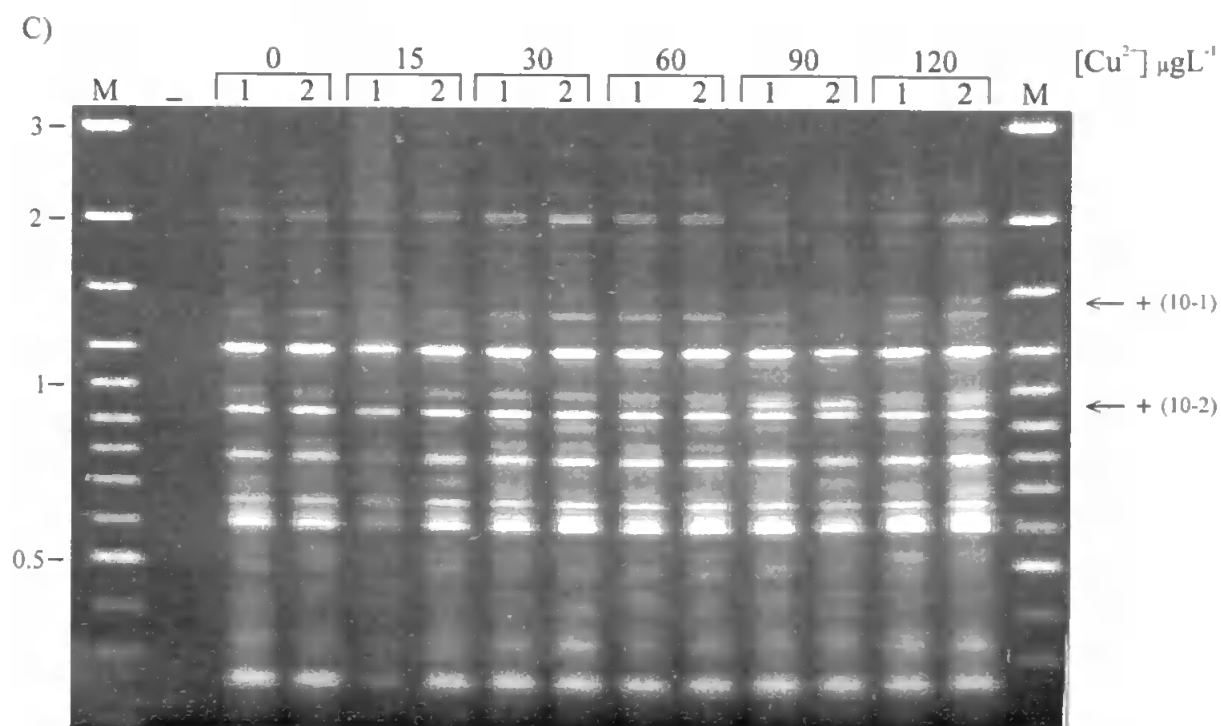


Figure 7.4 RAPD profiles of *Daphnia magna* exposed to copper. Concentrations as indicated at the top of the gels. M = DNA molecular size marker (GeneRuler™ 1000bp DNA ladder plus, Igi). The molecular sizes (in Kb) of selected bands are indicated on the left of each gel. - = no DNA control. RAPD profiles were generated using 10-mer primer



OPA9 (A), OPB7 (B), OPB 10 (C), and OPB14 (D) and a mixture of 4 *D. magna* for each concentration and replicate. 1 and 2 refer to replicate 1 and 2, respectively. Selected changes are indicated by arrows in comparison to control patterns; *: variation in band intensities, +: appearance of a new band, and -: disappearance of a band.

modifications. Although some changes in RAPD profiles arose at 15 and 30 μgL^{-1} Cu [e.g. band 9-2 (increase in band intensity), 9-4 (disappearance of bands)], most of the modifications occurred in the RAPD patterns obtained from *D. magna* exposed to 60, 90, and 120 μgL^{-1} Cu. Extra bands appeared only for the 3 highest Cu concentrations (e.g. bands 10-1, 10-2, 14-3) whereas band 9-4 was only present in the control profiles. In addition, an increase in band intensity was the major event arising from exposure to the 2 lowest Cu concentrations. In contrast, every type of modification was well represented in the patterns produced by *D. magna* exposed to the 3 highest Cu concentrations although the most frequent event was a decrease in band intensity. Figure 7.5 shows that band intensity (selected from 28 bands) followed 2 different tendencies. Intensity of 60 % of the bands (17 out of 28) increased and decreased in the ranges 15-60 and 90-120 μgL^{-1} Cu, respectively, compared to control intensities (curve b). In contrast, the average intensity of the remaining bands (40 %) increased at all Cu concentrations (curve a). Finally, some of the modification in profiles generated by a mixture of *D. magna* and primer OPB14 (Figure 7.6C) were similar to those obtained from single individuals in both replicates (Figure 7.6A and 7.6B). This is the case for bands 14-1, 14-2, and 14-3.

7.3.5 Comparison of fitness parameters with genomic template stability

To compare the sensitivity of the parameters presented in Figure 7.7, changes in each factor were calculated as a percentage of their control value (set to 100 %). All the parameters presented in Figure 7.7 were measured throughout the experiment except for *D. magna* exposed to 120 μgL^{-1} Cu for which the parameters were measured for 10 d. Changes in RAPD profiles were expressed as reductions in GTS in relation to profiles obtained from control *D. magna*. GTS calculated with 5 primers (OPA9, OPB7, OPB8, OPB10, and OPB17) is presented in Table 7.2. All parameters presented in Figure 7.7 were significantly different at 90 and 120 μgL^{-1} Cu ($p < 0.05$) except that I_x which was only

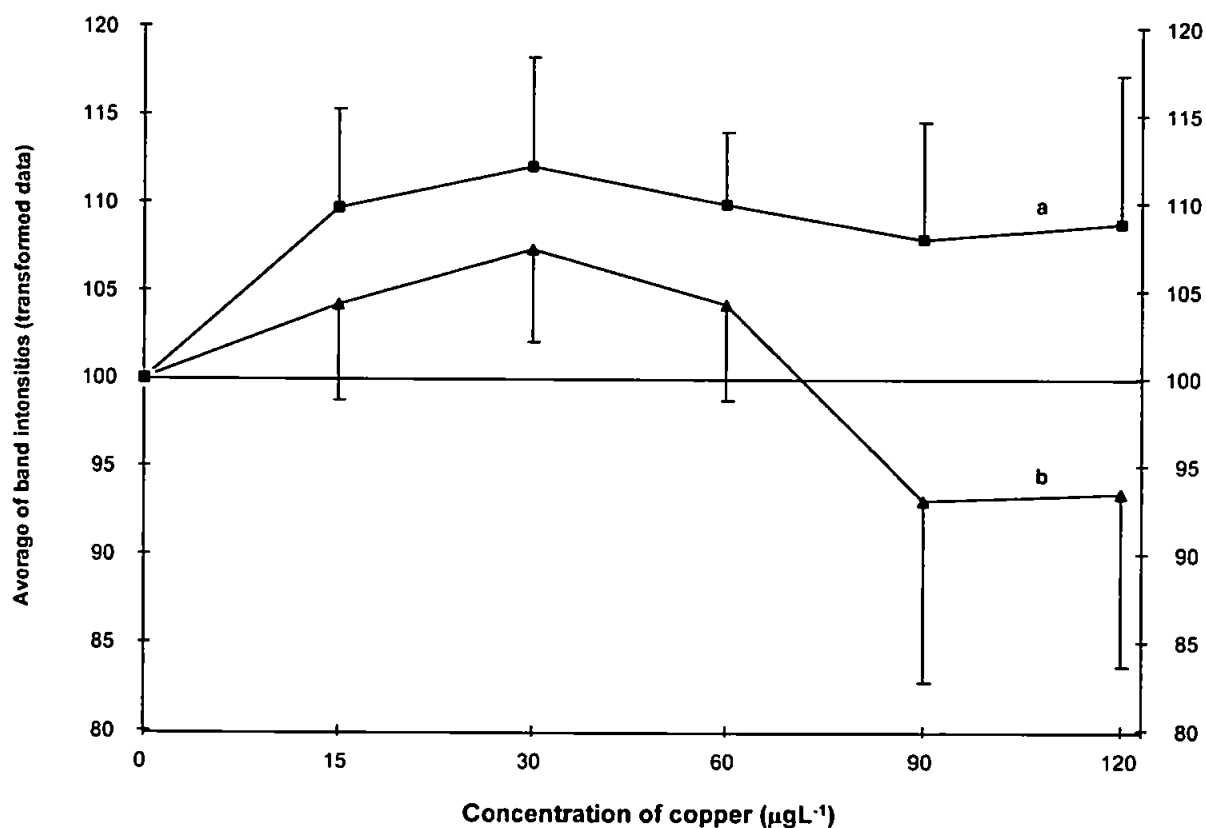
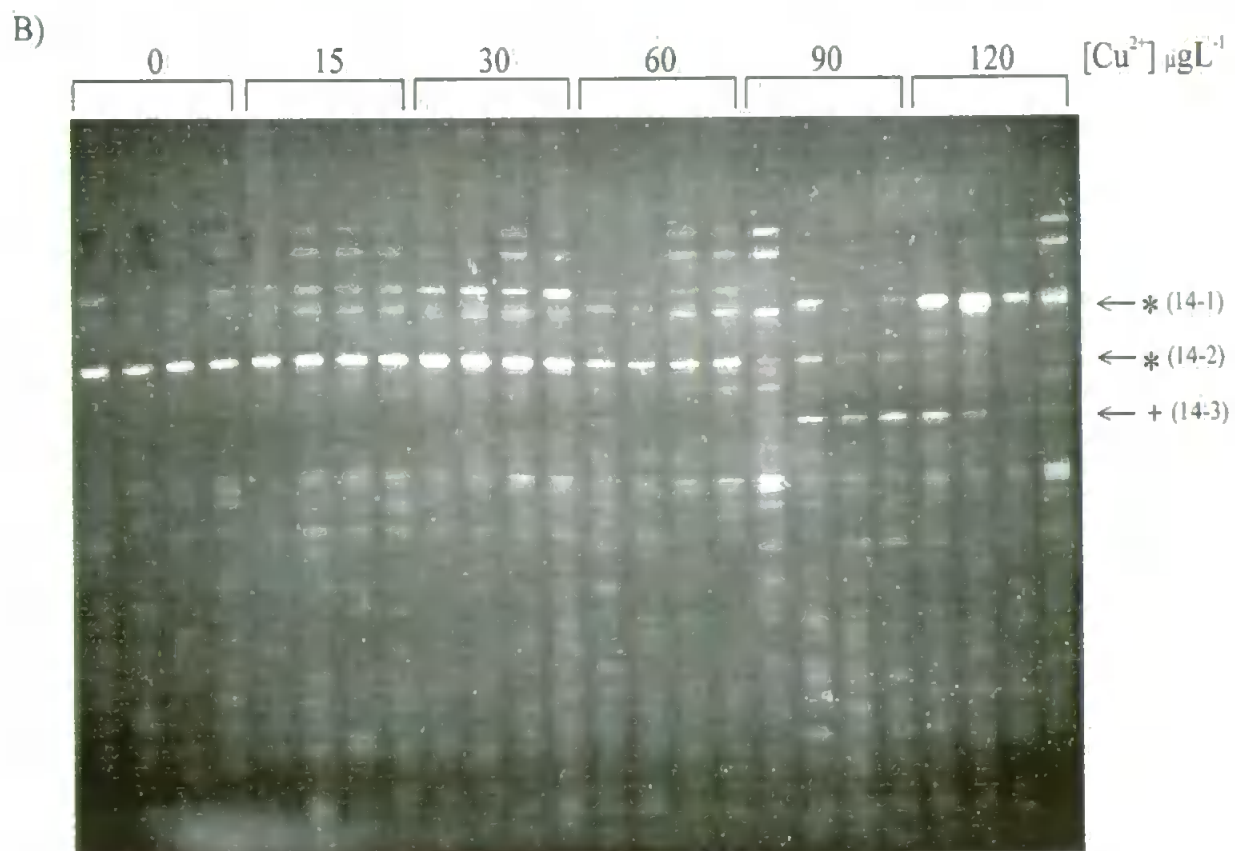
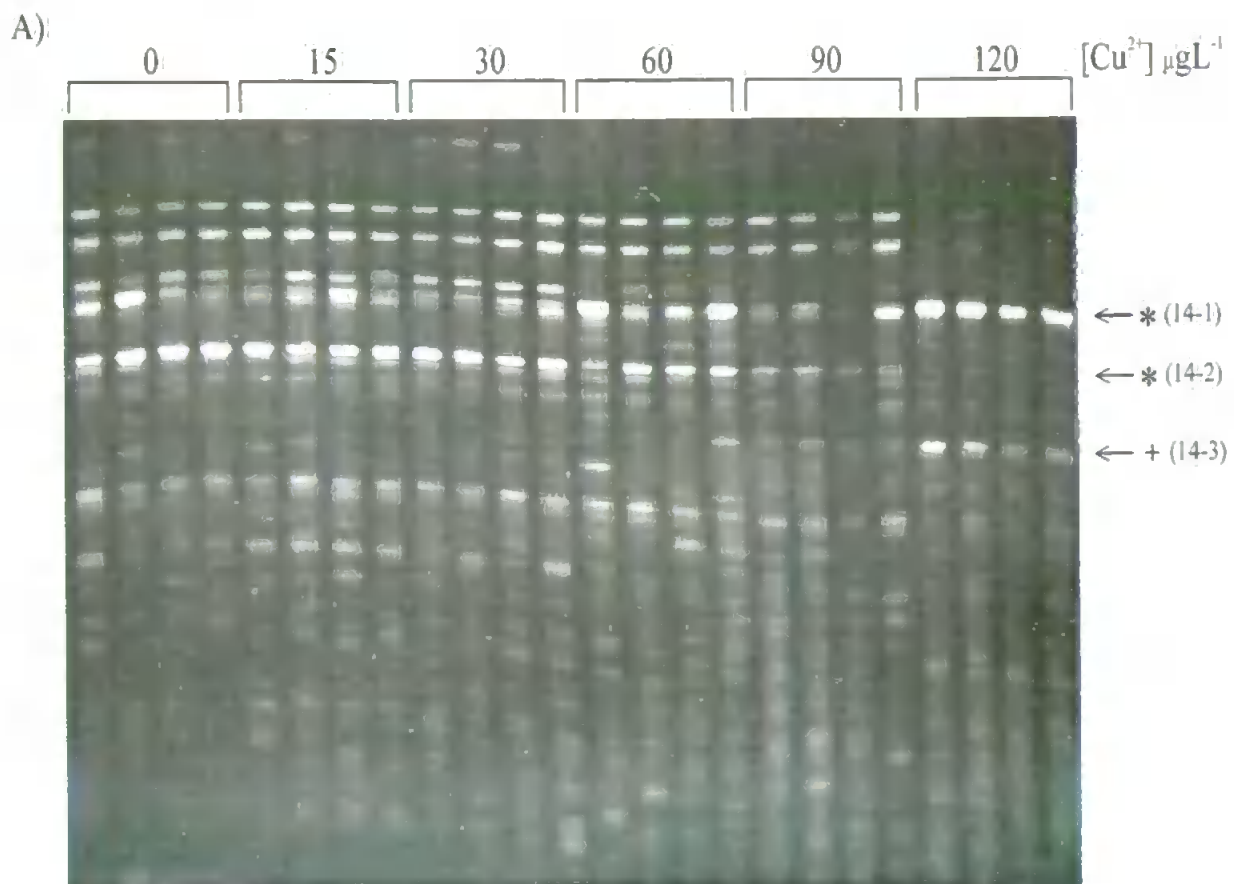


Figure 7.5 Variation of band intensities selected from RAPD profiles generated by *Daphnia magna* exposed to copper. A total of 28 relatively intense bands (generated by 5 primers) appearing across the width of the gels were arbitrarily selected. The band intensities were calculated as a percentage of its own control value (set to 100 %). As band intensity followed 2 different patterns, average and standard deviation were calculated with 11 (—■—, curve a) and 17 (—▲—, curve b) bands.



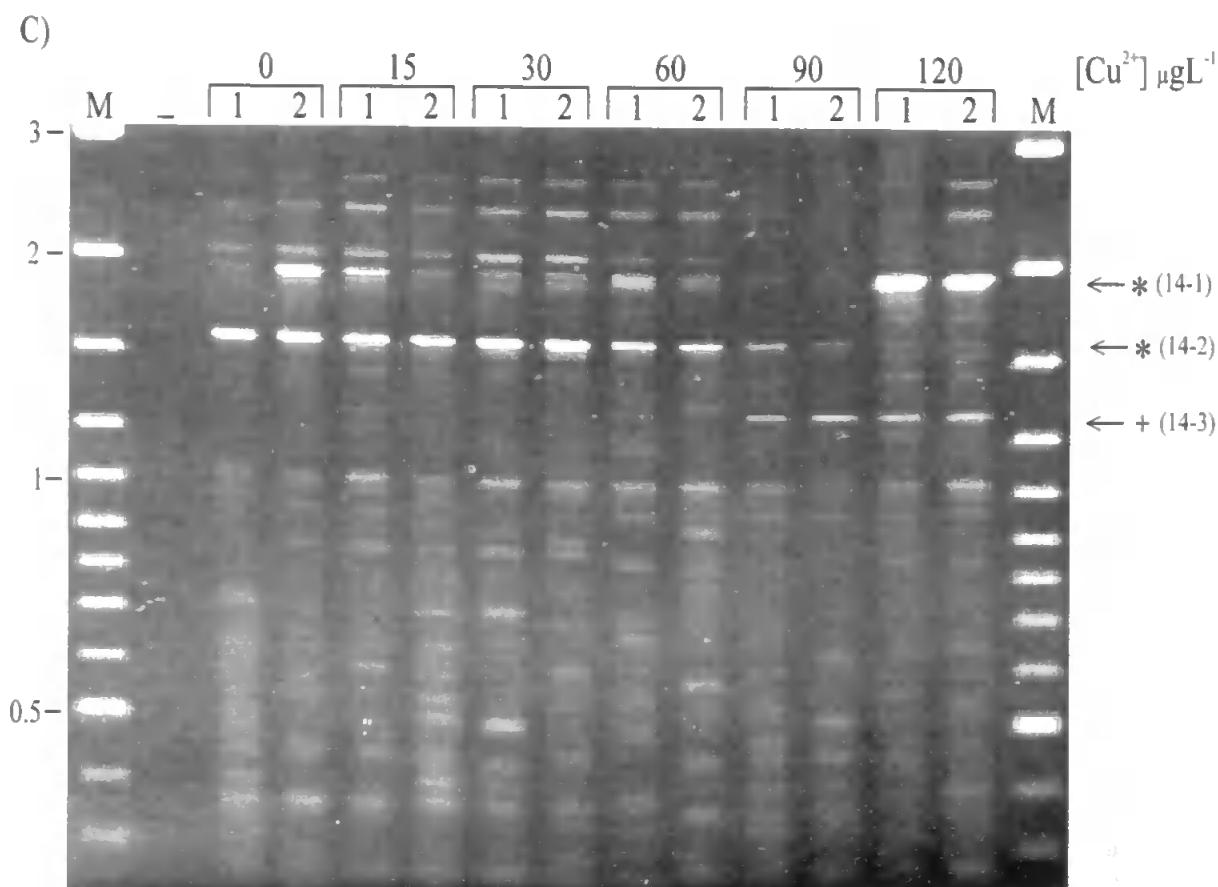


Figure 7.6 RAPD profiles of individuals and mixture of *Daphnia magna* exposed to copper. Concentrations as indicated at the top of each gel. Selected changes are indicated by arrows in comparison to control patterns; *: variation in band intensities, and + : appearance of a new band. PCR reactions were performed using primer OPB14. A) and B) RAPD patterns generated by replicate 1 and 2, respectively. Each line represents a single individual. C) profiles obtained from a mixture of 4 individuals using both replicates. 1 and 2 refer to replicate 1 and 2, respectively. M = DNA molecular size marker (GeneRuler™ 1000bp DNA ladder plus, Igi). The molecular sizes (in Kb) of selected bands are indicated on the left of each gel. - = no DNA control.

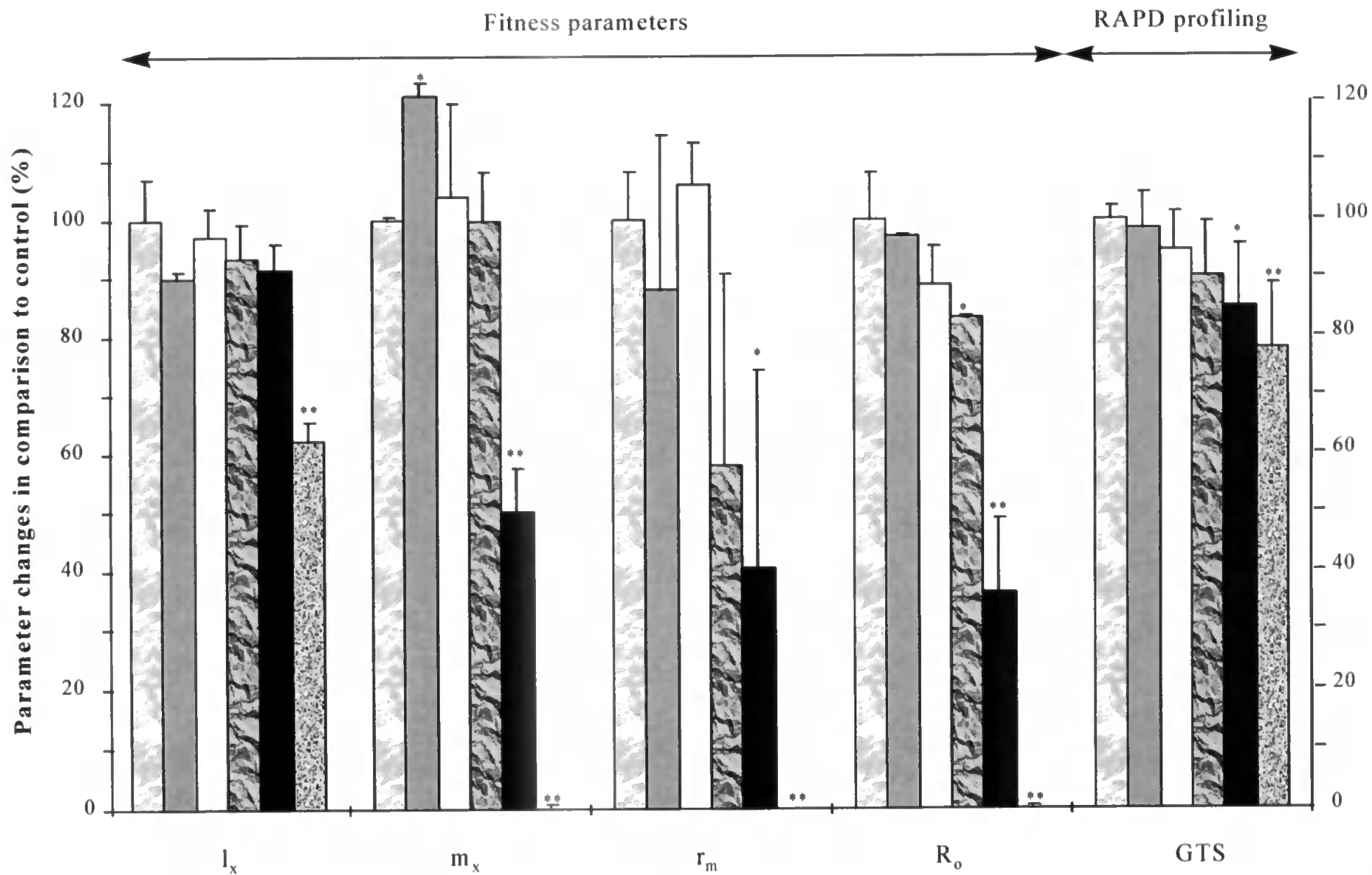








Figure 7.7 Comparison between key fitness parameters and genomic template stability (GTS) in populations of *Daphnia magna* exposed to copper. l_x = age-specific survival; m_x = age-specific fecundity; R_0 = net reproductive rate, and r_m = intrinsic rate of natural increase.  0,  15,  30,  60,  90, and  $120\mu\text{gL}^{-1}$ Cu. * and ** indicate a significant difference from control ($p < 0.05$ and $p < 0.01$, respectively). Error bars represent standard deviation.

Table 7.2 Genomic template stability calculated with five 10-mer primers.

[Cu] (µgL⁻¹)	Primer					Mean	SD
	OPA9	OPB7	OPB8	OPB10	OPB17		
0	95,3	100,0	100,0	100,0	100,0	99,1	2,1
15	86,0	100,0	100,0	100,0	100,0	97,2	6,2
30	86,0	94,7	87,5	100,0	100,0	93,7	6,7
60	74,4	89,5	91,7	90,0	100,0	89,1	9,2
90	67,4	84,2	83,3	90,0	95,4	84,1	10,5
120	60,5	78,9	75,0	80,0	90,9	77,1	11,0

significantly different at the highest Cu concentration. The general tendency of all measured parameters was a progressive decrease with increasing Cu concentration except for the age-specific fecundity which was significantly higher than control at $15 \mu\text{gL}^{-1}$ Cu ($p < 0.05$). Overall, m_x and R_0 were the most sensitive parameters. However, the GTS calculated from a single 10-mer primer (e.g. OPA9) was more sensitive for the detection of toxic effects than the fitness parameters because GTS differed significantly from the control at all Cu concentrations.

7.4 Discussion

To further evaluate the potential of the RAPD technique to detect DNA effects, *D. magna* were exposed to Cu, which was reported to induce diverse kinds of DNA damage (see section 7.1). However, besides the possible generation of DNA damage, it is well established that Cu is an essential element of living systems (Linder, 1991) and it is not surprising to find this essential element in Elendt's medium (Elendt and Bias, 1990; see section 7.2.2.2).

In the present study, the variation in band intensity, disappearance of bands, and appearance of new PCR products occurred in profiles generated from exposed organisms. Cu-induced DNA damage (e.g. Cu(I)-DNA complex, bulky adducts, oxidised bases) may significantly interfere with the PCR events and consequently affect the interaction of the primers with the priming sites and the activity of the *Taq* DNA polymerase. It has been reported that in naked double-stranded DNA the transition from weak complex DNA-Cu(II) to strong complex DNA-Cu(I) (George *et al.*, 1987) could induce conformational B-to-Z conversion in certain DNA fragments (Prütz *et al.*, 1990). In addition, dimers can cause alterations of the secondary and tertiary structure of the DNA (Wang and Taylor, 1991; 1993). If so, such structural effects are likely to have a significant effect on the

dynamics of PCR reactions. For instance, new PCR products can be amplified because some sites become accessible to the primers after structural changes. However, the loss of a band can only be the outcome if the same structural changes occur in nearly all the cells, which is very unlikely. DNA lesions such as bulky adducts are also expected to have detrimental effects on RAPD profiles. Not only can they induce structural changes, but they can also reduce the polymerisation of DNA and block the *Taq* DNA polymerase (Strauss, 1985) which will result in a decrease in band intensity or, alternatively, in a disappearance of amplified products. Moreover, new PCR products may reveal a change in the DNA sequence due to point mutations and/or large rearrangements (see section 5.4). Due to the rapid growth of *D. magna* and the presence of DNA damage, it is very likely that, in the present study, mutations occurred after replication (see section 5.4). Prütz *et al.* (1990) as well as Rodriguez *et al.* (1995) reported that the interaction between DNA and Cu is not random. This suggests that DNA damage which is created at specific spots may lead to identical mutations (among the exposed cells) after DNA replication. Finally, the increase in band intensity can be ascribed to DNA effects which alter the accessibility of *Taq* DNA polymerase and primers. For instance, the fact that the PCR enzyme is blocked at certain sites may allow a more efficient amplification of non-damaged genomic DNA. Alternatively, a greater availability of 10-mer primers could potentially increase band intensity.

The results of this study revealed that relatively high Cu concentrations (90 and 120 $\mu\text{g L}^{-1}$ Cu) induced a deleterious effect on the growth and reproduction of *D. magna*. Accordingly, genomic template stability was also significantly affected in the same range in comparison to the control (Figure 7.7). Thus, it seems that for the 2 highest Cu concentrations DNA replication was significantly reduced due to a high level of DNA effects. On the other hand, as growth and reproduction displayed similar values compared to the control in the range 15-60 $\mu\text{g L}^{-1}$ Cu, it could be assumed that DNA lesions were

efficiently repaired and that DNA replication was not significantly inhibited. Interestingly, increases and decreases in band intensity were the major events represented in the range 15-30 and 60-120 μgL^{-1} , respectively (Figure 7.5, curve b). Similar results were obtained when macroalgae were exposed to ultraviolet radiation (chapter 6). However, the present study revealed that increases in band intensity occurred even for the highest Cu concentrations tested. This suggests that DNA extracted from *D. magna* exposed to either 90 or 120 μgL^{-1} Cu was not saturated by Cu-induced DNA damage. This contrasts with the study performed in Chapter 6.

Winner and Farell (1976) revealed that survival of four species of *Daphnia* (including *D. magna*) was reduced at concentrations beyond 40 μgL^{-1} Cu. Winner and Farell (1976) also reported that the two larger species were significantly less sensitive to an acute Cu stress than were the two smaller species. The 48h-LC50 for *D. magna* to Cu reported in the literature was for instance, 0.03 μgL^{-1} Cu (Meador, 1991), 6.5 μgL^{-1} Cu (Dave, 1984), 25.8 μgL^{-1} Cu (Koivisto *et al.*, 1992), 31.8 μgL^{-1} Cu (Koivisto *et al.*, 1992), and 93 μgL^{-1} Cu (Khangorot *et al.*, 1987). In the present study, the 48h-LC50 of Cu for *D. magna* (clone 5) was 165.1 μgL^{-1} Cu. As already reported, such variability can be ascribed to different factors such as medium quality, amount of food and the use of different clones of the same species (Meador, 1991; Soares *et al.*, 1992; Koivisto and Ketola, 1995). The 48h-LC50 values obtained in the acute toxicity of B(a)P and Cu were generally much higher than those reported in the literature because *D. magna* (used in the different studies presented in this research project) were cultured in a rich medium and fed high levels of algae. In accordance with an earlier study (Dave, 1984), a low Cu concentration (i.e. 15 μgL^{-1} Cu in the present study) stimulated reproduction, as age-specific fecundity was significantly higher than control. However, the total number of neonates generated by a population of *D. magna* exposed to either 0 or 15 μgL^{-1} Cu was statistically identical because the number of living animals decreased at 15 μgL^{-1} Cu in comparison to control

and all other groups, except for *D. magna* exposed to the highest Cu concentration. Thus, despite the significant increase in reproduction (m_x) at $15 \mu\text{gL}^{-1}$ Cu in comparison to control ($p < 0.05$), overall there was no beneficial effect in *D. magna* exposed to this concentration of Cu. The increase in reproduction can be explained by hormesis, a nonspecific stimulation to a chemical or physical agent (Luckey, 1975). In ecotoxicology, the intrinsic rate of natural increase (r_m) is the more relevant measure of toxic effects as it integrates potentially complex interactions in life-history traits (Forbes and Calow, 1999). In the present study, r_m was significantly reduced at the 2 highest Cu concentrations compared to control. r_m at $15 \mu\text{gL}^{-1}$ Cu was also lower (but not significantly lower) than control because of the shorter expectancy of life at the lowest Cu concentration. The growth experiment showed that *D. magna* exposed to 90 and $120 \mu\text{gL}^{-1}$ Cu had a significantly reduced body size. The decrease in growth following sub-lethal Cu exposure has been also demonstrated in other species of crustaceans (Conradi and Depledge, 1998). However, despite the smaller size, the animals were able to attain reproductive maturity without no delay in comparison to control *D. magna* (Lynch, 1985). In addition, the effect on growth was not due to a limitation in food levels as the size of the neonates at first reproduction was not affected (Enserik *et al.*, 1993) but was due to a direct toxic effect of Cu. In this study, nearly all the measured parameters indicated that *D. magna* exposed to 90 and $120 \mu\text{gL}^{-1}$ Cu were significantly affected. Among fitness parameters, the age-specific fecundity and the net reproductive rate were the most sensitive indicators of toxicity. In contrast, age-specific survival was only significantly different at the highest Cu concentration suggesting that mortality is not a sensitive parameter. Finally, changes in RAPD profiles were detectable even at $15 \mu\text{gL}^{-1}$ Cu (e.g. primer OPA9) which means that the RAPD technique was more sensitive than fitness parameters for the measure of toxicity. It must be borne in mind that genomic template stability greatly depends on the primer used. For instance, primer OPA9 revealed a significant difference at all Cu concentrations in comparison to control. However, in order

to modulate the effect of each primer, genomic template stability was calculated with 5 primers.

Normal aerobic metabolism in organisms may be responsible for the formation of intracellular concentrations of reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide radical (O_2^\cdot) (Halliwell and Aruoma, 1991). In addition, exposure to numerous xenobiotic and ionising radiation can result in the production of the highly reactive and electrophilic hydroxyl radicals (OH^\cdot) (Cadet *et al.*, 1999) due to a Fenton-type reaction (Fenton, 1894) between reduced or oxidised forms of certain transition ions (copper and iron) and H_2O_2 (Prutz *et al.*, 1990). Oxygen radicals have the potential to damage diverse biochemical macromolecules and to induce lipid peroxidation (Halliwell and Gutteridge, 1985), carbohydrate degradation (Cheeseman *et al.*, 1988), protein fragmentation (Wolff and Dean, 1986), and DNA damage (see references in section 7.1). In this context, it is likely that some of the changes occurring in RAPD profiles of Cu-exposed *D. magna* (when compared to controls) were due to oxygen radicals. In the same way, B(a)P- (chapter 5) and UV- (chapter 6) induced genotoxicity could be partly attributed to oxygen radicals. In addition, Cu which can interfere with multiple essential enzymes (e.g. cytochrome c oxidase, superoxide dismutase) has vital functions in many tissues, including the brain, and the immune system in human (Linder, 1991) and possibly in *D. magna*. Consequently, Cu is likely to interact with the physiology and the metabolism of the cells. Oxygen radicals are also expected to have some effects on the physiology. It is now well established that cellular and molecular responses to physiological and environmental stress induces a rapid and transient change in gene expression associated with major changes in nuclear architecture that impacts on signals involved in cell growth (Jolly and Morimoto, 1999). It is hypothesised that variation in gene expression may have some effects on RAPD profile. Indeed some of the structural changes induced by modulation in gene expression may be stable during denaturation of the genomic DNA

during the PCR reaction. Consequently, these structural effects can induce changes in RAPD profiles.

In conclusion, the RAPD method has been successfully used as a sensitive means of detecting Cu-induced DNA effects (including DNA damage, mutations and possibly variation in gene expression). In addition, the measure of parameters at both molecular and population levels represents a valuable tool in ecotoxicology. In agreement with the studies using B(a)P and UV radiation, the nature and amount of DNA effects is open to speculation. At this stage, it was important not only to further evaluate the potential of the RAPD technique to detect DNA effects in aquatic organisms induced by a class of chemicals able to directly and/or indirectly interact with genomic DNA, but also to better understand the effect of oxygen radicals and variations in gene expression on RAPD profiles.

Chapter 8

EVALUATION OF THE RAPD METHOD FOR THE DETECTION OF DNA EFFECTS INDUCED BY OESTROGENS, XENO-OESTROGENS AND BY STRESSFUL CONDITIONS

8.1 Introduction

This chapter further evaluates the potential of the RAPD method to detect changes in patterns following the exposure of organisms to potent genotoxins and to stressful conditions. In particular, RAPD was used to assess the potential DNA effects induced by oestrogen and xeno-oestrogen compounds (study 1 and 2) and by stressful conditions in the absence of xenobiotic compounds (study 3). Oestrogens are particularly interesting because they are classified as non-genotoxic and non-mutagenic compounds (Barett *et al.*, 1981) although elevated levels of oestrogens are known to be carcinogenic (Marselos and Tomatis, 1992). A further objective was to evaluate whether stressful conditions such as prolonged physical activity, heightened levels of oxygen, and a lack of essential elements, in the absence of xenobiotic compounds may induce changes in RAPD profiles. This would allow an evaluation of the contribution of oxygen and oxygen radicals, as well as variations in gene expression, to any observed changes in RAPD patterns.

Xeno-oestrogens (or oestrogenic xenobiotics) represent a range of structurally diverse anthropogenic chemicals which interact with oestrogen receptors in vertebrates (White *et al.*, 1994). They may as well have an effect on hormone metabolism, synthesis, storage, release, transport and clearance (Phillips and Harrison, 1999). Xeno-oestrogens can disrupt normal hormonal activity and result in, for example, the feminisation of male fish (Jobling and Sumpter, 1993; Purdom *et al.*, 1994), developmental abnormalities in reptiles and invertebrates (Guillette *et al.*, 1994; Brown *et al.*, 1999), and infertility in birds (Fry *et al.*, 1987). However, numerous reports have revealed that hormonal disturbances also occur in invertebrates exposed to environmental pollutants such as copper (Depledge and Billingham, 1999). The induction of vitellogenin (Purdom *et al.*, 1994) and egg shell protein or zona radiata protein (Aruwke and Goksoyr, 1996) have been used as biomarkers of exposure to xeno-oestrogens. One of the best studied xeno-oestrogens is nonylphenol;

this is an alkylphenolic compound used in the preparation of lubricating oil additives, plasticisers, resins, detergents, and surface-active agents (White *et al.*, 1994). Nonylphenol is a relatively stable biodegradation product of nonylphenol ethoxylates generated during sewage treatment of common non-ionic surfactants (Ahel *et al.*, 1994; Bokern and Harms, 1997). White *et al.* (1994) demonstrated that although structural differences exist between alkylphenol and 17- β estradiol, alkylphenols are able to mimic the action of the natural hormone by inducing transcriptional activity of the receptor. Previous work demonstrated that several types of DNA damage (reviewed by Roy and Liehr, 1999) such as DNA single strand breaks [Han and Liehr, 1994a], chromosomal damage [Banerjee *et al.*, 1994], DNA adducts including 8-hydroxyguanine [Han and Liehr, 1994b], oestrogen-DNA adducts [Lutz *et al.*, 1982; Bhat *et al.*, 1994], endogenous indirect DNA adducts [Liehr *et al.*, 1986], and lipid DNA adducts [Wang and Liehr, 1995] can be induced by oestrogens such as 17 β -estradiol (natural oestrogen) and diethylstilbestrol (synthetic oestrogen). Genotoxicity of oestrogens must be an important contributor to the induction of adverse effects because oestrogen receptor-mediated events alone cannot explain the carcinogenic adverse properties of oestrogens (Roy and Liehr, 1999). However, the lack of mutagenic activity of oestrogens in bacterial and mammalian cell mutation assays (Barrett *et al.*, 1983; Glatt *et al.*, 1979) and the fact that oestrogen DNA adducts could not be always detected (Epe and Metzler, 1985) has led to the categorisation of oestrogens as non-genotoxic and non-mutagenic compounds. Nonylphenol has been demonstrated to accelerate the growth of mammary glands and alter epithelial cell-cycle kinetics in the mammary gland (Colerangle and Roy, 1996). Such effects are considered as important factors for the development of genetic instability (Preston-Martin *et al.*, 1990). Finally, the weak oestrogenic activity of nonylphenol does not explain its profound effect on cell proliferation (Colerangle and Roy, 1996).

In a study performed by Hartmann *et al.* (1994) the single cell gel electrophoresis assay was used to detect the presence of DNA strand breaks and alkali-labile damage in peripheral white blood cells from three volunteers after physical activity. The results indicated that physical activity above the aerobic-anaerobic threshold caused DNA breakages and reached its maximum 24 h later although no sister chromatid exchange was detected (Hartmann *et al.*, 1994). In a similar experiment, Tice *et al.* (1990) did not find any significant differences between samples taken before and after physical activity, presumably because the test was performed too early after the physical exercise (i.e. 6 min). More recently, it was confirmed that DNA damage occurs in human leucocytes after intensive endurance exercise, possibly because of the release of reactive oxygen species by neutrophils (Niess *et al.*, 1998). Lepage *et al.* (1999) reported that 8-hydroxyguanine is one of the major products formed by the reactive oxygen species which are generated in living cells as a consequence of either the normal metabolic pathways or exogenous chemical or physical stress. The results of other studies have indicated that physical stress can induce glucocorticoid receptor-mediated apoptosis of rat thymocytes (Concordet and Ferry, 1993). Bast (1986) emphasised the fact that oxygen is toxic to aerobic species. Reactive oxygen species, produced in cells by a variety of mechanisms, can damage DNA and cause mutations (Troll and Wiesner, 1985; Breimer, 1990; Feig *et al.*, 1994b). They are considered to be one of the major contributors to DNA damage and mutagenesis (Newcomb *et al.*, 1999) and have been linked to different diseases (Marx, 1987). Furthermore, tandem base damage has been proposed as a significant component of free-radical induced DNA damage (Box *et al.*, 1995; 1997).

The objective of the first and second studies was to examine whether the exposure of larval barnacles (*Elminius modestus*) (study 1) and adult crabs (*Carcinus maenas*) (study 2) to 4-*n*-nonylphenol (studies 1 and 2), 17- β oestradiol (study 1) and diethylstilbestrol (study 2) induces DNA effects which can be detected by RAPD methodology. In the third

study, the RAPD technique was used to assess the DNA effects in *D. magna* subjected to stressful treatments including the use of distilled water lacking essential elements, constant stirring of the medium forcing the *D. magna* to swim, and the raised concentration of dissolved oxygen in the medium.

8.2 Materials and methods

The following studies were performed in collaboration with Dr. Zoe Billingurst (study 1), and Dr Shaw Bamber (study 2) who exposed the organisms to the oestrogens and xeno-oestrogens.

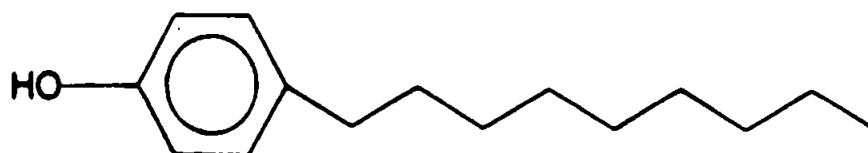
8.2.1 *Elminius modestus* exposed to 4-*n*-nonylphenol and 17- β oestradiol (study 1)

8.2.1.1 Culture of *Elminius modestus*

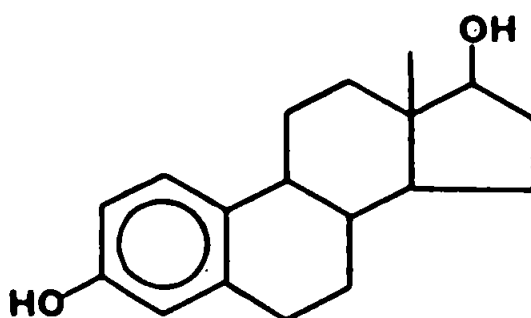
Adult barnacles were cultured at $22 \pm 2^{\circ}\text{C}$ in filtered seawater as described in section 3.1.7.

8.2.1.2 Experimental design

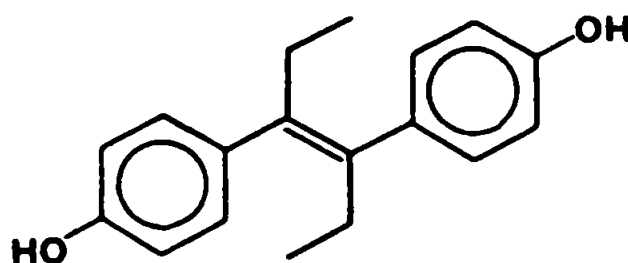
Test solutions were prepared from stock solutions of 4-*n*-nonylphenol and 17- β oestradiol (Figure 8.1) at a concentration of 1 mg/ml in acetone, prior to dilution in seawater. The maximum volume of the stock solution used was 10 μL in 1 L of seawater. A solvent control (barnacles exposed to 10 μL acetone in 1 L of seawater) was also included in the experiment. DNA extracted from the solvent control was the only control used in the RAPD experiments. Barnacle larvae (*Elminius modestus*) were exposed to 0, 0.1, 1, 5, and 10 $\mu\text{g L}^{-1}$ 4-*n*-nonylphenol as well as 10 $\mu\text{g L}^{-1}$ 17- β oestradiol for 8 d from release until the cyprid stage at 8 d. The larvae were maintained at 25°C , on a 8 h light / 16 h dark cycle and fed *Skeletonema costatum* daily (see section 3.1.7). The larvae were filtered daily and the water treatment renewed.



4-*n*-nonylphenol



17-β oestradiol



Diethylstilbestrol

Figure 8.1 Structures of natural (17-β oestradiol) and synthetic (4-*n*-nonylphenol, diethylstilbestrol) oestrogens. The only structural resemblance among these compounds is the presence of a hydroxyl group bound to benzene.

8.2.1.3 Generation of RAPD profiles

Total DNA was extracted and purified using a conventional phenol/chloroform method as described in section 3.2.2.1. The extracted DNA was electrophoresed on 1.2 % agarose gels at 100 V for 2 h and the DNA concentration of each sample was determined by comparison with known concentrations of Lambda phage DNA (see section 3.3.1). RAPD profiles were generated using 10-mer primers OPA9, OPB1, and OPB5 (see section 3.4.2) under the optimised conditions as described in section 3.4.1. PCR products were then electrophoresed on 1.2 % agarose gel at 90 V for 6 h, stained with ethidium bromide and visualised under UV light. A photograph was also taken and the image of the gel was saved electronically for further analysis when necessary (see section 3.5).

8.2.2 *Carcinus maenas* exposed to 4-*n*-nonylphenol and diethylstilbestrol (study 2)

8.2.2.1 Culture of *Carcinus maenas*

Crabs were maintained in aerated sea water at a temperature of $15 \pm 2^{\circ}\text{C}$ as described in section 3.1.5.

8.2.2.2 Experimental design

Separate stock solutions of 4-*n*-nonylphenol and diethylstilbestrol were prepared at a concentration of 2 mg/ml in methanol. Each stock solution was diluted to 1 ml in 20 L of seawater. The structure of diethylstilbestrol is presented in Figure 8.1. Individual groups of male and female crabs (*Carcinus maenas*) were exposed to $100 \mu\text{gL}^{-1}$ 4-*n*-nonylphenol and $100 \mu\text{gL}^{-1}$ diethylstilbestrol for 14 d. A solvent control consisting of 1 ml of methanol in 20 L of seawater was also included; no other control (i.e. crab in sea water) was performed. Haemolymph was collected at 0, 7 and 14 d following exposure from the same individual. In total, 64 animals (32 males and 32 females) were used throughout the experiment. Seawater was changed every 2 d and crabs were fed on cooked mussel flesh at a rate of 1 mussel per animal every 4 d. The number of animals utilised was limited to 3 males and 3

females for each of the three treatments i.e. solvent controls, animals exposed to $100\ \mu\text{gL}^{-1}$ 4-*n*-nonylphenol and diethylstilbestrol. In total 54 samples were analysed.

8.2.2.3 Generation of DNA profiles

Between 50-100 μl of haemolymph was used for the DNA extraction. The DNA extraction, determination of DNA concentrations, PCR conditions, and electrophoresis were identical to those described in section 8.2.1.3 except that primers OPA9, OPB6, OPB7, and OPB10 were used. In addition, the amount of DNA used in the PCR reactions was outside the optimal range. Please refer to section 8.2.4 for more details.

8.2.3 Daphnia magna exposed to stressful conditions (Study 3)

8.2.3.1 Culture of *Daphnia magna*

D. magna (clone 5) was cultured in M7 medium at $20 \pm 2^\circ\text{C}$ (see section 3.1.1).

8.2.3.2 Experimental design

D. magna were exposed to 3 different treatments, namely stirring of the medium, absence of essential elements and raised concentration of dissolved oxygen in medium, in order to induce stressful conditions. Five groups of 40 neonates less than 48 h post-hatch were placed in 1 L of either M7 medium [4 groups: no treatment (2 groups), stirring of the medium (1 group), and aeration of the medium (1 group)] or distilled water lacking essential elements (1 group). In one experiment, the M7 medium was stirred by using a stir plate (Ika[®]-Labortechnik, Staufen, Denmark) and a magnetic bar at a speed of approximately 60 rotations per min forcing the *D. magna* to swim continuously. In a separate experiment, the M7 medium was aerated using a single air stone. The bubbling which was kept at a relatively low level (approximately 5 bubbles per second) did not force the *D. magna* to swim. Each treatment lasted 24 h and the animals were not fed during the experiment. The temperature was maintained at $20 \pm 2^\circ\text{C}$. The concentration of the dissolved oxygen in the medium and pH were recorded before and after each treatment. The oxygen meter

(dissolved oxygen probe CelloX 325, Weillheim, Germany) was calibrated before use. 0.2 % oxygen (equivalent to $0.02 \text{ mgL}^{-1} \text{ O}_2$) was obtained by adding less than 1 g of sodium bisulfite (Sigma, Poole, UK) to 1 L of M7 medium. The highest dissolved oxygen concentration, which was of 94.4 % oxygen (equivalent to $8.99 \text{ mgL}^{-1} \text{ O}_2$), was obtained by using 3 air pumps connected to 3 air stones in 1 L of M7 medium. The pH meter (Mettler-Toledo Ltd., Halstead, UK) was calibrated according to the instructions of the manufacturer. At the end of each treatment the number of survivors was recorded.

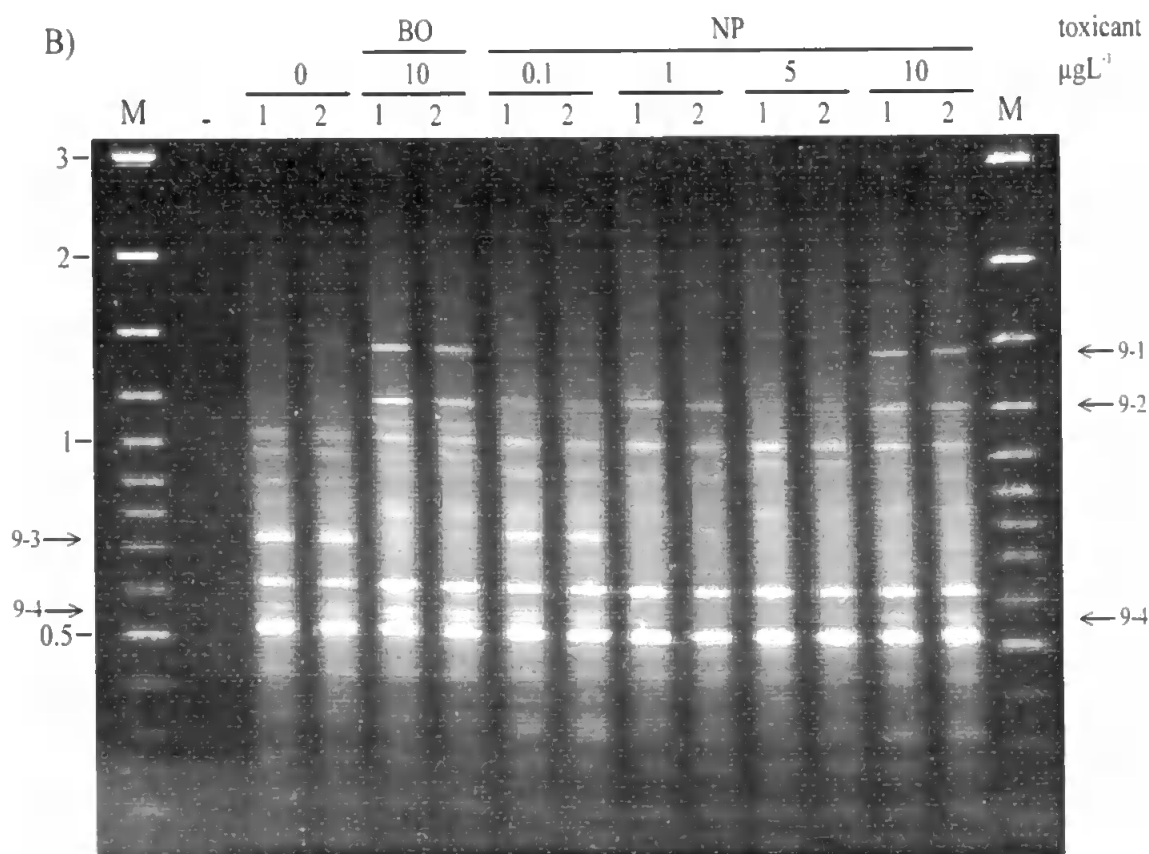
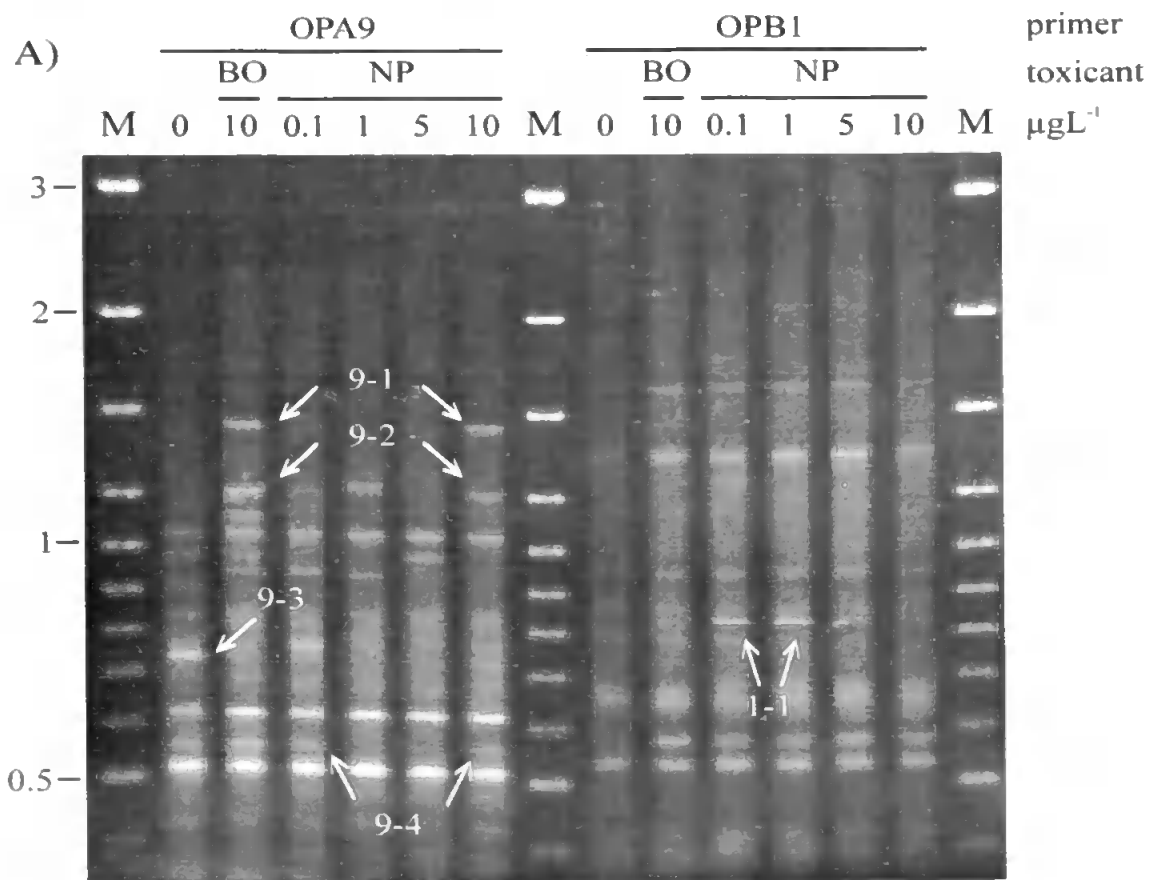
8.2.3.3 Generation of RAPD profiles

Total DNA was extracted and purified using a conventional phenol/chloroform method as described in section 3.2.1.1. The determination of DNA concentrations, PCR conditions, and electrophoresis were identical to those described in section 8.2.1.3 except that primers OPA9, OPB1, OPB5, OPB6, OPB7, OPB8, OPB10 and OPB17 were used.

8.3 Results

8.3.1 Barnacle larvae (*Elminius modestus*) exposed to 4-*n*-nonylphenol and 17- β oestradiol (study 1)

DNA extracted from larval barnacles that had been exposed to 4-*n*-nonylphenol and 17- β oestradiol produced RAPD profiles that displayed some differences in comparison to control patterns with primer OPA9 and OPB1 (Figure 8.2 and appendix III). For instance, bands 9-1 and 9-2 only appeared in profiles generated from barnacles exposed to $10 \text{ } \mu\text{gL}^{-1}$ 4-*n*-nonylphenol and 17- β oestradiol. In addition, band 9-3 which was present in the control profile was absent from all patterns obtained from the barnacles which had been exposed to either chemical, regardless of the concentration. Interestingly, band 9-4 was absent only in profiles generated by animals which had been exposed to 1 and $5 \text{ } \mu\text{gL}^{-1}$ 4-*n*-nonylphenol. The use of two DNA concentrations confirmed these results because the



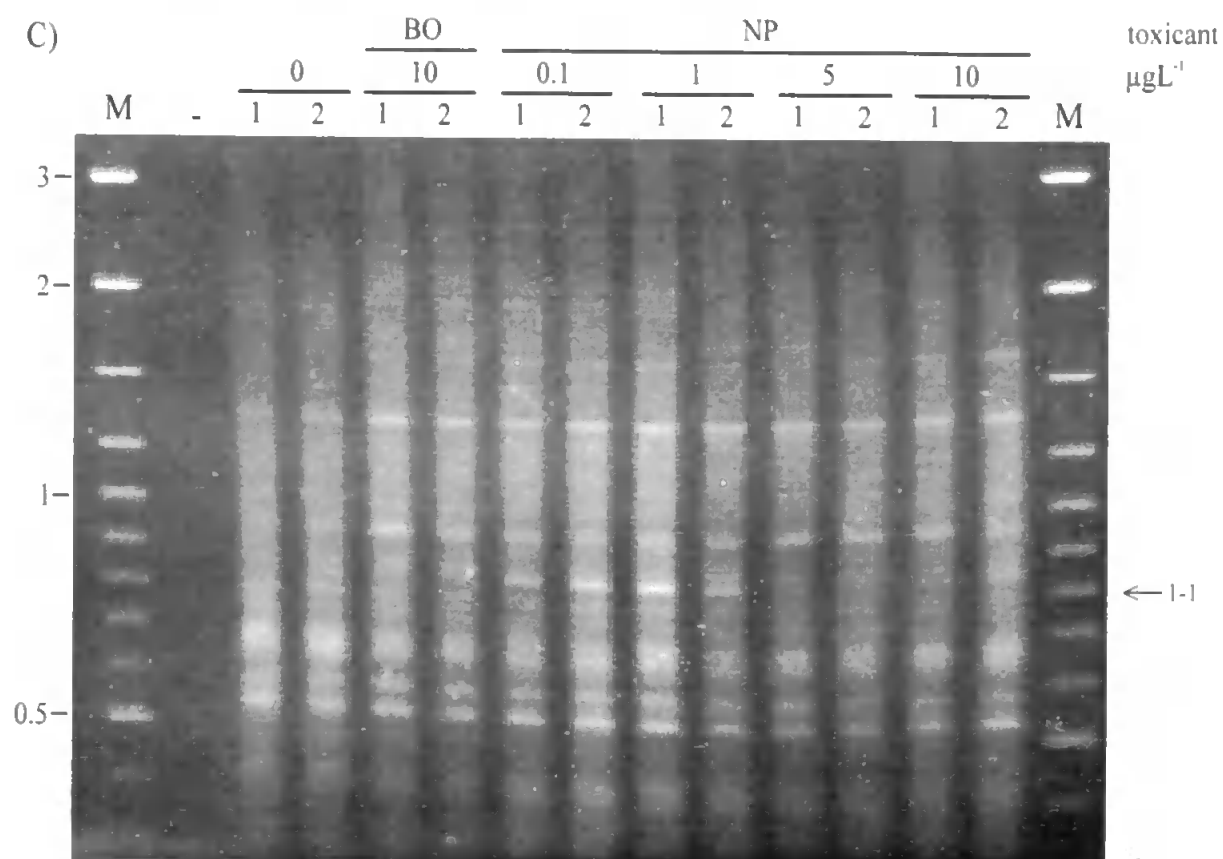


Figure 8.2 RAPD profiles of barnacle larvae exposed to 17- β oestradiol (BO) and 4-*n*-nonylphenol (NP). Concentrations as indicated at the top of each gel. O refer to the solvent control. In Figure 8.2A, primers as indicated at the top of the gel, in Figure 8.2B and 8.2C primers OPA9 and OPB1 were used, respectively. RAPD analysis was also performed using two DNA template concentrations (lanes 1 and 2: 10 and 2.5 ng genomic DNA, respectively), to check reproducibility of the profiles (B and C). M = 100 bp DNA ladder plus (IGi). The molecular sizes (Kb) of selected bands are indicated on the left of each gel. Selected changes are indicated by arrows in comparison to control patterns.

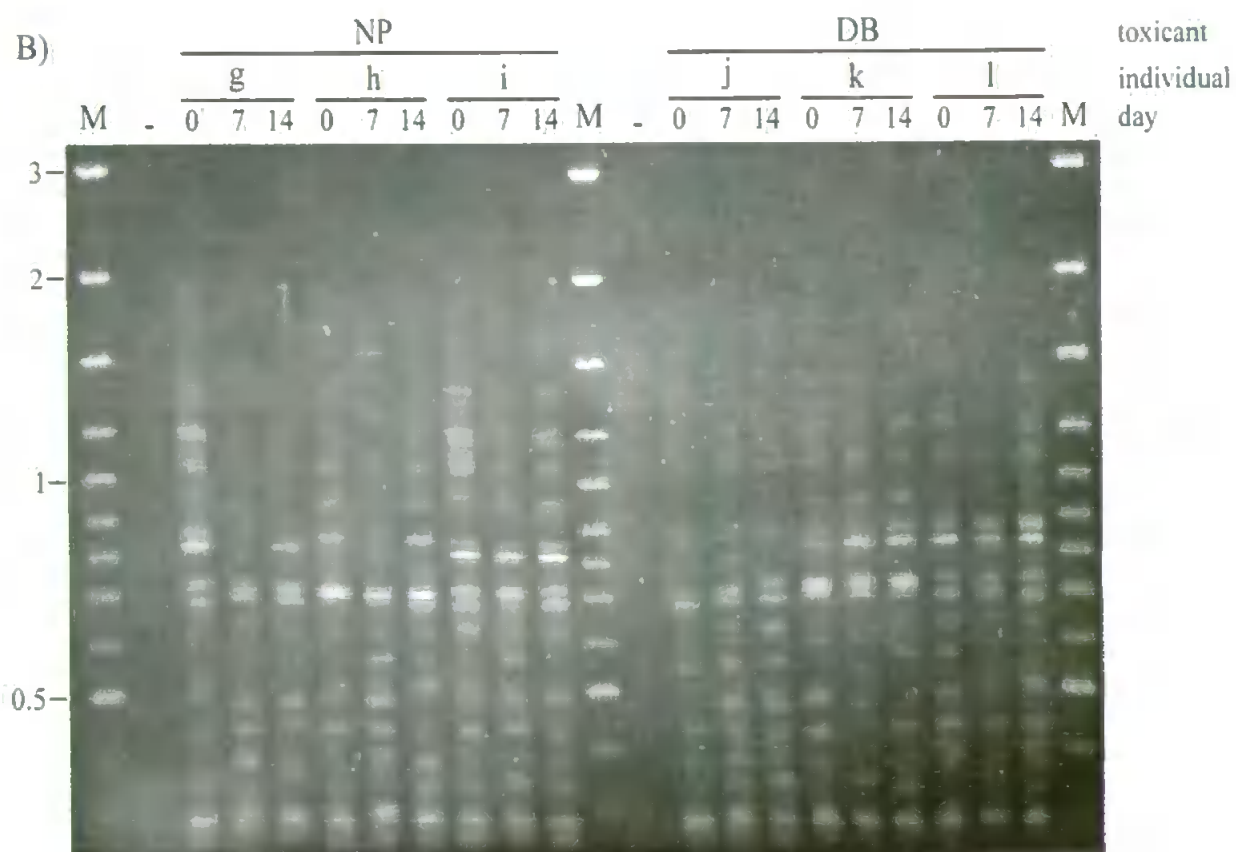
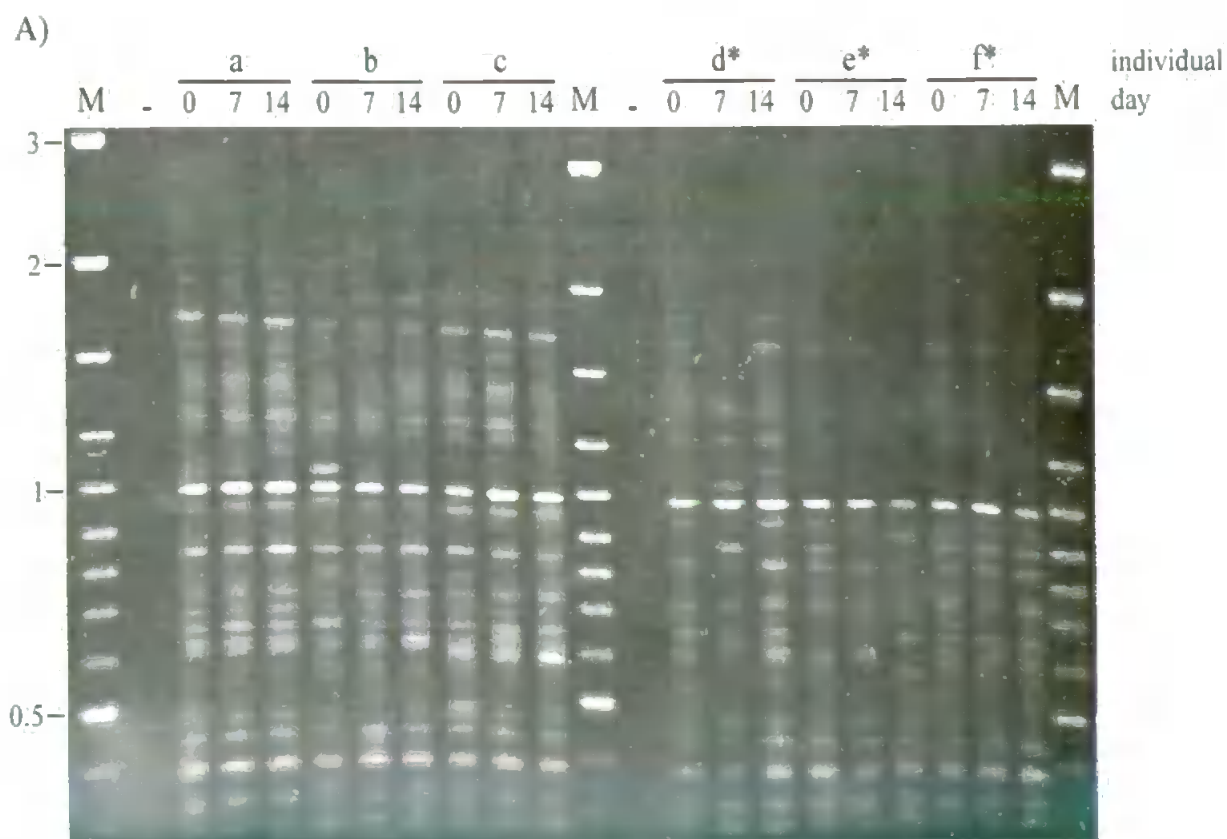
reproducibility of the RAPD profiles was optimal at both concentrations. Primer OPB1 also demonstrated that the profiles produced by the exposed barnacles differed from control animals but to a lesser extent than primer OPA9. For instance, using primer OPB1 band 1-1 appeared in patterns obtained from barnacles exposed to 0.1 and 1 μgL^{-1} 4-*n*-nonylphenol (Figure 8.2B). The use of two DNA template concentrations revealed that band 1-1 was reliably present at 0.1 and 1 μgL^{-1} 4-*n*-nonylphenol (Figure 8.2C). The RAPD profiles obtained with primer OPB5 and two DNA concentrations are presented in appendix III.

8.3.2 Adult crabs (*Carcinus maenas*) exposed to 4-*n*-nononylphenol and diethylstilbestrol (study 2)

Although some of the RAPD profiles generated using DNA extracted from control crabs were highly reproducible at days 0, 7, and 14 (e.g. individual 'a'), the quality of the RAPD patterns was poor for most of the samples (Figure 8.3A and appendix III). This was also the outcome when DNA template extracted from crabs exposed to 100 μgL^{-1} 4-*n*-nonylphenol and diethylstilbestrol was subjected to PCR (Figure 8.3B and appendix III). When RAPD reactions were performed using two DNA template concentrations the results clearly showed that the reproducibility of the assay was affected (Figure 8.3C).

8.3.3 *Daphnia magna* exposed to stressful conditions (study 3)

Table 8.1 shows the number of surviving *D. magna* as well as the variation in pH and the concentration of dissolved oxygen in the medium before and after each treatment. The number of surviving animals indicate that the two most stressful treatments were the stirring of the medium and the absence of essential elements (Table 8.1); only 37.5 % and 70 % of the *D. magna* survived these treatments, respectively. Although the rotation of the magnetic bar was as low as possible some of the *D. magna* might have died because of its



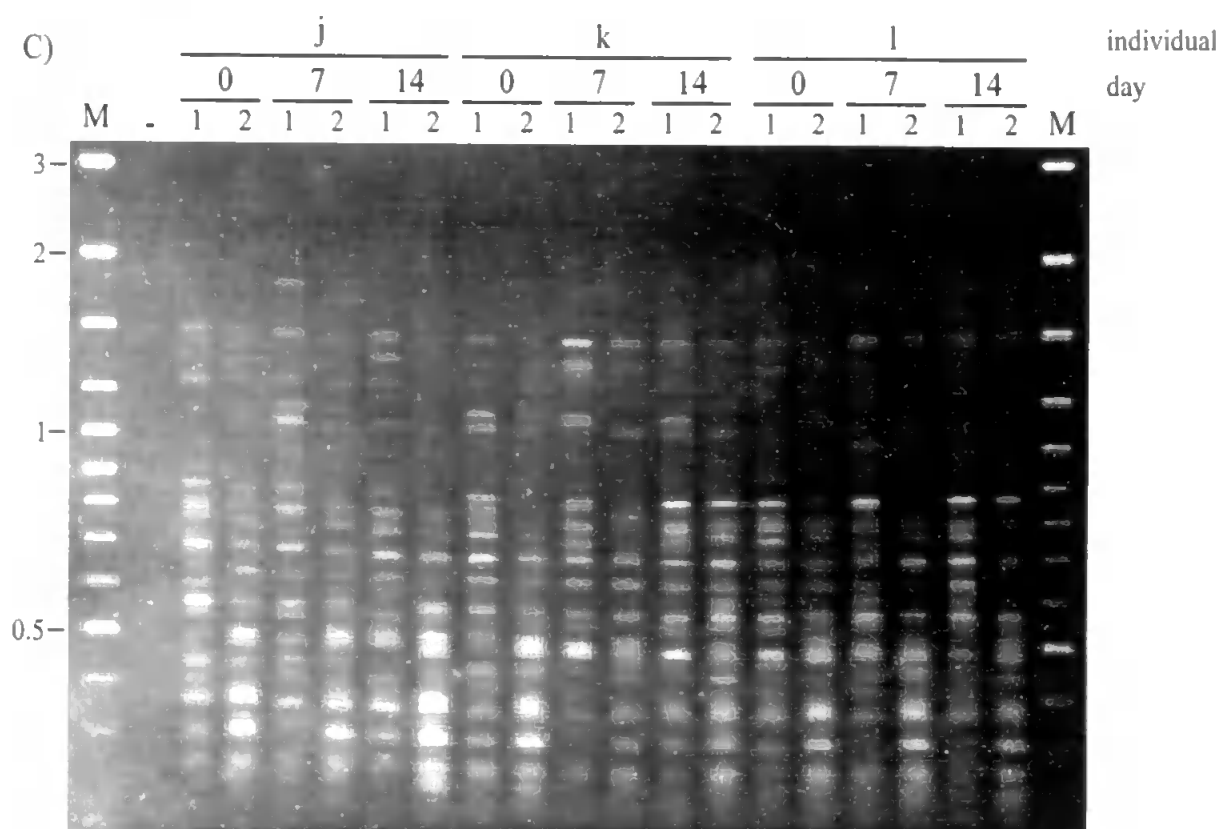


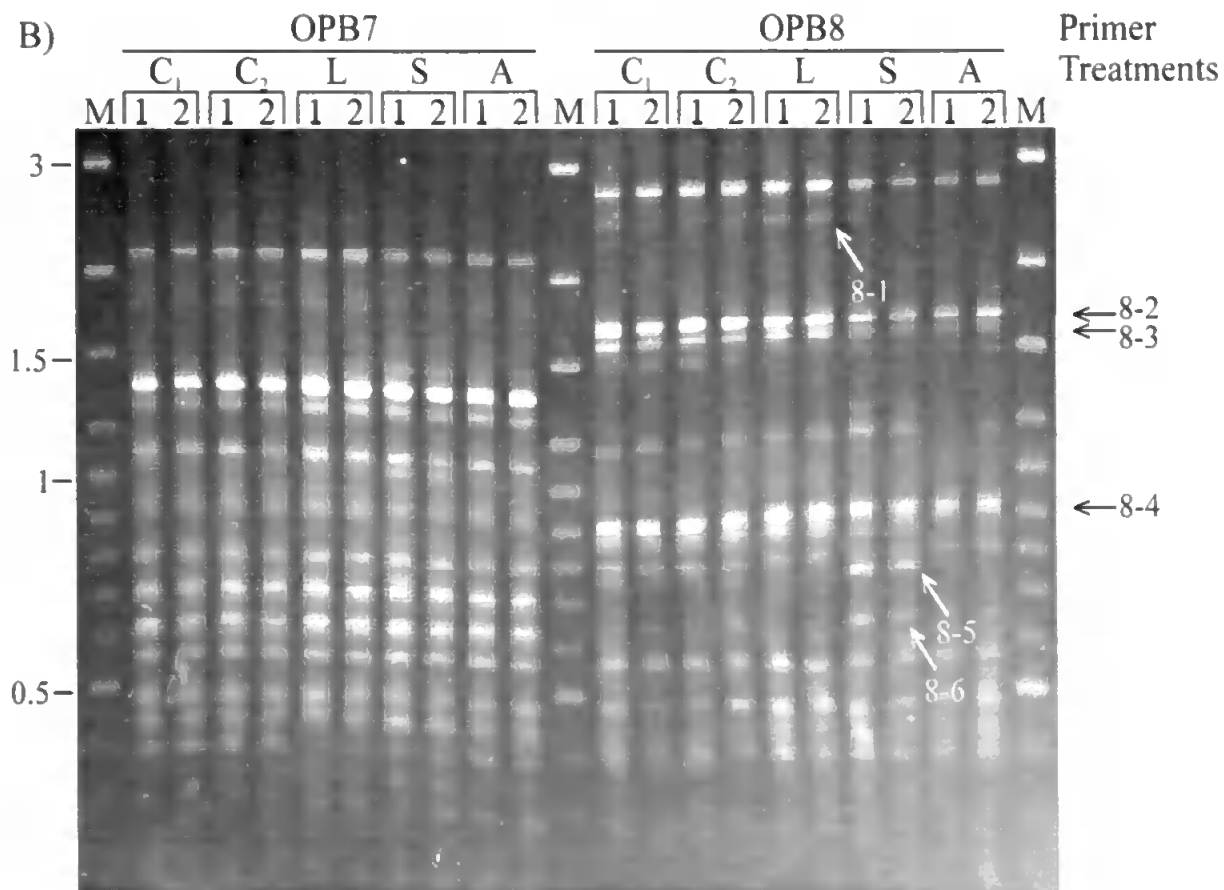
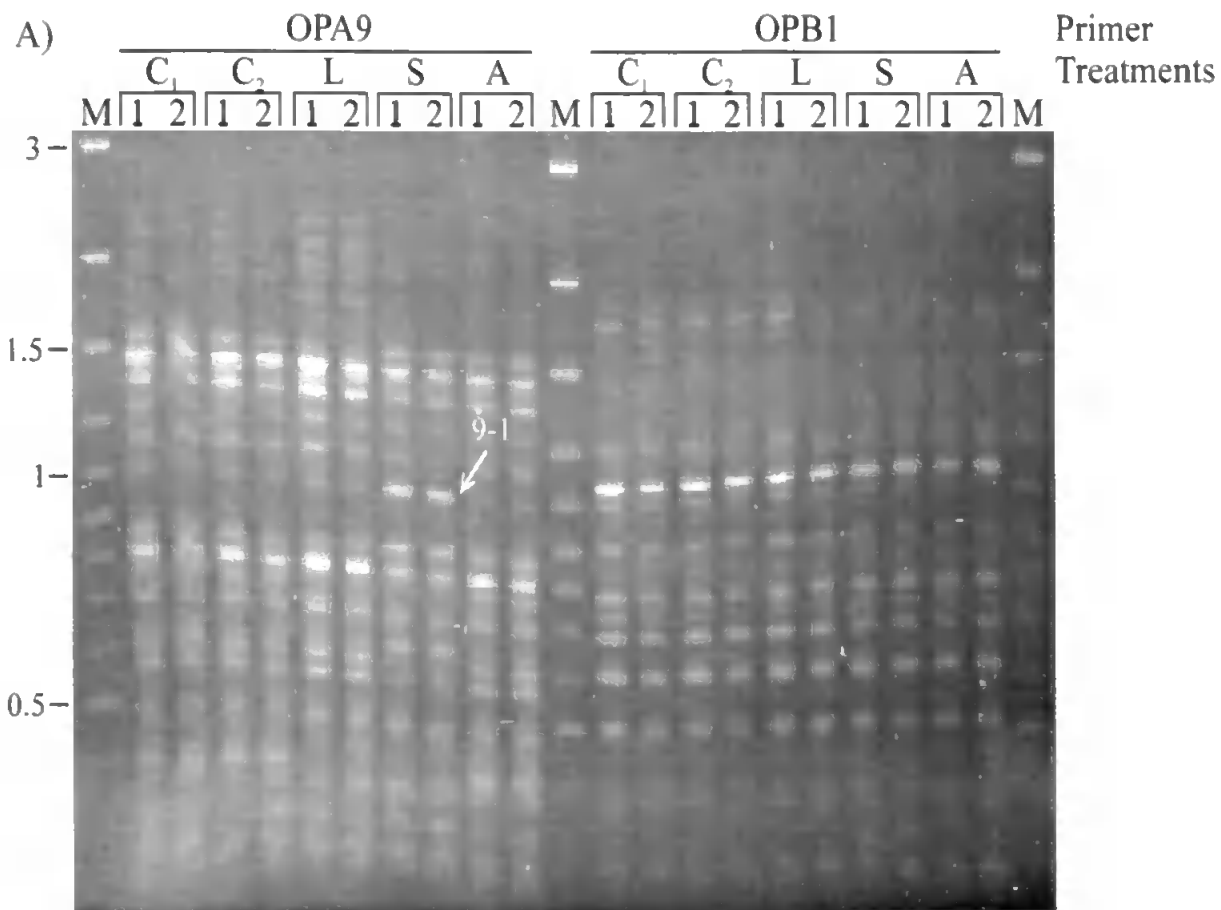
Figure 8.3 RAPD profiles of *Carcinus maenas* exposed to solvent, $100 \mu\text{gL}^{-1}$ of 4-n-nonylphenol (NP) or diethylstilbestrol (DB). A) control animals, B) treated animals, treatment as indicated at the top of the gels, C) reproducibility of RAPD profiles of animals exposed to $100 \mu\text{gL}^{-1}$ diethylstilbestrol using two DNA template concentrations , lane 1: $< 1 \text{ ng DNA}$, lane 2: $< 0.33 \text{ ng DNA}$. Profiles were generated at day 0, 7, and 14 as indicated in each gel. Primers used were OPB10 (A), OPB7 (B) and OPA9 (C). * indicates that female crabs were used. M = 100 bp DNA ladder plus (Igi). The molecular sizes (Kb) of selected bands are indicated on the left of each gel.

Table 8.1 Number of surviving *Daphnia magna*, pH, and concentration of dissolved oxygen in medium before (t = 0) and after (t = 24 h) each treatment. ^a and ^b indicate the value measured before and after each treatment, respectively. [O₂] refers to the concentration of dissolved oxygen. Although two control groups were used the results presented in this table represents only one of them. Nevertheless, these 2 control groups presented very similar values.

	No treatment	Stirring (~1 rotation/s)	Aeration (~5 bubbles/s)	Lack of essential elements (distilled water)
number of surviving <i>D. magna</i>	40 ^a 39 ^b	40 ^a 15 ^b	40 ^a 40 ^b	40 ^a 28 ^b
[O ₂] mgL ⁻¹	6.26 ^a 5.86 ^b	6.77 ^a 8.12 ^b	6.33 ^a 8.56 ^b	6.11 ^a 6.18 ^b
pH	7.69 ^a 7.77 ^b	7.69 ^a 7.85 ^b	7.61 ^a 7.94 ^b	6.35 ^a 6.40 ^b

direct action (e.g. animals crushed). On the other hand, increasing the level of dissolved oxygen from 6.33 to 8.56 mgL⁻¹ O₂ by aeration of the medium, had no effect on the survival of the *D. magna*. Finally, the variation in pH was minimal for each treatment (Table 8.1).

Figure 8.4 and appendix III display the RAPD profiles obtained from each treatment using two DNA template concentrations and 10 primers. Changes in RAPD profiles obtained from the populations of *D. magna* exposed to various sources of stress occurred compared to control patterns (e.g. Figure 8.4A primer OPA9; Figure 8.4C primer OPB10). Some of the variations arising in RAPD profiles are described in Table 8.2. More changes were apparent in RAPD profiles produced by the stirred *D. magna* rather than those generated from the two other treatments. For instance, the profiles generated using DNA extracted from the stirred animals revealed that the intensity of certain bands decreased (Figure 8.4B bands 8-2 to 8-4; Figure 8.4C bands 17-1 to 17-6) and that new bands appeared (Figure 8.4A band 9-1, Figure 8.4B bands 8-5 and 8-6, Figure 8.4C band 10-1). When *D. magna* were placed in distilled water for 24 h, the number of changes in RAPD patterns were very low compared to control profiles. The two only obvious changes were an increase in the intensity of bands 8-1 (Figure 8.4B) and 10-2 (Figure 8.4C) compared to control patterns. *D. magna* exposed to high levels of dissolved oxygen due to the aeration of the medium also revealed some changes compared to control RAPD profiles despite the fact that extra bands never appeared. The main modification occurring in the profiles generated by the population of *D. magna* exposed to raised levels of dissolved oxygen was a decrease in band intensity (Figure 8.4B bands 8-2 to 8-4; Figure 8.4C bands 17-1 to 17-6). Interestingly, stirring and aeration of the medium induced some common RAPD profile changes (Table 8-2).



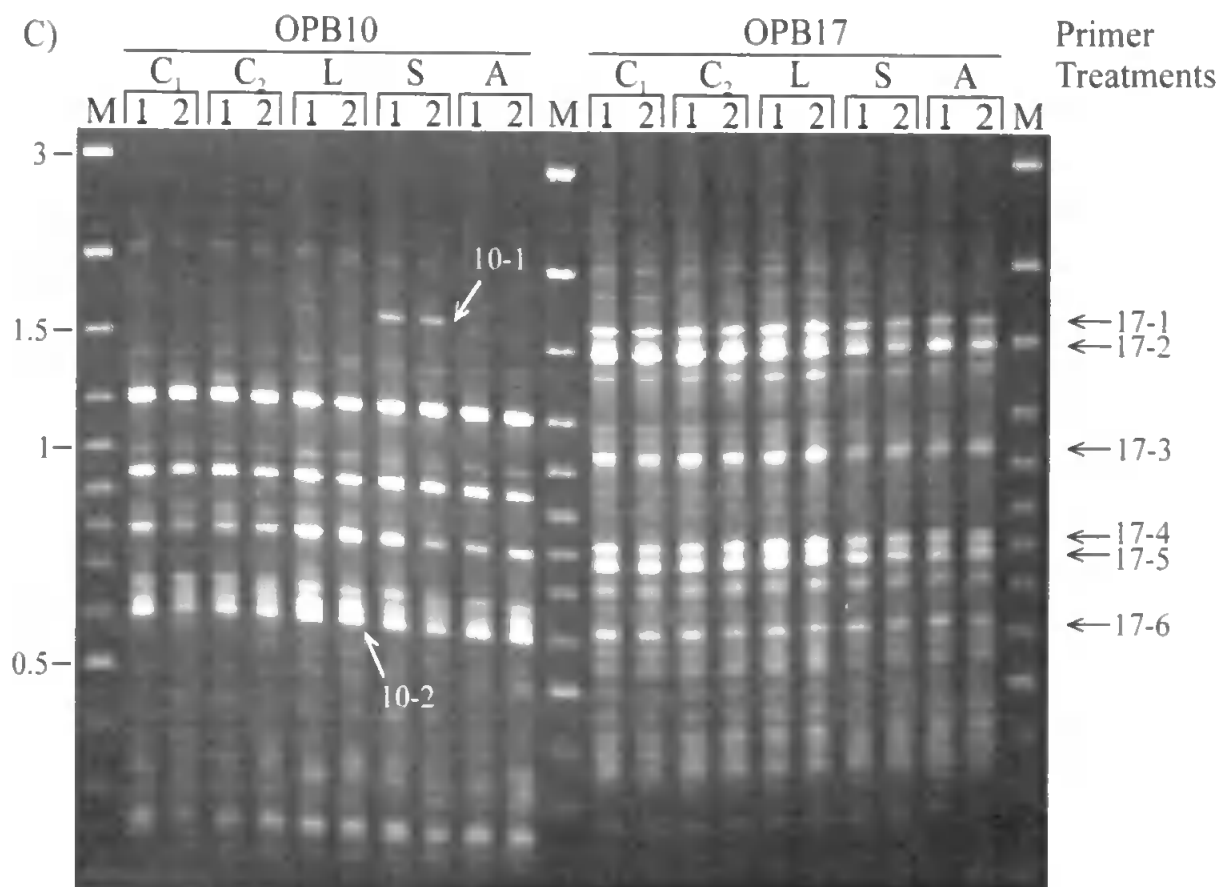


Figure 8.4 RAPD profiles of *Daphnia magna* exposed to different treatments. C1 and C2: no treatment (control), L: lack of essential elements (distilled water), S: stirring of the medium, and A: aeration of the medium. Primers as indicated at the top of each gel. The reproducibility of the RAPD profiles was determined using two DNA template concentrations (lanes 1 and 2: 4 and 1 ng genomic DNA, respectively). Some of the changes are indicated by arrows. M = 100 bp DNA ladder plus (IGi). The molecular sizes (Kb) of selected bands are indicated on the left of each gel.

Table 8.2 Examples of some of the changes occurring in RAPD profiles due to various treatments. Treatments as indicated in the table.

Band(s)	No treatment	Lack of essential elements (distilled water)	Stirring (1 rotation/min)	Aeration (air stone)
9-1, 8-5, 8-6, 10-1	Absent or very faint	Absent or very faint	more intense	Absent or very faint
8-1	very faint	more intense	very faint	very faint
8-2 to 8-4, 17-1 to 17-6	Relatively intense	Relatively intense	Less intense	Less intense
10-2	Relatively intense	very intense	Relatively intense	Relatively intense

In conclusion, the lack of essential elements induced hardly any modifications in RAPD patterns in contrast to the stirring and aeration of the medium. However, the greatest number of changes in profiles were obtained from the stirred *D. magna*.

8.4 Discussion

4-*n*-nonylphenol and 17- β oestradiol were used in study 1 because oestrogens and possibly xeno-oestrogens are able to induce diverse type of DNA effects as described in section 8.1. They are also known to induce production of vitellogenin in some vertebrates (reviewed by Depledge and Billinghamurst, 1999). 17- β oestradiol, a naturally occurring oestrogen, was used as a positive control (Billinghurst *et al.*, 1998). The results clearly indicated that changes occurred in RAPD profiles generated by the exposed larvae even at concentration as low as 0.1 μgL^{-1} 4-*n*-nonylphenol. These changes in patterns indicated 4-*n*-nonylphenol and 17- β oestradiol -induced DNA effects. Interestingly, when primer OPA9 was used similar modifications were observed in the RAPD profiles generated by barnacles exposed to either 4-*n*-nonylphenol or 17- β oestradiol. For instance, bands 9-1 and 9-2 appeared in profiles generated at 10 μgL^{-1} 4-*n*-nonylphenol and 17- β oestradiol only. The tantalising possibility exists that both chemicals induced the same end point at the DNA level which had significant effect on PCR and led to new amplified products of an identical size. These changes could be attributed to 4-*n*-nonylphenol and 17- β oestradiol -induced DNA adducts at specific sites. For instance, it has been reported that guanine is the main target base of steroid compounds possibly due to the carbonyl group (CO-CH₂OH) which seems to play a crucial role in the formation of DNA adducts (Seraj *et al.*, 1996). However, because the structures of 4-*n*-nonylphenol and 17- β oestradiol are different (Figure 8.1), it seems unlikely that they would bind to the same locus. Previous studies have failed to prove the presence of oestrogen DNA adducts (Epe and Metzler, 1985)

probably because this type of adducts can be chemically unstable and thus can be rapidly lost from the genomic DNA (Roy and Liehr, 1999). An alternative and more likely explanation is that 4-*n*-nonylphenol and 17- β oestradiol and/or their metabolites interact with the same compound possibly after binding to the receptor which would bind directly to the DNA and thus create the same set of adducts. Liehr *et al.* (1986) demonstrated that structurally diverse oestrogens (e.g. 17- β oestradiol, diethylstilbestrol, hexestrol, and 11 β -methyl-17 α -ethinylestradiol) indirectly induced an identical set of covalently modified nucleotides in target tissue. Alternatively, the same set of different DNA adducts (e.g. lipid DNA adducts) and/or mutations can be responsible for the common RAPD profile changes induced by 17- β oestradiol and 4-*n*-nonylphenol when compared to control patterns. This suggests that the mechanistic processes mediating DNA effects are common (at least partly) between these two chemicals. This could explain why xeno-oestrogens can mimic the same effects produced by natural oestrogens.

Changes occurring in RAPD profiles can be ascribed to DNA effects including DNA damage such as DNA strand breaks, 8-hydroxylation of guanine, DNA adducts, and chromosome aberrations. Point mutations which may accumulate after the replication of damaged bases (Strauss, 1985; Ide *et al.*, 1991; Hoffmann *et al.*, 1995) may also have significant effects on RAPD profiles as well as large genomic rearrangements. In the experiments reported here, barnacles were exposed to low concentrations of 4-*n*-nonylphenol and 17- β oestradiol and it is therefore likely that any resultant damage to genomic DNA was efficiently repaired (Sancar and Sancar, 1988) but possibly slowly (Tornaletti and Pfeifer, 1994). Nevertheless, it has been demonstrated that DNA damage such as DNA adducts (Varanasi *et al.*, 1981) and micronuclei (Majone *et al.*, 1987) can also persist long after the cause has been removed. Profiles generated following the exposure of barnacles to 4-*n*-nonylphenol and 17- β oestradiol revealed as well the disappearance of bands (e.g. Figure 8.3A band 9-3). This band probably did not disappear

as a direct result of adduct formation because the complete block of the DNA polymerase would require massive adduction. Since relatively low concentrations of the chemicals were used in this study such a situation would not be expected to arise. However, structural changes to the DNA could induce a disappearance of a band if the changes occurred in the DNA contained within a majority of cells. Finally, it is difficult to fully explain the reasons for some of the changes which were shown to occur in RAPD profiles as a consequence of exposure to these chemicals (e.g. Figure 8.3A band 9-4 which disappears at 1 and 5 μgL^{-1} 4-*n*-nonylphenol or band 9-1 which only appears at 0.1 and 1 μgL^{-1} 4-*n*-nonylphenol).

In conclusion, the RAPD technique detected DNA effects in the DNA extracted from barnacles exposed to 4-*n*-nonylphenol and 17- β oestradiol at all concentrations. In the past few years, numerous effects of endocrine disrupting chemicals on wildlife have been documented including changes in the sex of riverine fish, reproductive failure in birds and abnormalities in the reproductive organs of creatures as diverse as alligators and polar bears (Depledge and Billingham, 1999). The mechanisms have yet to be discovered. The present study suggests that the detection of DNA effects in organisms exposed to oestrogens deserves more attention. It seems likely that some of the effects which can be observed at the DNA level may explain the changes that have been documented in populations which have been exposed to potential endocrine disrupters.

In the second study which used crabs, the results clearly demonstrated the need to produce genomic DNA of satisfactory quality for reliable RAPD reactions. If this is not achieved then artefacts can be produced. Small differences in the quality and concentrations of two templates can lead to spurious amplifications (Welsh *et al.*, 1995a). If RAPD profiles which are amplified from the same genomic template DNA, or from different individuals of the same species vary, then the reproducibility of the assay should be confirmed by repeating the PCR reaction using two DNA template concentrations, differing by at least two fold (Welsh *et al.*, 1995a). If following this, the profiles still vary,

then the results should always be treated with scepticism. As this simple requirement was not met, no analysis could be performed on the data. In this study, the quality of the DNA extracted from some of the samples was not satisfactory enough to allow reproducible amplification of RAPD profiles. Thus, it was impossible to determine if DNA effects occurred in crabs exposed to 4-*n*-nonylphenol and diethylstilbestrol. Although it was possible to amplify RAPD profiles from crab DNA extracts, the DNA pellet obtained from crab samples appeared to be tiny. Indeed, often no band could be detected in agarose gels when 20% of the total extracted DNA was electrophoresed. As the minimum amount of genomic DNA detectable in agarose gels by ethidium bromide staining is approximately 5 ng, the highest concentration in the samples was of 5/20 ng per μl (as 20 μl of each sample was loaded into the gel) or 0.25 ng/ μl DNA. Although 1 ng of genomic DNA (equivalent to 4 μl of each sample) has been shown to be within the optimal range for successful amplification of genomic DNA extracted from organisms other than crabs (chapter 4), most of the samples had to be diluted 10 fold in order to obtain RAPD profiles. The problems encountered may be ascribed to protein contamination which is capable of interfering with the PCR process. This situation is very probable as protein concentrations in crab haemolymph can be high and fluctuate among individuals in the range 30-60 mg/ml protein (Dr Shaw Bamber, personal communication). Other sources of contamination, such as carbohydrates, polysaccharide, or DNases which might have been present could also affect the reproducibility of the results. Alternatively, it is possible that the pH or salt concentration in crab haemolymph may interfere with the phenol/chloroform DNA extraction method. Numerous studies have demonstrated that genomic DNA of good purity has to be used in RAPD reactions (Benter *et al.*, 1995; Welsh *et al.*, 1995a). In contrast, the amplification of a single PCR product does not always require the extraction and purification of the genomic DNA (Mercier *et al.*, 1990; Liu *et al.*, 1995; McEwan and Wheeler, 1995). Finally, it has to be stressed that the intensity of an amplified band

depends on the efficiency of the interaction of the genomic sequence with the primer during the initial steps (Welsh *et al.*, 1995a). Thus, the presence of any contaminants in the samples might compromise the fidelity of the RAPD assay.

In the third study, *D. magna* were subjected to 3 different treatments, stirring of the medium, raised levels of dissolved oxygen, and lack of essential elements in order to induce stress. The stirring of the medium forced the *D. magna* to swim which meant that they were physically active for 24 h. In another treatment, the medium was aerated to increase the concentration of dissolved oxygen. In addition, as *D. magna* require not less than 26 chemicals in the medium for long term culture (Elendt and Bias, 1990), the lack of essential elements induced a stress despite the fact that the treatment was applied only for 24 h.

Hartmann *et al.* (1994) demonstrated that physical activity in humans above the aerobic-anaerobic threshold caused DNA breakages. Niess *et al.* (1998) showed that DNA damage also occurs in human leucocytes after prolonged exercise. The cause of DNA damage after physical activity is not clear but some form of oxidative mechanism may be involved (Hartmann *et al.*, 1994). Niess *et al.* (1998) suggested that reactive oxygen species released by the neutrophils might be implicated. Exercise causes an increase oxygen consumption *in vivo* and a small part of the oxygen consumed leads to formation of reactive oxygen species (Aruoma, 1994). Reactive oxygens have beneficial effects but when produced in excess, beyond the antioxidant capacity, they can cause tissue damage. Hartmann *et al.* (1995) demonstrated that vitamin E prevented exercise-induced DNA damage in human. Following prolonged endurance exercise, 8-hydroxyguanine was detected in the human urine (Aruoma, 1994; Poulsen *et al.*, 1996) and Lepage *et al.* (1999) reported that 8-hydroxyguanine is one of the major products formed by the reactive oxygen species which are generated in living cells as a consequence of physical stress. In the present study, physical activity induced some changes in RAPD profiles suggesting that

effects on DNA occurred. The main changes were a decrease in band intensity as well as the appearance of new amplicons. It is conceivable that these changes were the result of diverse DNA damage induced by oxygen radicals as discussed below.

In the environment, the oxygen concentration of surface waters is influenced by the diurnal changes of the photosynthetic oxygen production and the respiratory oxygen consumption (Liepelt *et al.*, 1995). Fluctuations of the oxygen production are particularly pronounced in waters with high eutrophication. In addition, within several hours following sunrise, the oxygen concentration may increase from hypoxic to hyperoxic conditions. In this study, the concentration of dissolved oxygen was 6.33 and 8.56 mgL⁻¹ O₂ before and after aeration of the M7 medium (Table 8.1). Thus, the raised oxygen levels in the medium may have resulted in a greater diffusion of oxygen through the carapace of the *D. magna* which constitutes the major site of respiratory gas exchange (Pirow *et al.*, 1999). This could lead to specific DNA damage. Indeed, the oxygen molecule (O₂^{••}) is a free radical as it has two unpaired electrons in its molecular orbitals (Cadenas, 1989). The two unpaired electrons have the same spin quantum number (parallel spin), and if O₂ attempts to oxidise another atom or molecule by accepting a pair of electrons from it, both new electrons must be of parallel spin so as to fit the vacant spaces of the orbital. Hence the reaction of oxygen is spin restricted and thus the molecules of oxygen will react specifically with certain type of molecules. Transition metals, such as Fe or Cu, frequently have unpaired electrons and are excellent catalysts of O₂ reduction (Cadenas, 1989). The reaction could be summarised as follow: $X^{II} + O_2 \rightarrow X^{III} + O_2^{\cdot}$ (X = Fe or Cu). In addition, O₂[•] is produced when electrophiles, such as quinoid compounds, are reduced to semiquinones by cellular electron-transfer flavoproteins, which subsequently reduce O₂ via one electron, within a process termed redox cycling (Cadenas, 1989). This situation will lead to the formation of various radical species which will react with diverse macromolecules such as DNA to create DNA damage. In addition, an increase in oxygen concentration can be associated

with the conversion of xanthine to uric acid by the enzyme xanthine oxidase (Liepelt *et al.*, 1995). This reaction produces superoxide anions which are responsible for the induction of oxidative stress. Against all this background, it seems very likely that an increase in oxygen concentration leads to an increase in oxygen radicals and thus to damage to macromolecules such as DNA (see below).

Hemoglobin (Hb) in crustaceans is known to decrease concomitantly with an increase in the oxygen concentration (Kobayashi and Nezu, 1986) and *vice versa*. Fox and Phear (1953) reported that 50% of the Hb in *D. magna* was lost after 7 d in air-saturated water. Furthermore, Kobayashi and Nezu (1986) revealed that the decrease in the Hb concentration in *D. magna* only occurred after more than 24 h of exposure to aerated water. It is likely that the Hb in the *D. magna* exposed to the aerated medium was saturated suggesting a protective role of the Hb if it is considered that the oxygen will not be released unless it is needed. However, the excess of oxygen might lead to the formation of more reactive radicals. As already mentioned, oxygen radicals can lead to diverse DNA damage and mutations (Troll and Wiesner, 1985; Breimer, 1990; Feig *et al.*, 1994a; 1994b; Box *et al.*, 1997; Lloyd *et al.*, 1997; Newcomb *et al.*, 1999). It is likely that such effects will induce structural alterations (Wang and Taylor, 1991; Wang and Taylor 1993) which could interfere with the PCR process. In addition, the formation of dimers may also block or reduce the polymerisation of DNA in the PCR reaction (Donahue *et al.*, 1994; Nelson *et al.*, 1996a). Consequently, the intensity of some bands in RAPD profiles could be partially or completely abolished. Interestingly, the intensity of the same bands decreased after *D. magna* were either stirred or exposed to raised concentrations of dissolved oxygen. This may have occurred because the stirring of the medium also increased the concentration of dissolved oxygen to a level similar to the one reported after aeration of the medium (Table 8.2). However, it was also clear that the aerated *D. magna* exposed to raised concentrations of dissolved oxygen were not forced to swim and thus not subjected to any physical

activity. Hence, the appearance of bands which were present in the profiles produced by the amplification of the DNA extracted from the stirred but not aerated *D. magna* may represent the result of physical activity. As reactive oxygen species may be implicated in both experiments, this means that the mechanisms which contributed to DNA effects following either the increase in oxygen levels or physical activity were different.

When *D. magna* were placed in distilled water, the animals experienced considerable difficulties illustrated by the death of 30 % of the population (Table 8.1). However, hardly any changes were evident in RAPD profiles as compared to controls suggesting that no or very few detectable DNA effects occurred. The populations of *D. magna* might have been expected to respond to the stress by modulating gene expression. It is well known that the control of gene expression is strongly influenced by environmental factors. For instance, starved bacteria (e.g. *E. coli*) inoculated in a broth will grow rapidly after a lag phase (Siegele and Kolter, 1992). Indeed, the bacteria may have been in a dormant state, and time is required before their metabolism begins to work efficiently. Miralles and Serrano (1995) reported that four genes in *Saccharomyces cerevisiae* are regulated by osmotic stress. Water deficit in plant elicits a complex of responses beginning with stress perception, which initiates a signal transduction pathway(s) and is manifested in changes at the cellular, physiological, and developmental levels (Bray, 1993). Genes expressed during stress are anticipated to promote cellular tolerance of dehydration through protective functions in the cytoplasm, alteration of cellular water potential to promote water uptake, control of ion accumulation, and further regulation of gene expression (Bray, 1993). All living cells display a rapid molecular response to adverse environmental conditions, a phenomenon commonly designated as the heat shock response (Mager and Ferreira, 1993; Welsh, 1993). In eukaryotes, transcription of heat shock genes is regulated through the activation of the heat shock transcription factor which interacts with its cognate nucleotide element, the heat shock responsive element (Sorger, 1991).

Recently, Jolly and Morimoto (1999) reported that the cellular and molecular response to physiological and environmental stress induces a rapid and transient change in gene expression associated with major changes in nuclear architecture that impacts on signals involved in cell growth. Against this background, modulation of gene expression may have occurred in *D. magna* as a response to the stressful conditions (i.e. distilled water). However, very few detectable DNA effects occurred in RAPD profiles produced by the stressed population in comparison to control patterns. This could be ascribed to two main reasons. Firstly, it is possible that most of the structural changes induced by a change in gene expression are undone during denaturation of the genomic DNA at 94°C for 5 min, the first step of the PCR reaction. The same situation probably occurs for damaged genomic DNA with the exception that DNA lesions such as DNA adducts will induce structural changes which are not necessarily lost during denaturation. This statement is probably true at least where the DNA lesion is localised and possibly at other loci because a constraint in one position induces constraints in other areas. In this context, it seems that the contribution of non-genotoxic effects, such as changes in gene expression, to variations in RAPD profiles is possible but probably of limited importance. Secondly, it is possible that the *D. magna* were unable to adapt to the stressful conditions as 30 % of the population died after 24 hours. This could be due to an absence or a delay in the production of heat shock proteins. Stress-inducing agents are characterised by their ability to cause the appearance of misfolded proteins whose accumulation and aggregation can be damaging to the cell (Jolly and Morimoto, 1999). In other words, it is possible that the population of *D. magna* did not induce a rapid (i.e. within 24 h) and transient change in gene expression in response to the stress. The lack of tolerance may be related to the fact that *D. magna* is considered to be a species which is sensitive to toxic agents. Soares *et al.* (1992) reported that genotype-environment interactions played a key role in determining chronic responses of *D. magna* to toxicants. The difference in stress sensitivity of genotypically different

clones of *D. magna* could depend on the ability to rapidly and appropriately respond in order to compensate the stressful effects.

In conclusion, oxygen radicals seem to have the potential to significantly affect RAPD profiles due to the fact that they generate diverse types of DNA damage. As numerous xenobiotics can produce highly reactive radicals (Cadet *et al.*, 1999), it is likely that substantial changes occurring in RAPD patterns may be the result of the action of radicals on genomic DNA. Finally, the results obtained in this chapter suggest that DNA effects such as variation in gene expression may affect RAPD profiles but probably to a lesser extent than DNA damage.

Chapter 9

DETECTION OF SPECIFIC AND DIVERSE TYPES OF DNA EFFECTS USING THE RAPD ASSAY

9.1 Introduction

So far the research project has clearly suggested that chemical and physical agents which interact with genomic DNA, either directly or indirectly, are likely to induce significant effects on RAPD profiles. In the previous chapters the changes in patterns were attributed to DNA effects and more specifically to DNA damage, mutations and possibly variations in gene expression. However, as already mentioned, the nature and the amount of DNA effects could only be speculated. At this stage, it was primordial to prove that various types of specific DNA effects can significantly alter RAPD profiles. In particular, the following questions needed to be answered:

- (i) Are mutations, and DNA damage such as DNA adducts, DNA breakages easily detected by the RAPD technology?
- (ii) Do genotoxin-induced DNA structural changes have an important effect on RAPD profiles?
- (iii) Does a single base change in the 10-mer primer sequence induce any detectable change in RAPD patterns at high annealing temperature?
- (iv) What is the minimum level of mutated/altered genomic DNA that can be detected using the RAPD protocol?

To answer some of these questions, DNA lesions had to be individually generated under *in vitro* conditions using extracted DNA, bearing in mind that genotoxins induce a wide range of DNA damage and mutations under *in vivo* conditions. For instance, *in vivo* studies have shown that DNA adducts lead to generation of a variety of mutations including base substitutions, frameshift mutations, insertions, and deletions (Rodriguez and Loechler, 1993; Loecher, 1995; Ross and Nesnow, 1999; Roy and Liehr, 1999). By using *in vitro* studies it would be possible to better understand the effects of DNA damage and mutation on the RAPD technology. Although the creation of specific types of DNA damage such as B(a)P DNA adducts, pyrimidine dimers, and DNA breakages was not technically complex,

it seemed difficult to induce mutations under *in vitro* conditions, to the best of our knowledge and competence. It was attempted to create mutations by using three different strategies. The first one consisted of using a bacterial strain of *E. coli* which was mutated in the mismatch repair gene. Thus, the deficient cells were expected to accumulate mutations after multiple replication *in vivo* in comparison to the parental strains. In the second study, RAPD was performed using a set of primers which differed by a single base (in comparison to the original primer) to monitor any detectable changes among the produced profiles. By extrapolation, this would allow to analyse the effect of point mutations in genomic DNA on RAPD patterns. In the third study, isolates of the bacterium *Renibacterium salmoninarum* from world wide locations were used. Briefly, *R. salmoninarum* is a genospecies that is an obligate bacterial pathogen of salmonid fish and is capable of intracellular survival. This organism causes bacterial kidney disease (BKD) which can be fatal under the appropriate conditions (Evenden *et al.*, 1993). This bacterium appears to possess remarkable biochemical uniformity, and no reliable serological means of distinguishing between isolates has been found (Goodfellow *et al.*, 1985). Thus this study allowed an assessment of the potential of the RAPD technique to detect genetic diversity in the absence of xenobiotic-induced DNA damage. Finally, it was attempted to evaluate the minimum level of mutated/altered genomic DNA that can be detected using the RAPD protocol. The strategy consisted in evaluating the sensitivity of the RAPD assay by mixing different proportions of genomic DNA of two different origins. The objective was to evaluate the minimum amount of one genomic DNA required to allow the detection of an amplicon specific to this genomic DNA. By extrapolation, genomic DNA of the first origin could be considered to be the result of genotoxin-induced effect arising in the genomic DNA of the second origin, and *vice versa*. Thus, the evaluation of the minimum relative amount of one source of genomic DNA would give an estimation of the minimum level of mutated/altered genomic DNA that is required to be detected using the RAPD protocol.

9.2 Materials and methods

The objectives were to create individual types of DNA damage such as DNA breakages using, separately, restriction enzymes and sonication, B(a)P adducts, pyrimidine dimers, and to induce mutations by performing the three different studies that have been succinctly described in section 9.1.

9.2.1 Creation of DNA breakages

9.2.1.1 Restriction enzyme digestion of genomic DNA

Restriction enzymes were used to induce non-random DNA double strand breakage (dsb) in genomic DNA. Caesium chloride purified DNA extracted from *E. coli* and calf thymus was obtained from Sigma (Poole, UK). The concentration was adjusted to 100 ng/ μ l in TE buffer and DNA was restricted using *EcoR* I, *Hind* III, *Not* I, *Pme* I (New England BioLabs, Hitchin, UK). Table I.3 (appendix I) shows the recognised sequence as well as the average fragment size generated by each endonuclease in *E. coli* and mouse. The average fragment size generated by each endonuclease in mouse and calf thymus was expected to be similar. Reactions were performed under the following conditions: 22.5 μ l of either *E. coli* or calf thymus genomic DNA (100 ng/ μ l TE buffer) was mixed with 2.60 μ l buffer (10X; see appendix I for the composition of the buffer) and 10 units of restriction enzyme in a final volume of 26 μ l and incubated for 0, 0.5, 1.5, 3 and 15 h. Samples were then incubated at 70°C for 20 min to inactivate the restriction enzymes. A volume of 2.88 μ l 10X TE buffer was then added to each reaction mixture to obtain a final concentration of 1X TE. Each DNA sample was diluted to two final concentrations of 5 and 20 ng/4 μ l. PCR was then performed under the optimised conditions (see section 3.4.1) using 5 and 20 ng of each sample as DNA template, and 10-mer primers OPB1, OPB5, OPB7, and OPB8 (see section 3.4.2). PCR products were electrophoresed in 1.2 % agarose gels at 90 V for 6

h, stained with ethidium bromide and visualised under UV light. A photograph was also taken and the image of the gel was saved electronically for further analysis when necessary (see section 3.5).

9.2.1.2 Sonication of genomic DNA

Sonication was performed to induce random DNA breakage in genomic DNA. Total DNA from *P. palmata* (see section 3.1.3) was extracted and purified using a conventional phenol/chloroform method as described in section 3.2.2.1. The extracted DNA was electrophoresed on 1.2 % agarose gels and the DNA concentration of the sample was determined by comparison with known concentrations of Lambda phage DNA (see section 3.3.1). A volume of 265 μl of a solution at a concentration of 70 ng DNA/ μl was sonicated for 0, 5, 10, 20, 30, 40, 50, 60, and 90 s. After each time point, 20 μl of the DNA solution was sampled; 10 μl were electrophoresed in 1.2 % agarose gel at 100 V for 2 h to check the extent of DNA breakage. The remaining 10 μl were diluted in TE buffer so that the final concentration was 20 ng/4 μl . PCR was then performed under the optimised conditions (see section 3.4.1) using 10-mer primers OPA9, OPB5, and OPB8 (see section 3.4.2). Genomic DNA was also extracted from *D. magna* (see section 3.1.1) as previously described in section 3.2.1.1. A volume of 150 μl of a solution at a concentration of 50 ng DNA/ μl was sonicated for 0, 2, 5, 10, 15, and 20 s. After each time point, 10 μl of the DNA solution was diluted in TE buffer to obtain a final concentration of 20 ng/4 μl . In this experiment, genomic DNA was not electrophoresed. PCR was performed under the optimised conditions (see section 3.4.1) using 10-mer primer OPA9 (see section 3.4.2). PCR products obtained from *P. palmata* and *D. magna* were then electrophoresed and analysed as described at the end of section 9.2.1.1. Both experiments were performed using a ultrasonic processor W-385 (Heat systems-ultrasonics, New York, USA) under the following conditions: cycle continuous, % duty cycle: 70, output control: 2.75.

9.2.2 Induction of DNA adducts using B(a)P

Part of the study (DNA adduct induction) was performed by Dr Paola Venier at the University of Padoa (Italy). Mediterranean mussels (*Mytilus galloprovincialis*) were collected from a relatively unpolluted site of the Venice lagoon (mussel farm at the Lagoon Inlets, Italy). Mussels were maintained as previously reported in Venier and Canova (1996). Genomic DNA was extracted from the mussel gill as described in Venier and Canova (1996). Briefly, the gill tissue was homogenised in a solution containing SDS and EDTA and treated using proteinase K, RNase, and perchlorate; DNA was then extracted with sevag (24:1 chloroform:isoamyl alcohol) and resuspended in TE buffer. Purified DNA was then reacted with the 3H-anti(+/-)-trans-7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydro-benzo(a)pyrene (3H-BPDE) (NCI, Chemical Carcinogen Standard Repository, Bethesda, MD) as follow. A volume of 200 µl volume of the DNA solution containing 500 ng DNA/µl (in 2 mM Tris-HCl buffer pH = 7.5) was reacted with 25 mM 3H-BPDE solution for 1 h at 37°C. Samples were then extracted twice in sevag and genomic DNA was precipitated using 0.1 volume of 2.5 M sodium acetate (pH 7) and 2 volumes of cold ethanol. After a single washing step by salt ethanol precipitation to remove free ³H-BPDE, DNA pellet was resuspended in analytical grade water and UV quantification of the samples was performed as described in section 3.3.2. The radioactivity in samples were determined by using a scintillation counter (Camberra Packard) and DNA adducts were detected by ³²P-postlabelling as described by Reddy and Randerath (1986). For more details refer to Venier and Canova (1996).

Control and modified genomic DNA were precipitated using 0.1 volume of 2.5 M sodium acetate (pH 7) and 2 volumes of cold ethanol, dried and sent to Franck Atienzar at the University of Plymouth (UK). On arrival, the DNA pellets were resuspended in 100 µl of 1X TE buffer. Different volumes of control and adducted solutions were then electrophoresed on 1.2 % agarose gels and the DNA concentration of the samples was

determined by comparison with known concentrations of Lambda phage DNA (see section 3.3.1). PCR conditions, electrophoresis, and analysis of the data were identical to those described at the end of section 9.2.1.1 except that primers OPA9, OPB1, OPB5, OPB7, OPB8, OPB10, OPB11, OPB12, OPB14, and OPB17 (see section 3.4.2) were used.

9.2.3 Creation of UVC-induced DNA damage

Caesium chloride purified DNA extracted from *E. coli* and calf thymus was obtained from Sigma (Poole, UK). The concentration was adjusted to 50 ng/μl in TE buffer and 20 μl of this solution was exposed to UV-C radiation (Philips UV-C 15 W, Holland) for 0, 5, 10, 20, 40, 60, or 80 s. The source of UV-C was provided using a PCR cabinet (Holten laminAir, Heto-Holten A/S, Denmark). Samples were placed at a distance of 5 cm from the UV-C lamp. After the irradiation the DNA samples were diluted to a final concentration of 5 and 20 ng/4 μl. PCR conditions, electrophoresis, and analysis of the data were identical to those described at the end of section 9.2.1.1 except that primers OPA9, OPB1, OPB5, and OPB7 (see section 3.4.2) were used.

In order to verify the presence of pyrimidine dimers, phage Lambda DNA [single-stranded DNA; Sigma (Poole, UK)] adjusted at a concentration of 250 ng/μl in sterile analytical grade water (Sigma, Poole, UK) was exposed to the same conditions of UV-C irradiation and for the same time series (as previously mentioned) except that 5s was not included. The samples were treated with the T4 endonuclease V (Worthington biochemical corporation, New Jersey, USA) under the following conditions: 5 μl DNA phage Lambda + 3.6 units of T4 endonuclease V + 15 μl of T4 endonuclease V buffer incubated for 1 h at 37°C. Samples were then kept on ice and electrophoresed in 0.8 % agarose gels at 100 V for 2 h, stained with ethidium bromide and visualised under UV light. A photograph was also taken and the image of the gel was saved electronically for further analysis when necessary (see section 3.5).

9.2.4 Production of mutations in genomic DNA

9.2.4.1 The use of strains of *Escherichia coli* deficient in mismatch repair genes (study 1)

9.2.4.1.1 Description of the strains and culture conditions

The strains of *E. coli* used in this study (C600, SMR346, and SMR687) were provided by Dr Susan Rosenberg (University of Alberta, Canada). SMR346 and SMR687 are two strains in which a transposon carrying an antibiotic resistant gene, i.e. kanamycin and tetracycline for SMR346 and SMR687, respectively, has been inserted in the mismatch repair gene of the parental strain C600. C600 displays a fully functional mismatch repair gene. The three strains were sent in separate tubes and, on arrival they were kept at 4°C. The strains were separately grown on agar (with or without antibiotics) at 37°C overnight and were kept at 4°C until the experiment started (see appendix I).

9.2.4.1.2 Experimental design

For each *E. coli* strain, C600, SMR 346, and SMR 687, a single colony was picked from an agar plate and incubated in 100 ml of Luria-Bertani (LB) broth, 100 ml of LB broth + 25 mg/ml kanamycin, and 100 ml of LB broth + 15 mg/ml tetracyclin, respectively (see appendix I). The cultures were agitated at 37°C and the optical density (OD) was measured in a spectrophotometer (UV4-100, Unicam, UK) at 260 nm every 30 min. When the OD was approximately 0.5, 40 ml of each solutions were sampled, and stored at -20°C. This step was repeated when the OD reached the value of 1.4, and 2, but the volume sampled was of 5 and 1 ml, respectively. Genomic DNA was extracted from the bacterial strains using a DNA extraction kit as described in section 3.2.3.2. Briefly the method of extraction was based on cell lysis, enzymatic digestion, and salt precipitation. The extracted DNA was electrophoresed in 1.2 % agarose gels at 80 V for 2 h and the DNA concentration of the samples was determined by comparison with known concentrations of Lambda phage DNA (see section 3.3.1). Samples were diluted in 1X TE buffer to a final concentration of 5 and 20 ng/4 µl. PCR conditions, electrophoresis, and analysis of the data

were identical to those described at the end of section 9.2.1.1 except that primers OPB1, OPB5, OPB6, OPB7, OPB8, and OPB10 (see section 3.4.2) were used.

9.2.4.2 Influence of a single base change in the primer sequence on RAPD profiles (study 2)

This experiment was carried out with caesium chloride purified genomic DNA extracted from *E. coli* and calf thymus (Sigma, Poole, UK) using a series of primers each of which contained a single base substitution such that the percentage of guanine plus cytosine was unchanged with respect to the original sequence (Table 9.1). DNA pellets were resuspended in 1X TE buffer and the stock solution was diluted in 1X TE buffer to obtain solutions at 5 and 20 ng/4 µl DNA. PCR was then performed under the optimised conditions (see 3.4.1) using 5 and 20 ng DNA for each sample and primers OPB5, OPB5-1 to OPB5-10 as well as primers OPB7, OPB7-1 to OPB7-10 (Table 9.1) in two distinct experiments. Electrophoresis, and analysis of the data were identical to those described at the end of section 9.2.1.1.

In a second set of experiments, PCR products generated by primers OPB5 and OPB7 were diluted (i.e. 4 µl in 1500 µl of 1X TE buffer) and reamplified under the optimised conditions (see section 3.4.1) using primers OPB5, OPB5-1 to OPB5-10 as well as OPB7, OPB7-1 to OPB7-10, respectively (see Table 9.1). Electrophoresis, and analysis of the data were identical to those described at the end of section 9.2.1.1.

9.2.4.3 Genetic diversity among *Renibacterium salmoninarum* isolates (study 3)

9.2.4.3.1 Bacterial isolates

Nineteen isolates of *R. salmoninarum* were used in this study. The isolates were sent to Dr Thomas Grayson (University of Plymouth, UK) from world-wide locations. Five isolates originated from Scotland (MT410, MT417, MT420, MT1363, and BA99), five from the United states (Marion Forks, Little Goose, Round Butte, NCIMB2196, and ATCC 33209), four from Canada (DR-128, DR143, DR384, and RS-TSA), three from Iceland (F-

Table 9.1 Sequence of 10-mer primers (5'→3') used in the present study. Bases in bold represent a modification in the sequence so that the % of GC is unchanged in comparison to the original primer (sequence underlined at the top of the gel). The number after the name of the primer indicates the position of the base which has been changed compared to the original primer.

OPB5	<u>TGCGCCCTTC</u>	OPB7	<u>GGTGACGCAG</u>
OPB5-1	AGCGCCCTTC	OPB7-1	CGTGACGCAG
OPB5-2	TCCGCCCTTC	OPB7-2	GCTGACGCAG
OPB5-3	TGGGCCCTTC	OPB7-3	GGAGACGCAG
OPB5-4	TGCCCCCTTC	OPB7-4	GGTCACGCAG
OPB5-5	TGCGGCCTTC	OPB7-5	GGTGTCGCAG
OPB5-6	TGCGCGCTTC	OPB7-6	GGTGAGGCAG
OPB5-7	TGCGCCGTTC	OPB7-7	GGTGACCCAG
OPB5-8	TGCGCCCATC	OPB7-8	GGTGACGGAG
OPB5-9	TGCGCCCTAC	OPB7-9	GGTGACGCTG
OPB5-10	TGCGCCCTTG	OPB7-10	GGTGACGCAC

138-87, F-273-87, and S-182-90) and, finally, two from Northern England (W2 and W6). On arrival each isolate was cultured in selective kidney disease medium broth supplemented with 5% spent culture broth at 15°C as described by Austin *et al.* (1983). The full description of the origin and identity of each isolate has been published by Grayson *et al.* (1999).

9.2.4.3.2 Generation of RAPD profiles

Genomic DNA was isolated by Dr Grayson using a Puregene D-6000 DNA isolation kit according to the instructions of the manufacturer (Gentra Systems Inc., Minneapolis, USA) as described in Grayson *et al.* (1999). Briefly, the method of extraction was based on cell lysis, enzymatic digestion, and salt precipitation. The extracted DNA was electrophoresed in 1.2 % agarose gels at 100 V for 2 h and the DNA concentration of unknown samples was determined by comparison with known amounts of Lambda phage DNA (see section 3.3.1). PCR conditions, electrophoresis, and analysis of the data were identical to those described at the end of section 9.2.1.1 except that primers OPA9 and OPB1 (see section 3.4.2) were used.

9.2.5 Sensitivity of the RAPD method

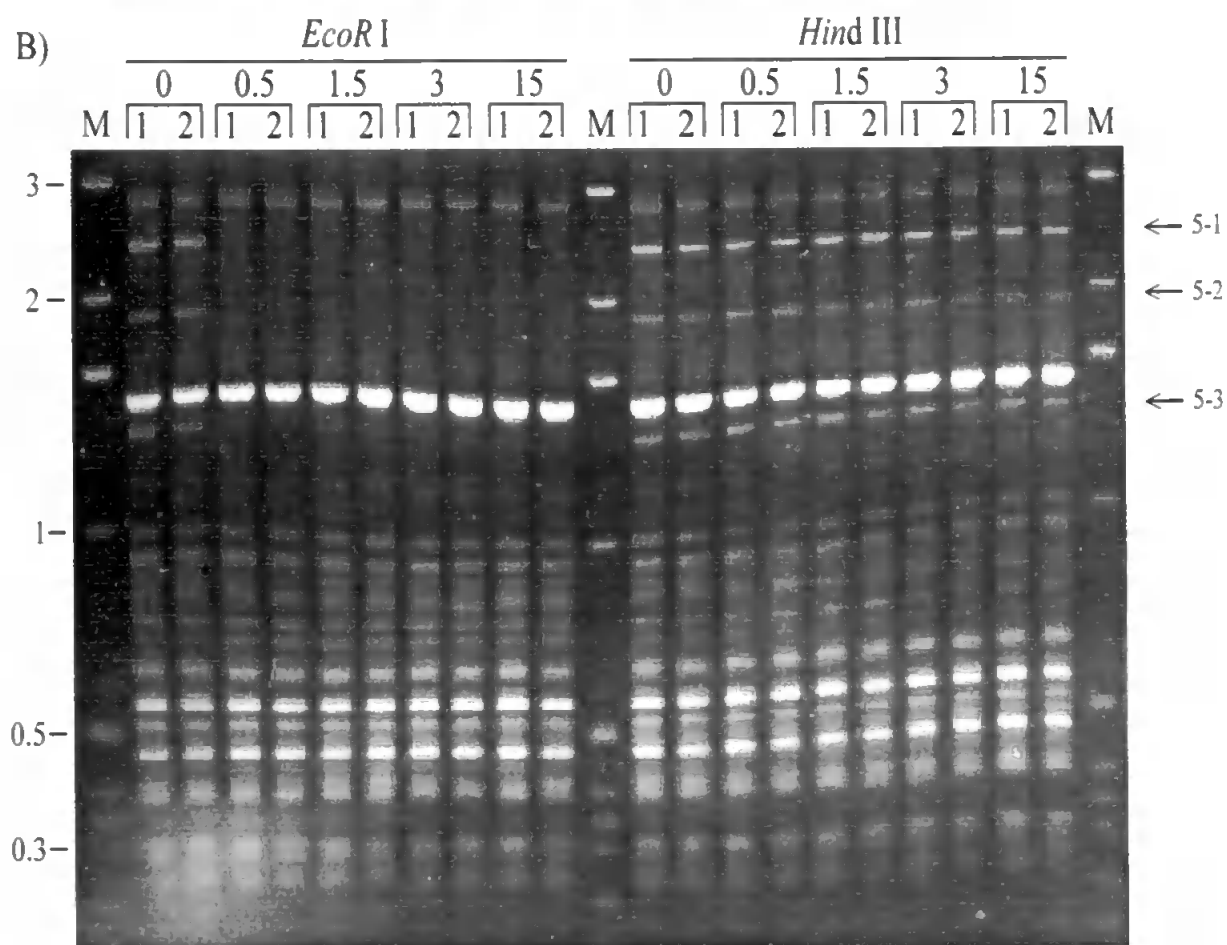
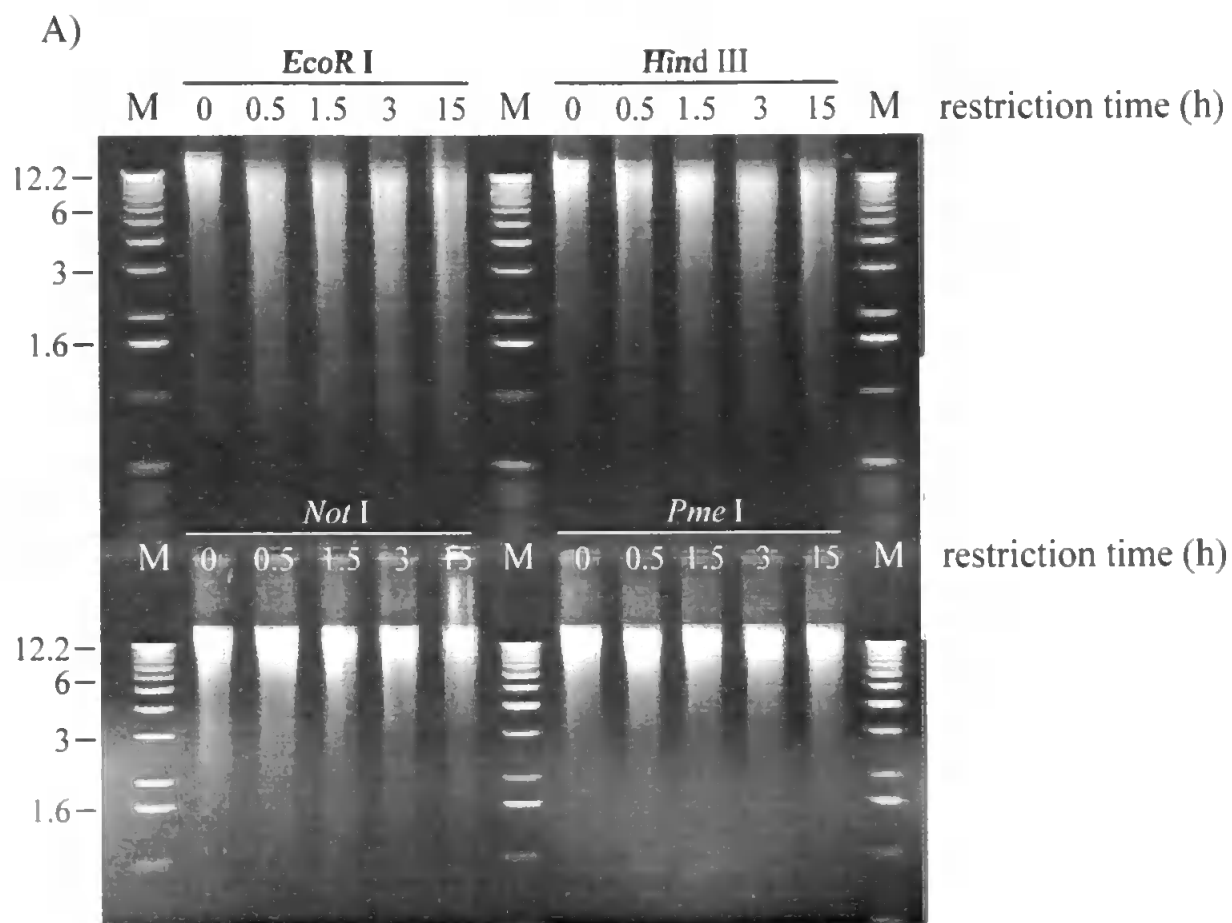
DNA used in this study was extracted from *D. magna* (see section 3.1.1) and *E. intestinalis* (see section 3.1.2) as described in sections 3.2.1.1 and 3.2.2.1, respectively. The extracted DNA was electrophoresed in 1.2 % agarose gels at 100 V for 2 h and the DNA concentration of unknown samples was determined by comparison with known amounts of Lambda phage DNA (see section 3.3.1). The concentrations of both solutions were adjusted to 20 ng/4 µl in 1X TE buffer. A series of solutions which contained mixtures of genomic DNA extracted from *D. magna* and *E. intestinalis* in differing relative proportions were prepared in order to evaluate the sensitivity of the RAPD technique for the detection of each template. The solutions contained *D. magna* and *E. intestinalis* DNA in the

following relative proportions: 100 + 0, 95 + 5, 85 + 15, 75 + 25, 65 + 35, 50 + 50, 35 + 65, 25 + 75, 15 + 85, 5 + 95, and 0 + 100 % of each template, respectively. RAPD reactions were then performed using 20 ng of genomic DNA in total in the proportions mentioned above under the optimised conditions (see section 3.4.1) using primers OPA9 and OPB5 (see section 3.4.2). PCR reactions were run in duplicate. Electrophoresis and analysis of the data were identical to those described previously at the end of section 9.2.1.1.

9.3 Results

9.3.1 Effect of enzymatically restricted DNA on RAPD profiles

Electrophoresis of restricted *E. coli* and calf thymus DNA revealed that *EcoR* I and *Hind* III cut DNA more frequently than *Not* I and *Pme* I (Figure 9.1A and 9.2A). This was in agreement with the data presented in Table I.3 (appendix I). After PCR was carried out on *E. coli* DNA which had been restricted by *EcoR* I, bands 5-1, 5-2, and 5-3 disappeared in comparison to control (Figure 9.1B). On the other hand, no bands disappeared when *E. coli* DNA was restricted using *Hind* III, *Not* I, and *Pme* I (Figure 9.1B and 9.1C) and subjected to PCR amplification. After restriction of calf thymus DNA using *Hind* III and PCR amplification, band 8-1 disappeared after 3 and 15 hours digestion (Figure 9.2B). However, no obvious changes occurred when calf thymus DNA was restricted with *EcoR* I, *Not* I and *Pme* I (Figures 9.2B and 9.2C). Results presented in appendix III show that no obvious modifications arose among RAPD profiles produced by control and restricted (*EcoR* I, *Hind* III, *Not* I, and *Pme* I) genomic DNA (*E. coli* and calf thymus) using primers OPB1 and OPB7.



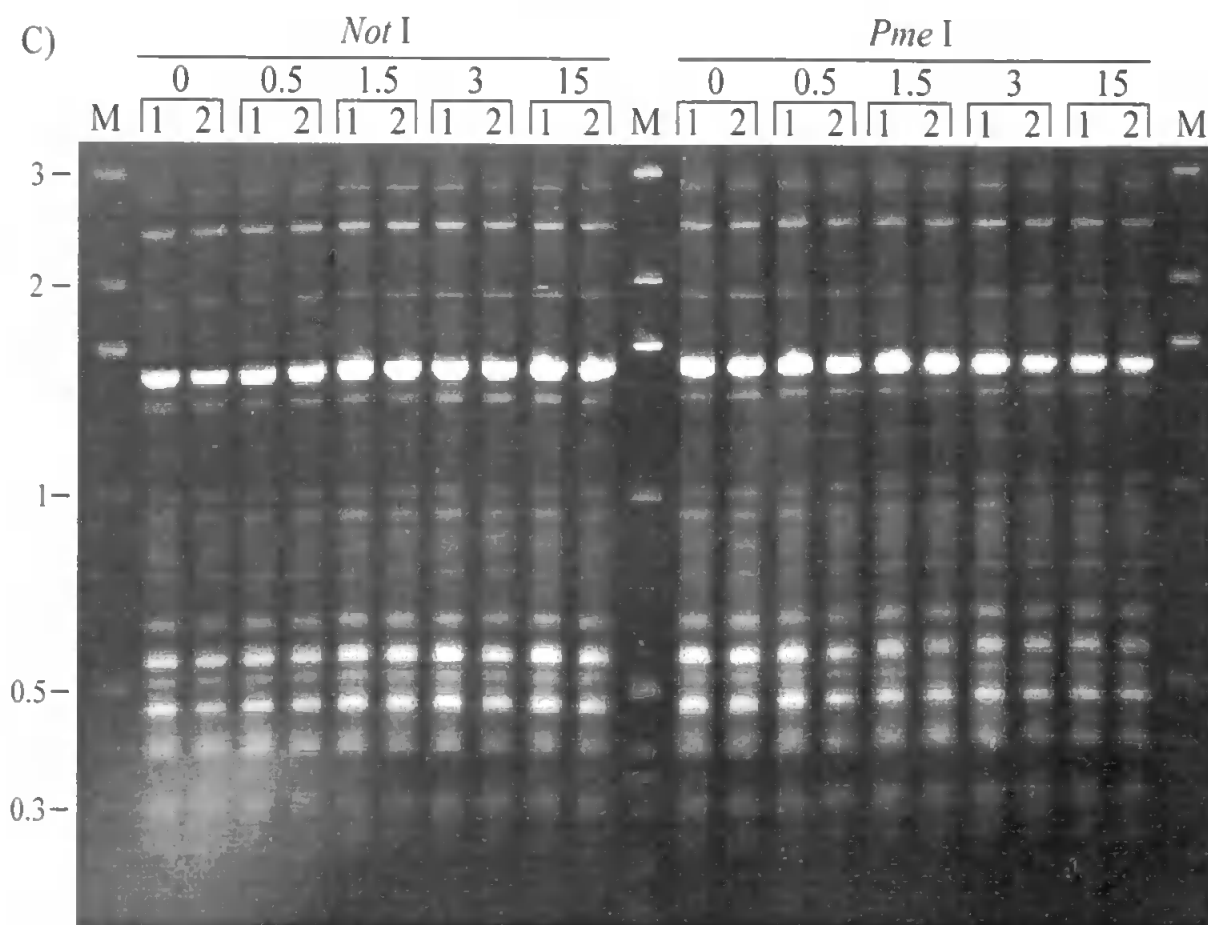
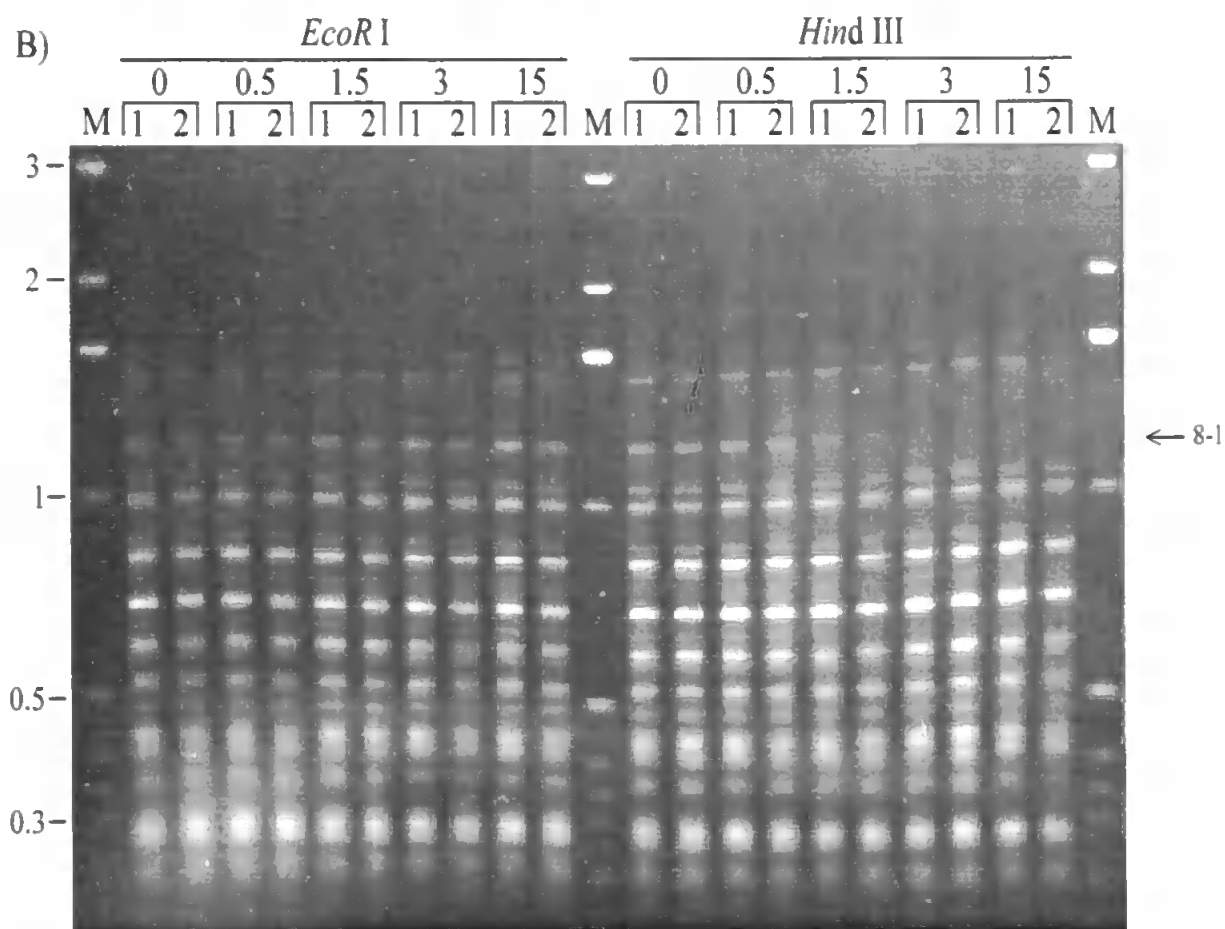
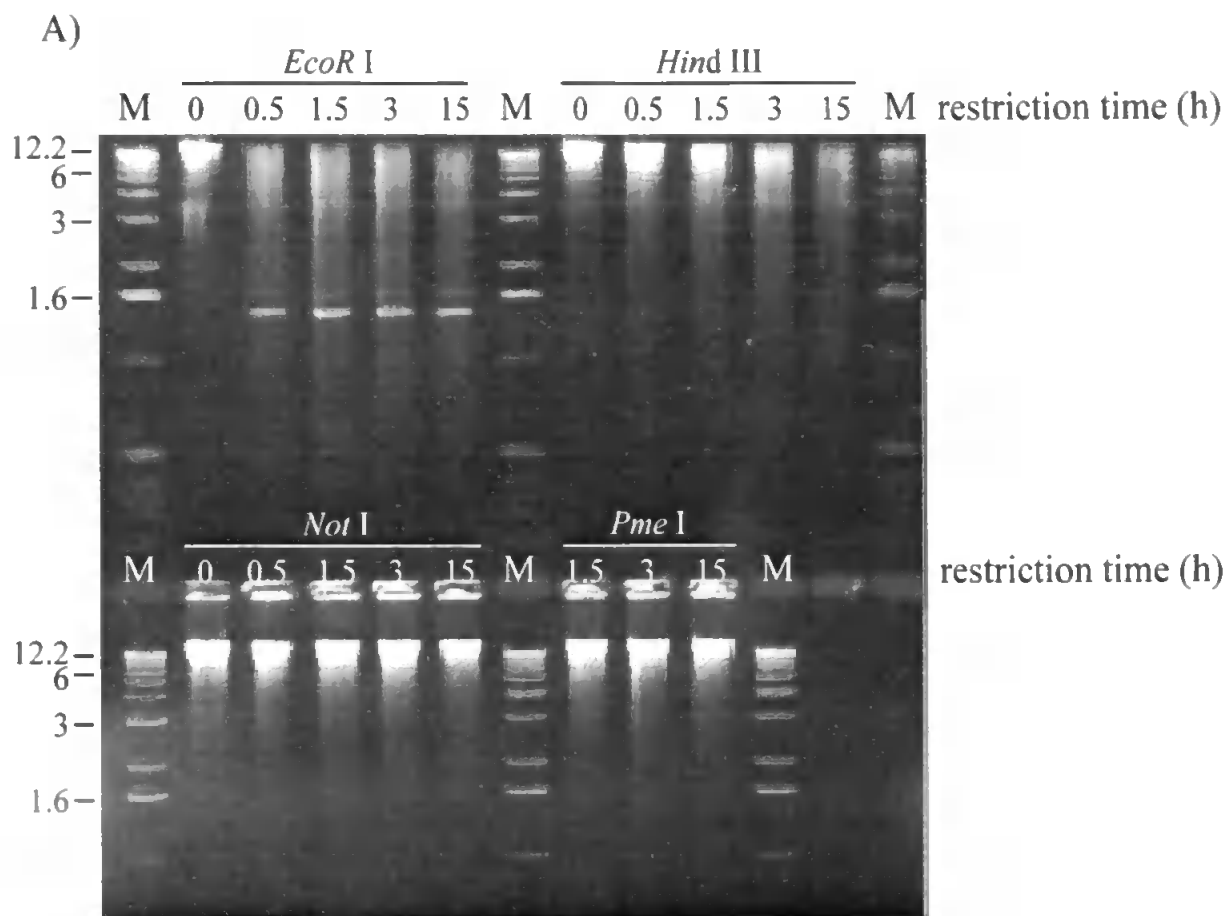


Figure 9.1 Effect of restricted *Escherichia coli* DNA on RAPD profiles. A) Genomic DNA was restricted by enzymes *EcoR* I, *Hind* III, *Not* I, and *Pme* I for 0, 0.5, 1.5, 3 and 15 h as indicated at the top of the gel. B) and C) RAPD profiles were generated by restricted DNA using 2 DNA template concentrations (lanes 1 and 2: 20 and 5 ng DNA, respectively) with primer OPB5. Time of restriction as indicated on the top of each gel. M = 1Kb DNA ladder (BRL). The molecular sizes (Kb) of selected bands are indicated on the left of each gel. Disappearing bands are indicated by arrows in comparison to control patterns.



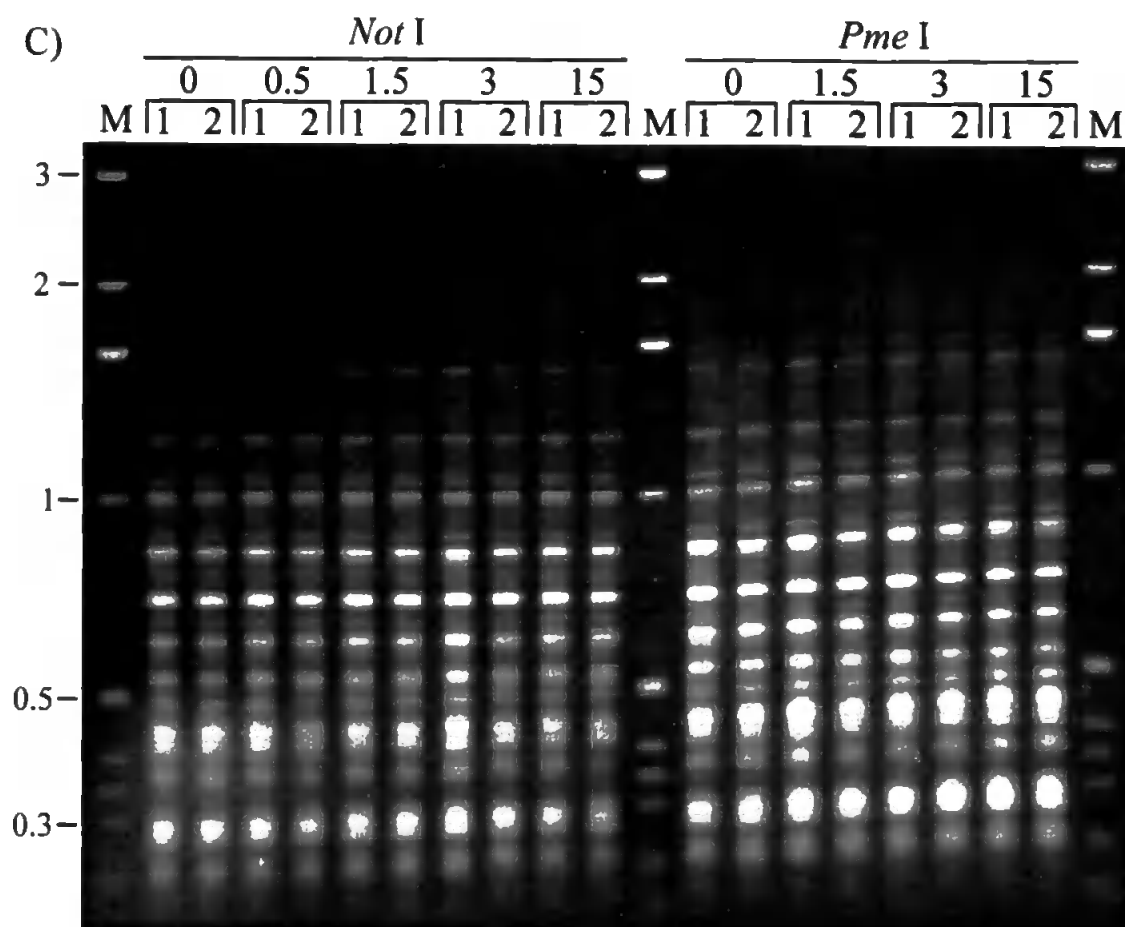


Figure 9.2 Effect of restricted calf thymus DNA on RAPD profiles. A) Genomic DNA was restricted by enzymes *EcoR* I, *Hind* III, *Not* I, and *Pme* I for 0, 0.5, 1.5, 3 and 15 h as indicated at the top of the gel. B) and C) RAPD profiles were generated by restricted DNA using 2 DNA template concentrations (lanes 1 and 2: 20 and 5 ng DNA, respectively) with primer OPB8. Time of restriction as indicated at the top of each gel. M = 1Kb DNA ladder (BRL). The molecular sizes (Kb) of selected bands are indicated on the left of each gel. Disappearing bands are indicated by arrows in comparison to control patterns.

9.3.2 Effect of randomly restricted DNA on RAPD profiles

Electrophoresis of sonicated *P. palmata* genomic DNA indicated that even after 5 s of sonication approximately 90 % of the DNA was fragmented as determined by eye (Figure 9.3A). The longer the time of sonication, the smaller the average size of genomic DNA fragments produced. For instance, after 30 s of sonication almost all DNA fragments were less than 1000 bases. After PCR amplification, it was observed that bands of the highest molecular weights disappeared progressively as the time of sonication increased (Figure 9.3B, 9.3C and appendix III). Major PCR products disappeared after 20 s sonication. However, bands whose molecular weights were lower than 800 bases were generally present in RAPD patterns. In another experiment, PCR using sonicated *D. magna* DNA showed the disappearance of bands after 20 s of sonication. However, two bands also appeared after 20 s of sonication (Figure 9.3D).

9.3.3 Effect of B(a)P adducts on RAPD profiles

Autoradiography of ^3H -BPDE treated genomic *Mytilus galloprovincialis* DNA showed the presence of bulky DNA adducts in the treated DNA (Figure 9.4B). In contrast, no adducts were present in the autoradiography of the control sample (Figure 9.4A). RAPD profiles produced by the adducted DNA revealed that the main events were the disappearance of some bands and the decrease in band intensity (Figure 9.4C and 9.4D). The bands of the highest molecular weights disappeared first in comparison to shorter amplicons and generally the decrease in band intensity concerned mainly the longer amplification products. However, the disappearance of some bands was not only dependent on the size of the amplicons. For instance, RAPD profile generated by primer OPB6 indicated that band 6-2 (~1.1 Kb) disappeared whereas band 6-1 (~1.3 Kb) was still present in comparison to control RAPD profiles. Similar results were obtained with primer OPB8 (see bands 8-1 and 8-2). In addition, the intensity of some bands (e.g. 5-1 and 10-1)

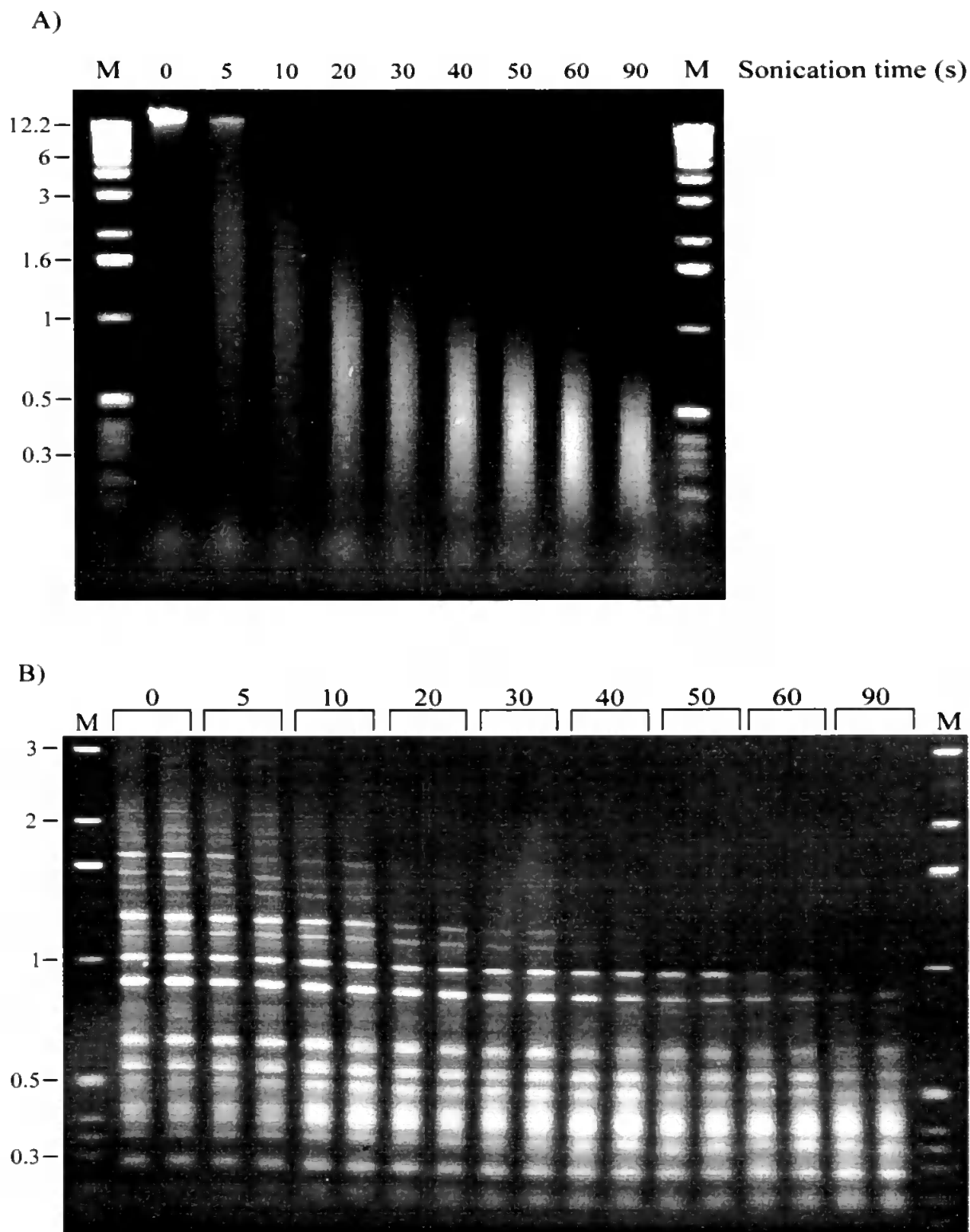
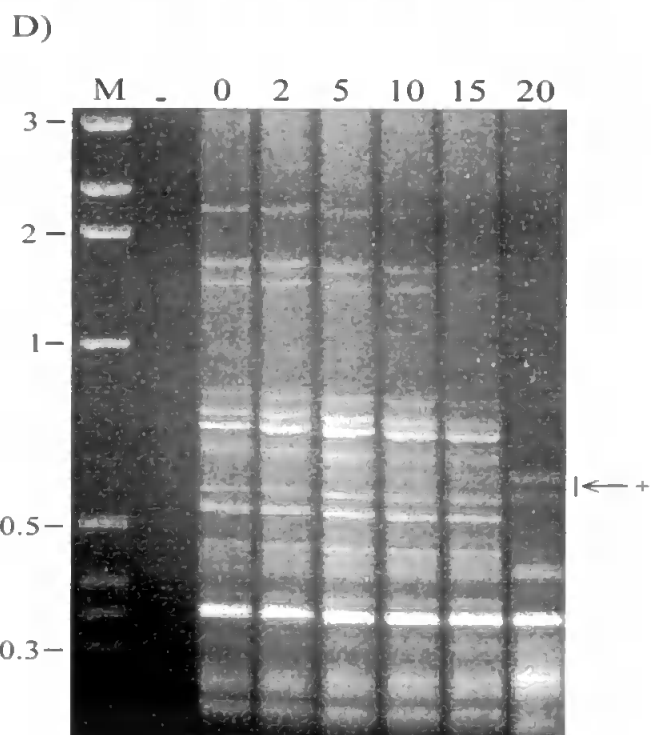
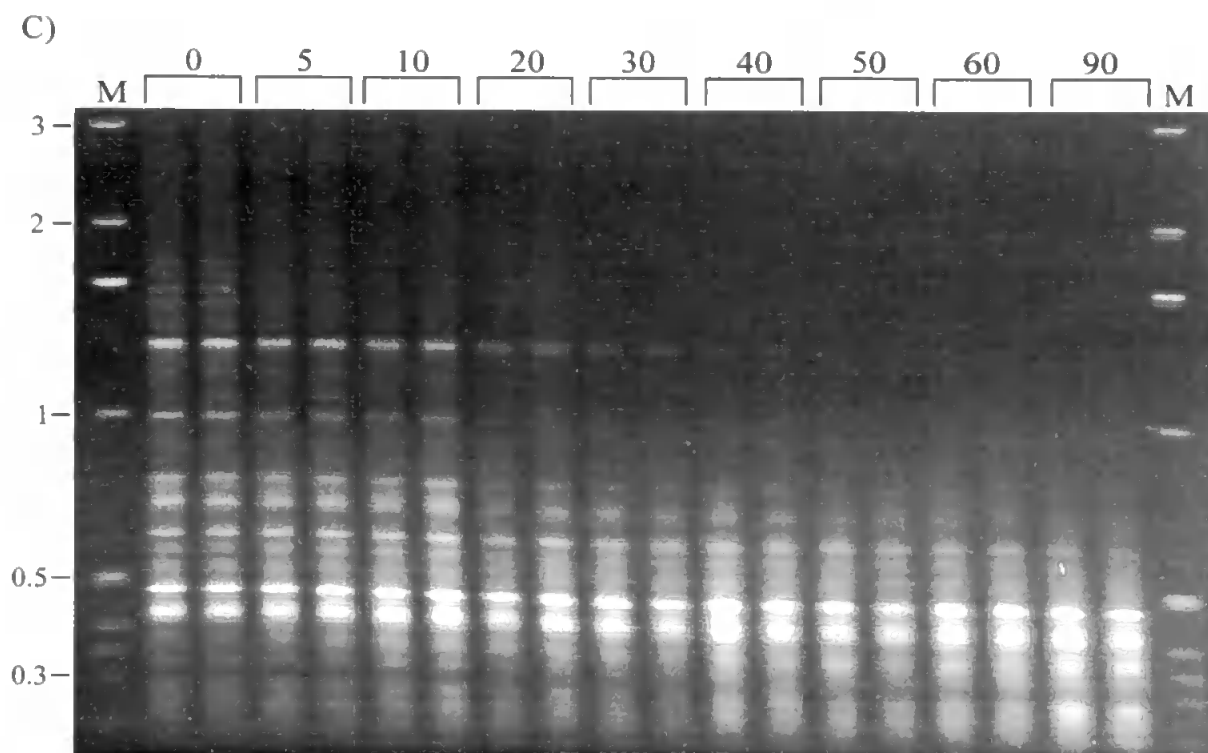


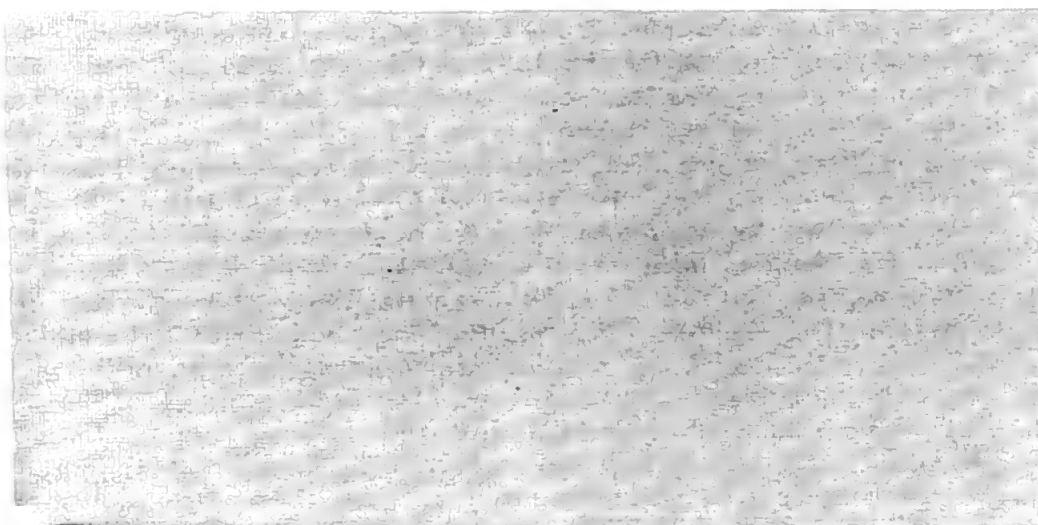
Figure 9.3 Effect of sonicated DNA on RAPD profiles. A) *P. palmata* genomic DNA was sonicated for 0, 5, 10, 20, 30, 40, 60, and 90 s as indicated at the top of the gel. B) and C) RAPD profiles of sonicated *P. palmata* DNA generated with primer OPB5 and OPB8, respectively. Sonication time (s) as indicated on the top of each gel. Each PCR



was run in duplicate using 20 ng DNA. D) RAPD profiles of sonicated *D. magna* DNA generated with primer OPA9. Time of sonication (s) as indicated on the top of the gel. The arrow shows the appearance of 2 new bands (+). M = 1Kb DNA ladder (BRL). The molecular sizes (Kb) of selected bands are indicated on the left.

A)

(e)

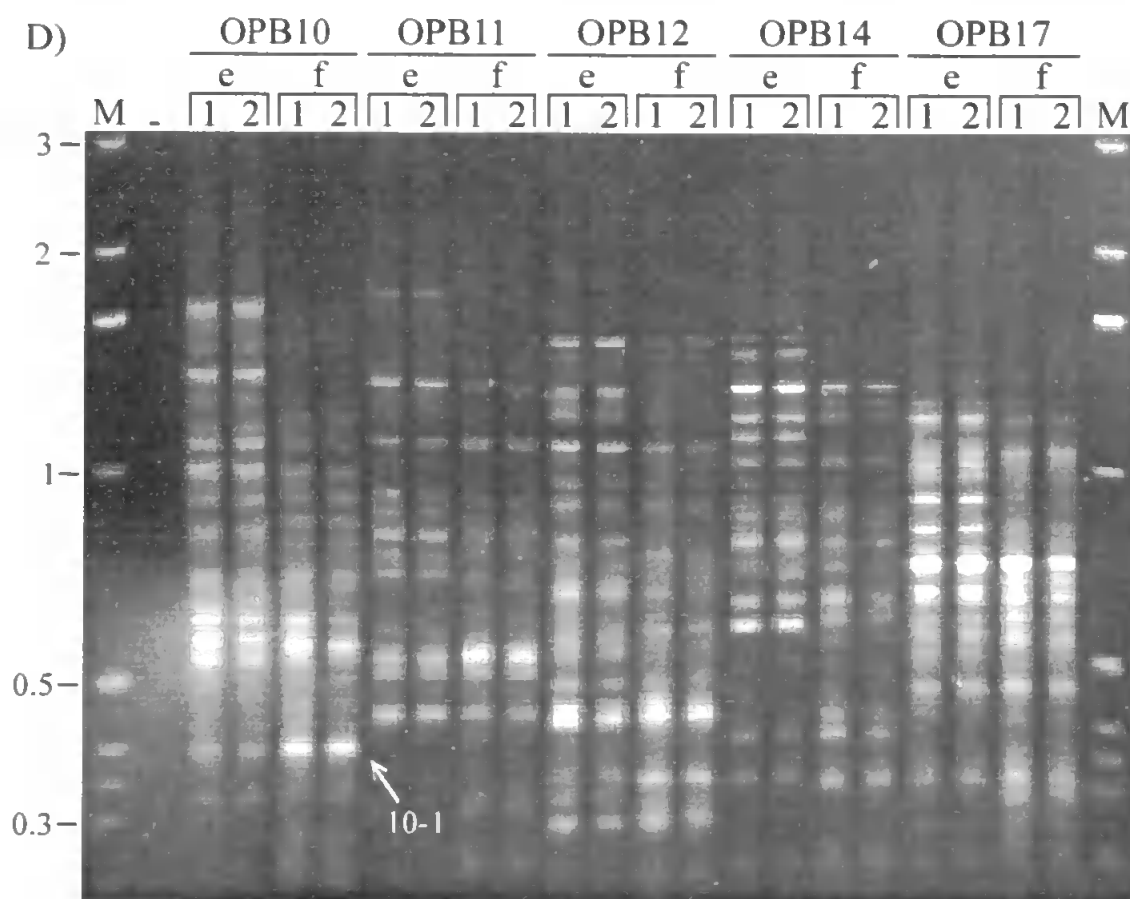
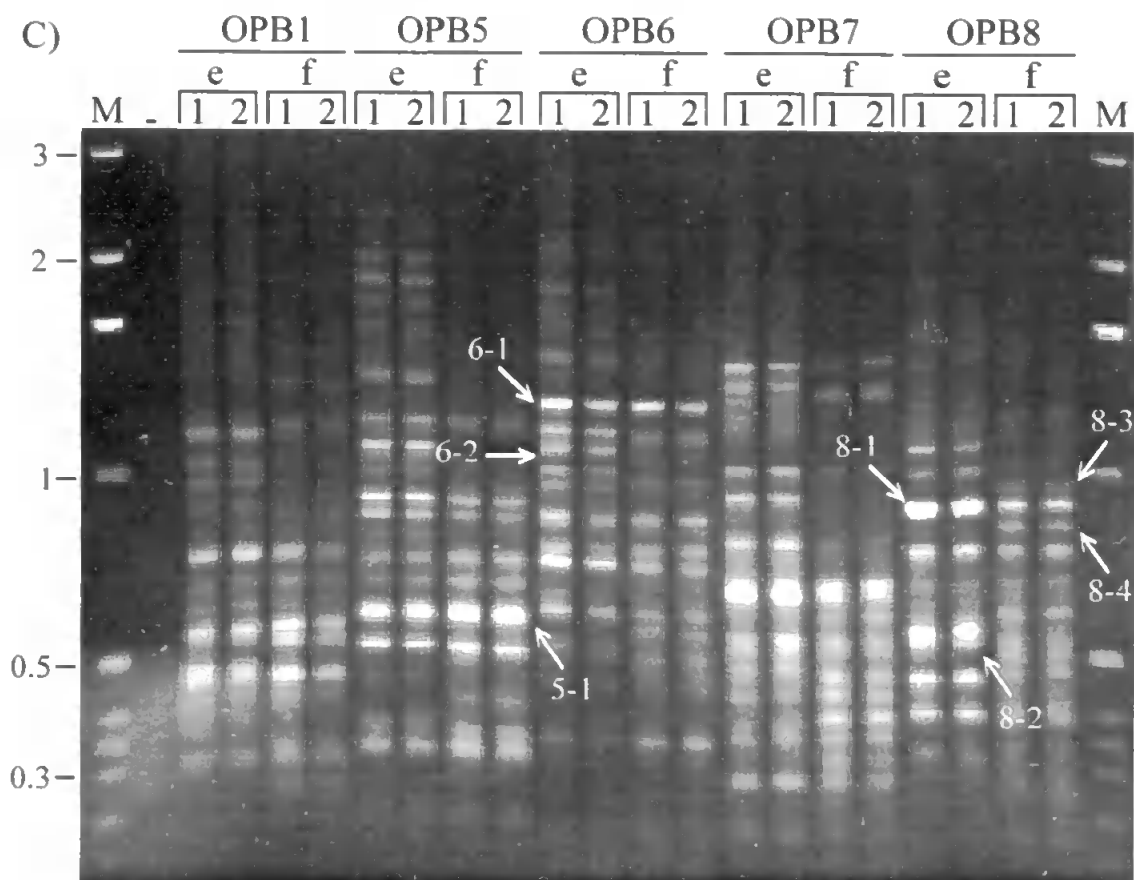


B)

(f)



Figure 9.4 Effect of control and adducted *Mytilus galloprovincialis* genomic DNA on RAPD profiles. A) and B) Autoradiographs of control and ^3H -BPDE treated genomic DNA, respectively. C) and D) RAPD profiles were generated by control (e) and adducted (f) DNA using 2 DNA template concentrations (lanes 1 and 2: 20 and 5 ng DNA, respectively) with different primers as indicated at the top of the gels. -: no DNA control. M = 1Kb DNA ladder (BRL). The molecular sizes (in kilobases) of selected bands are indicated on the left.



increased in B(a)P exposed samples. It was also observed that some bands appeared in the profiles produced using the adducted DNA in comparison to those generated by the control DNA (e.g. bands 8-3 and 8-4).

9.3.4 Effect of UVC-induced DNA damage on RAPD profiles

When the UV irradiated phage Lambda was restricted with the T4 endonuclease V and subjected to electrophoresis the results did not allow an estimation of the proportion of pyrimidine dimers as most of the DNA was trapped in the wells of the gel (Figure 9.5A). However, the general tendency was an increase in the number of pyrimidine dimers with raising UV-C radiation. For instance, after 80 s of radiation it seemed that at least a fraction of the DNA was saturated with pyrimidine dimers as the average size after digestion was about 3 Kb. RAPD profiles generated from UV-C treated *E. coli* and calf thymus DNA clearly showed that, compared with control patterns, there was a decrease in band intensity followed by a disappearance of the PCR products of highest molecular weight as the period of UV-C exposure increased (Figure 9.5 B, 9.5C, 9.5D and appendix III). Amplicon 1-1 (Figure 9.5D) however followed a special pattern. The intensity of this band decreased until $t = 20$ s of UV-C radiation and increased afterwards in comparison to the control band intensity. In addition, the intensity of some bands (e.g. 9-1, Figure 9.5 C) also increased with raising UV-C exposure compared to the band intensity displayed in the control but this event was rare. Finally, the results clearly showed that raising the time of exposure of DNA to UV-C radiation generally increased the number of changes occurring in RAPD profiles.

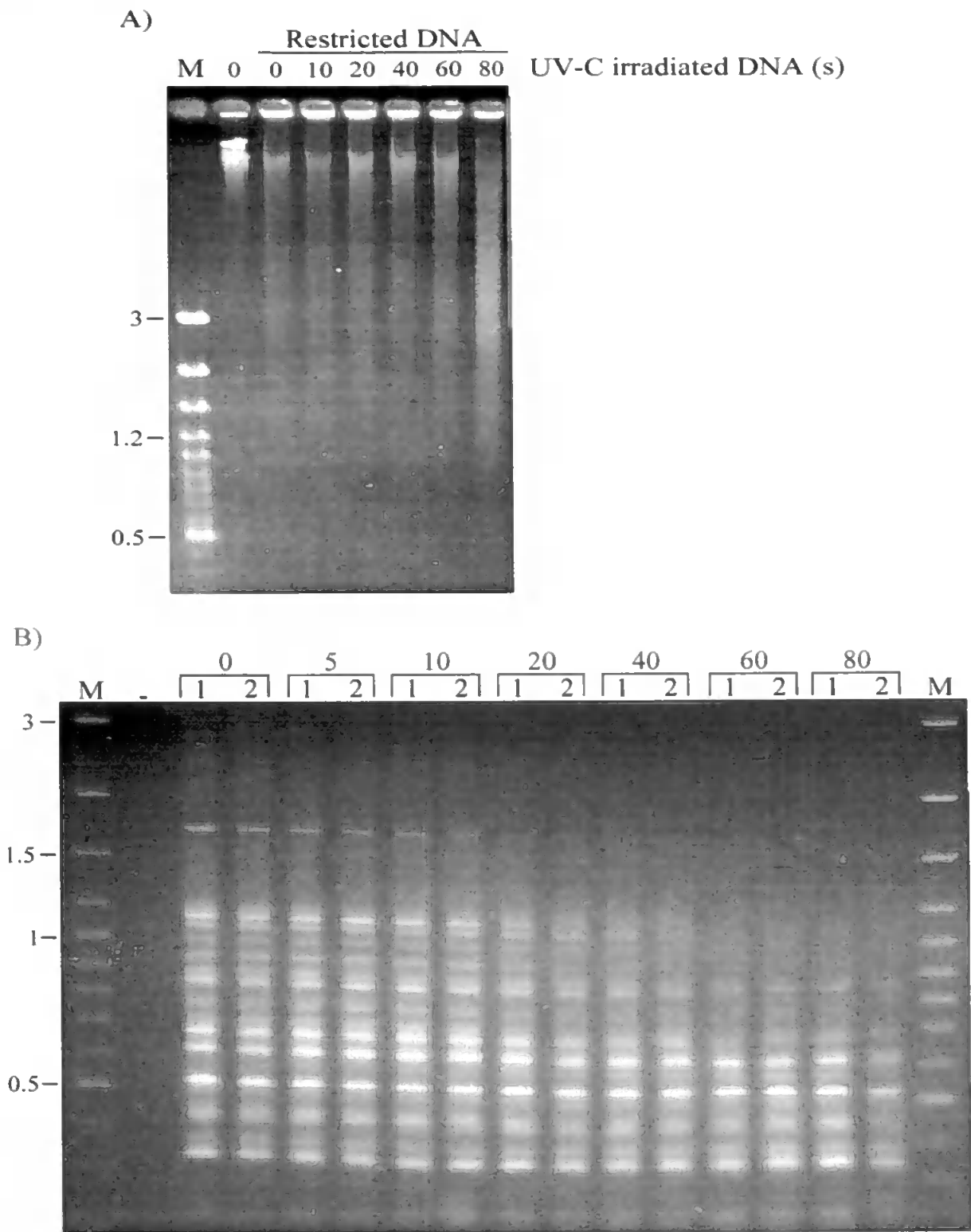
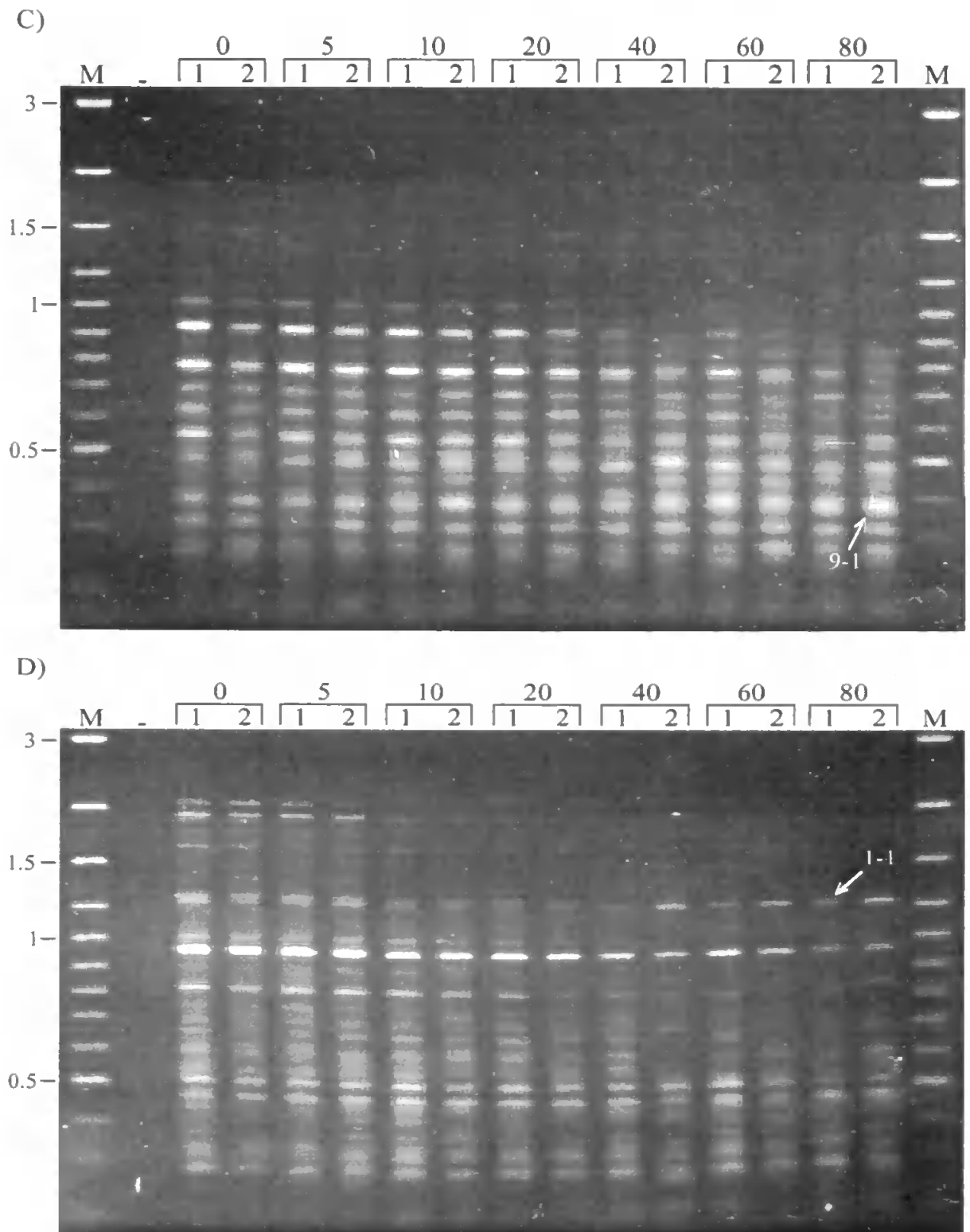


Figure 9.5 Effect of UV-C irradiated genomic DNA on RAPD profiles. A) Phage Lambda DNA irradiated for 0, 10, 20, 40, 60, and 80 s and digested by T4 endonuclease V (except for the first lane after the marker). B) RAPD profiles of UV-C irradiated *E. coli* genomic DNA using primer OPA9. C) and D) RAPD patterns of UV-C irradiated calf



thymus genomic using primer OPA9 and OPB1, respectively. Each PCR was run using 2 DNA template concentrations (lanes 1 and 2: 20 and 5 ng DNA, respectively). Some of the changes are indicated by arrows. M = 1Kb DNA ladder (BRL). The molecular sizes (Kb) of selected bands are indicated on the left of each gel.

9.3.5 Effect of mutations on RAPD profiles

9.3.5.1 The use of strains of *Escherichia coli* deficient in mismatch repair genes (study 1)

The Marmur's method was initially used to extract DNA from the bacterial strains (see section 3.2.3.1). However as this method was not optimal when the extraction was performed using 1 ml of bacteria ($OD > 2$), the Puregene DNA isolation kit was finally used. Figure 9.6 shows the growth curves obtained from the 3 strains which followed the same type of curve. The lag or latent phase which lasted for about 3.5 h was followed by the log or exponential phase which proceeded for 4 h. Finally after 8 h the bacteria entered the stationery phase as the OD stabilised until $t = 31$ h. The results obtained from PCR reactions showed that very similar RAPD patterns were produced by the 3 strains whenever the sampling was performed using primer OPB5 and OPB7 (Figure 9.7). These results were confirmed with other primers (appendix III).

9.3.5.2 Influence of a single base change in the primer sequence on RAPD profiles (study 2)

Changing a single base in the sequence of the 10-mer primer often induced a different RAPD profile in comparison to the pattern generated by the unmodified primer (i.e. primer OPB5 or OPB7) (Figure 9.8A, 9.8C, and appendix III). However, the profiles generated by primers OPB5 and OPB5-1 were very similar although the intensity of bands were not necessarily identical (Figure 9.8C). Nevertheless, for the other modified primers tested (i.e. OPB5-2 to OPB5-10 and OPB7-1 to OPB7-10) a single change in the original sequence dramatically changed the RAPD profiles. PCR products generated by primers OPB5 (Figure 9.8C) and OPB7 (Figure 9.8A) were also reamplified using primers OPB5, OPB5-1 to OPB5-10 as well as OPB7, OPB7-1 to OPB7-10, respectively. PCR products could be reamplified even when the template DNA/primer match was not perfect. However, when the 4th base was substituted in the original primer the reamplification process was reduced (Figure 9.8C, 9.8D, and appendix III). Figure 9.8D showed as well

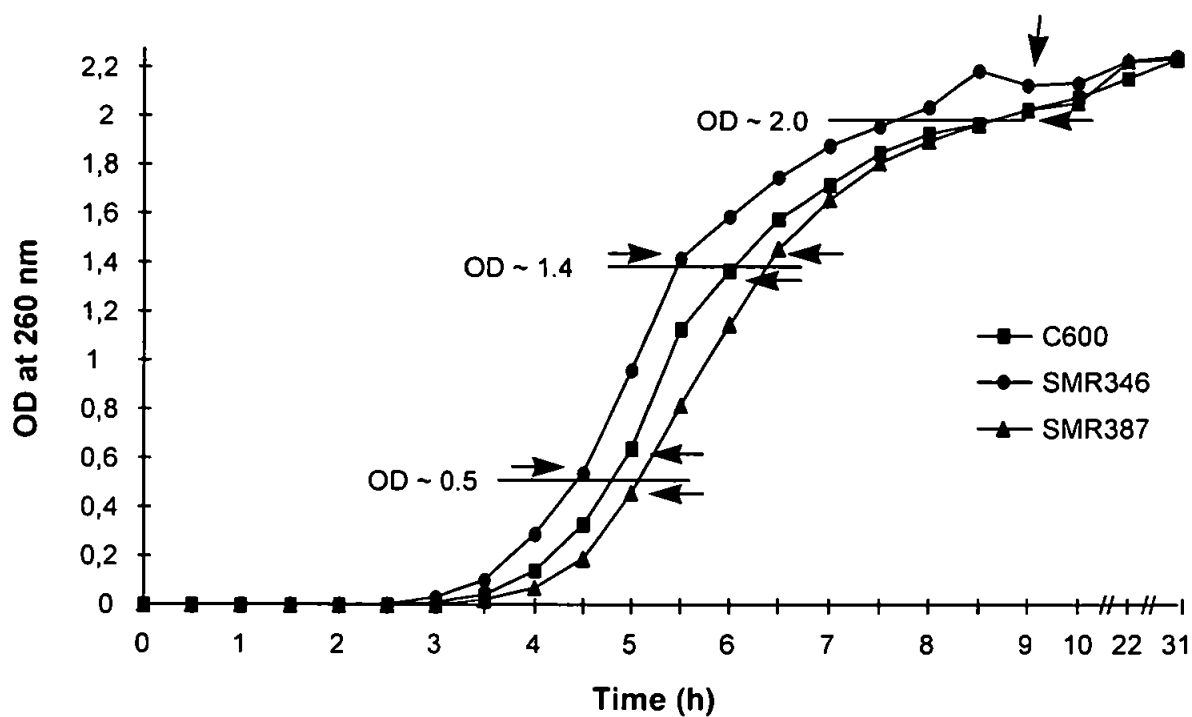


Figure 9.6 Growth of *Escherichia coli* strains C600, SMR346, and SMR387 at 37°C under constant agitation. Bacterial cells were sampled when the OD_{260nm} was approximately of 0.5, 1.4, and 2 for each strain. Arrows indicate when the sampling was carried out.

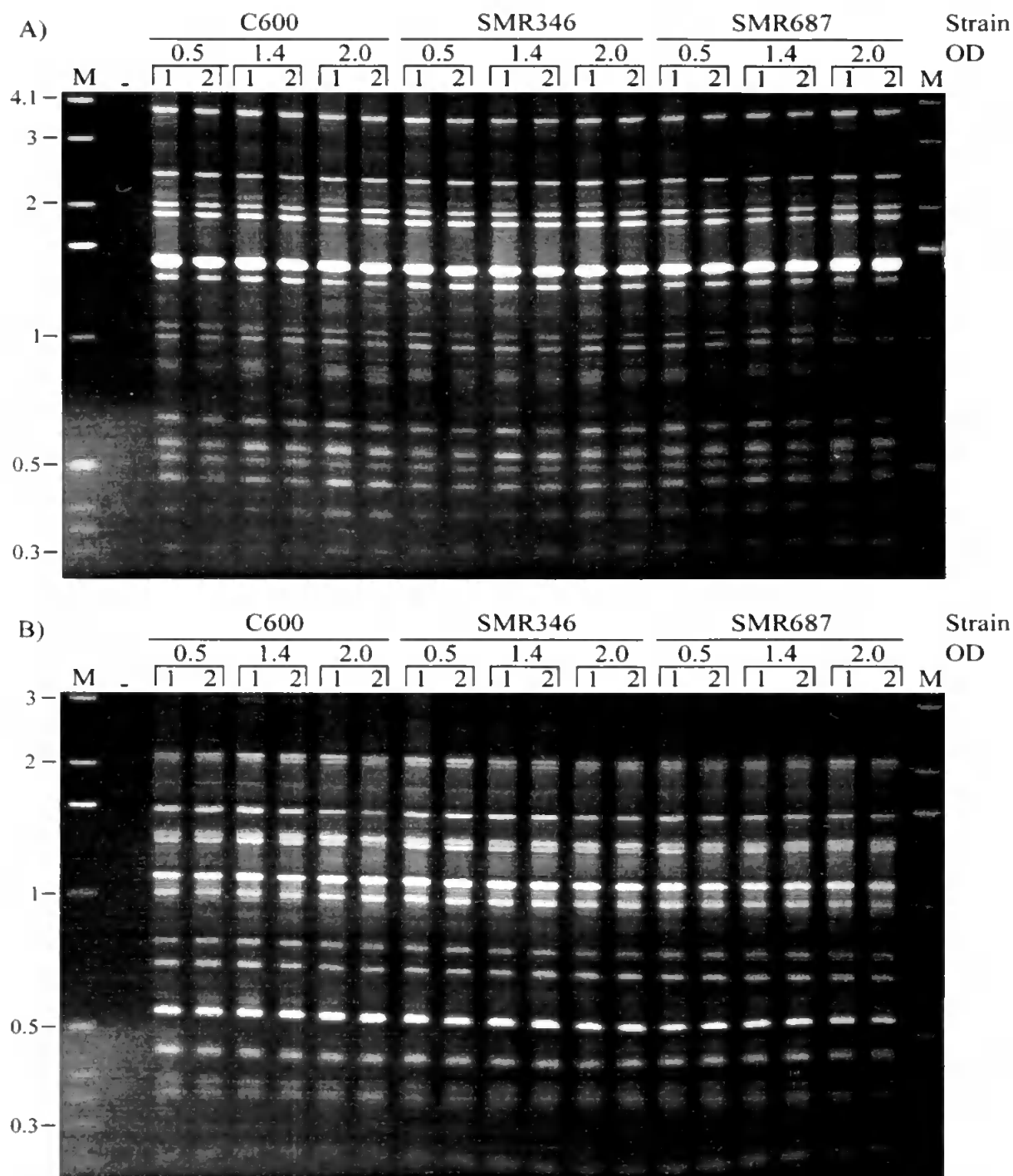


Figure 9.7 RAPD profiles of *Escherichia coli* control (C600) and mismatch repair defective strains (SMR346 and SMR687) at an approximate OD_{260nm} of 0.5, 1.4, and 2. Patterns were produced using primers OPB5 (A), OPB7 (B) and two template DNA concentrations (lanes 1 and 2: 20 and 5 ng DNA, respectively). -: no DNA control. M = 1Kb DNA ladder, (BRL). The molecular sizes (Kb) of selected bands are indicated on the left of each gel.

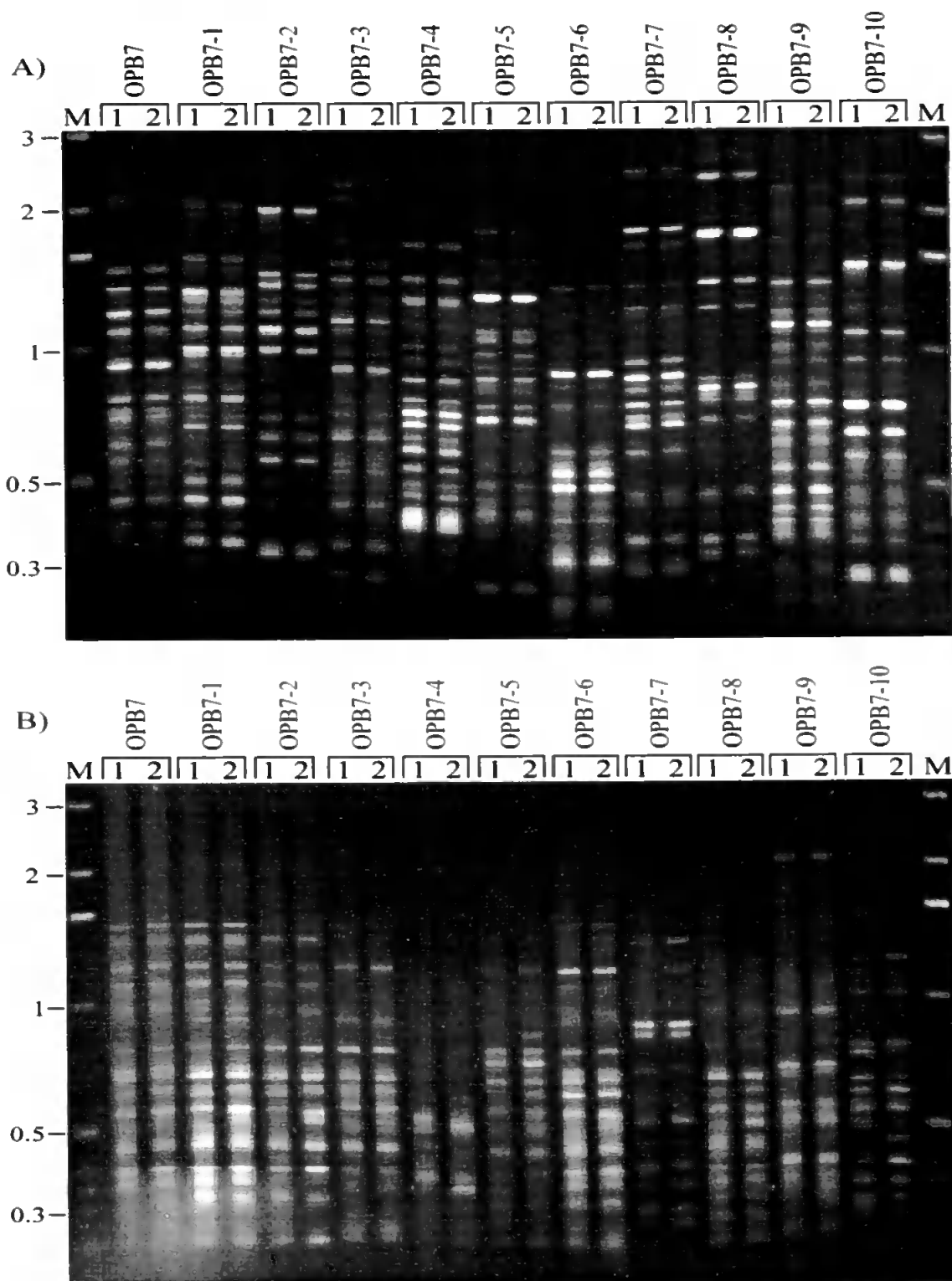
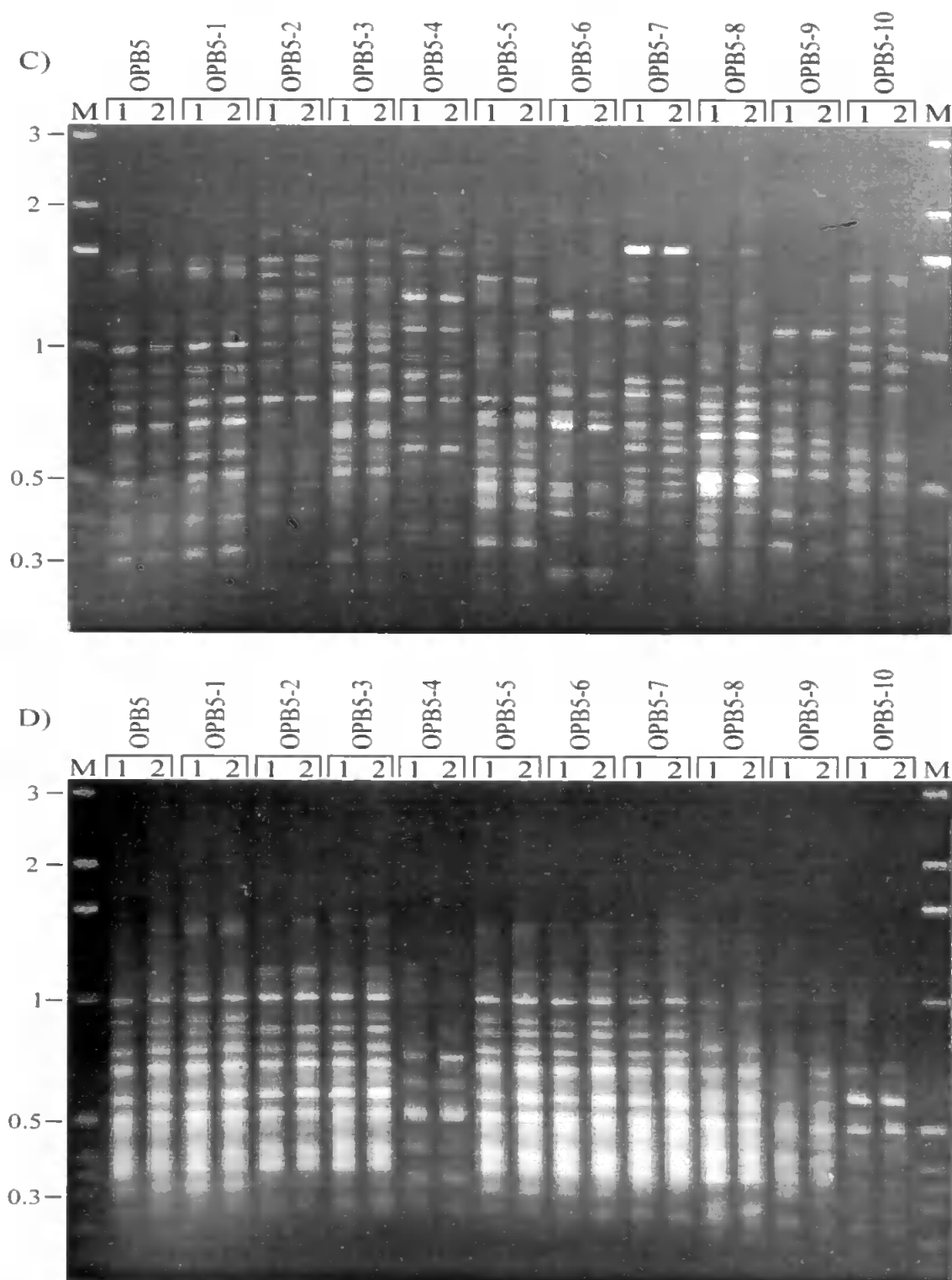


Figure 9.8 Effect of single nucleotide substitutions in 10-mer primers on RAPD profiles. Primers as indicated at the top of figure. A) and C) patterns were obtained using *E. coli* and calf thymus DNA, respectively. B) and D) represent re-amplification of PCR products generated by primers OPB5 and OPB7, respectively. PCR reactions were



performed using two template DNA concentrations (lanes 1 and 2: 20 and 5 ng DNA, respectively). M = 1Kb DNA ladder (BRL). The molecular sizes (Kb) of selected bands are indicated on the left of each gel.

that a change in the last 3 bases (at the 3' end) in the original primer had some importance as less bands were generated by these primers. The reproducibility of the RAPD profiles after reamplification was usually satisfactory except for some bands (see for example appendix III).

9.3.5.3 Genetic diversity among *Renibacterium salmoninarum* isolates (study 3)

The results generated by primer OPA9 and OPB1 are presented in Figure 9.9A and 9.9B, respectively. Although most of the bands were shared by the isolates, the profiles clearly revealed some differences. The patterns produced by the different isolates cultured in laboratory conditions originating from the same country shared a lot of bands with the exception of the USA. According to Figure 9.9A and 9.9B, 3 different groups emerged for the United States isolates: group 1: ATCC 33209 and Round Butte (lines c and d respectively), group 2: Little Goose and NCIMB2196 (lines g and o respectively), group 3: Marion Forks (lines p). The patterns generated suggested that groups 1 and 3 shared more similarities with each other than with group 2. In addition, although the profiles produced by the other isolates (Canada, Scotland, Iceland and England) were very similar they were not identical to those generated from group 2 of the USA. RAPD reactions were also performed using two DNA concentrations with primers OPA9 and OPB1 to assess the reproducibility of profiles (Welsh *et al.*, 1995a). Figure 9.9C and 9.9D which displays the RAPD patterns generated by primer OPA9 using 2.5 and 10 ng genomic DNA with all isolates showed that the profiles were very reproducible. The results for primer OPB1 which are presented in appendix III showed the same level of reproducibility.

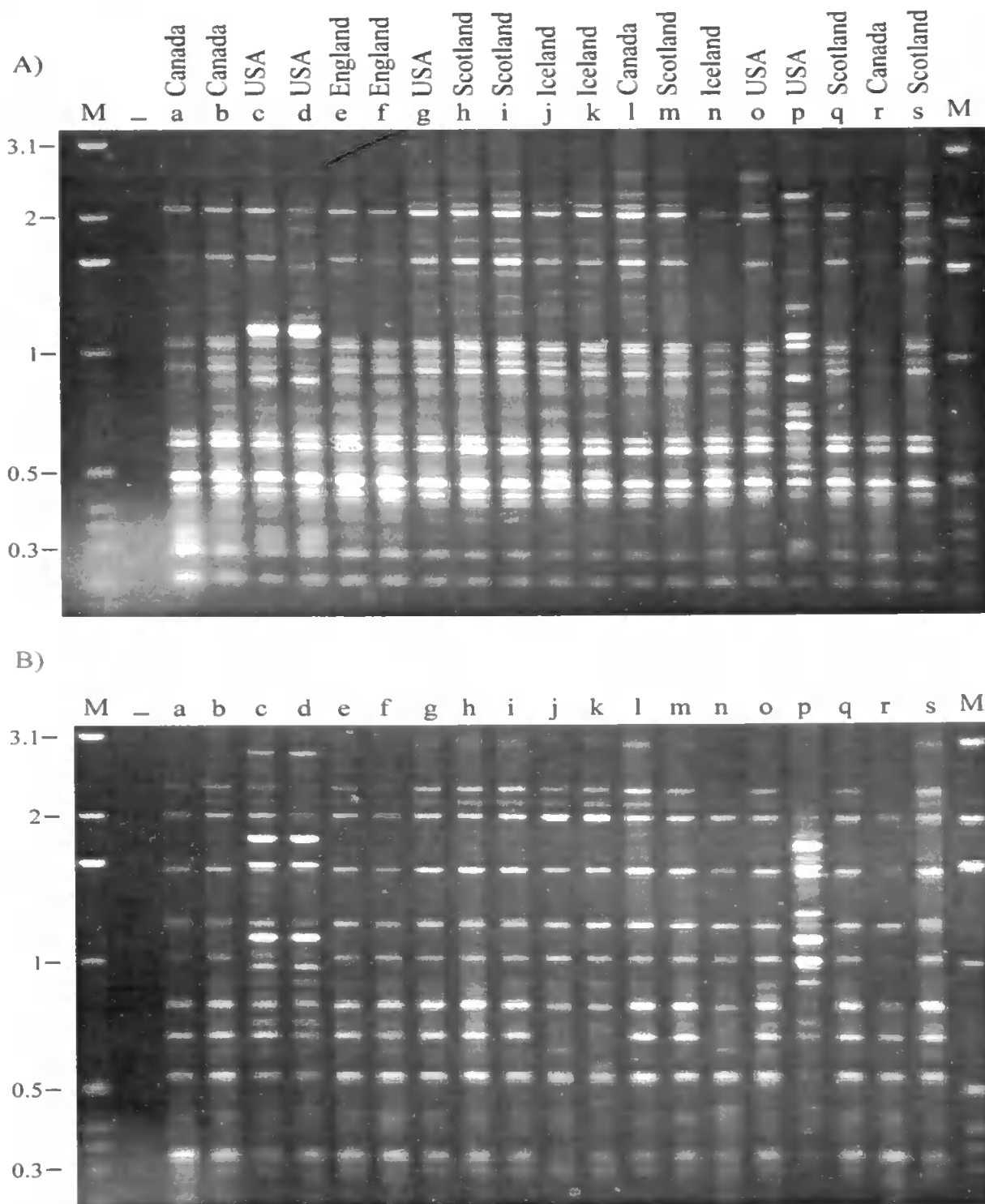
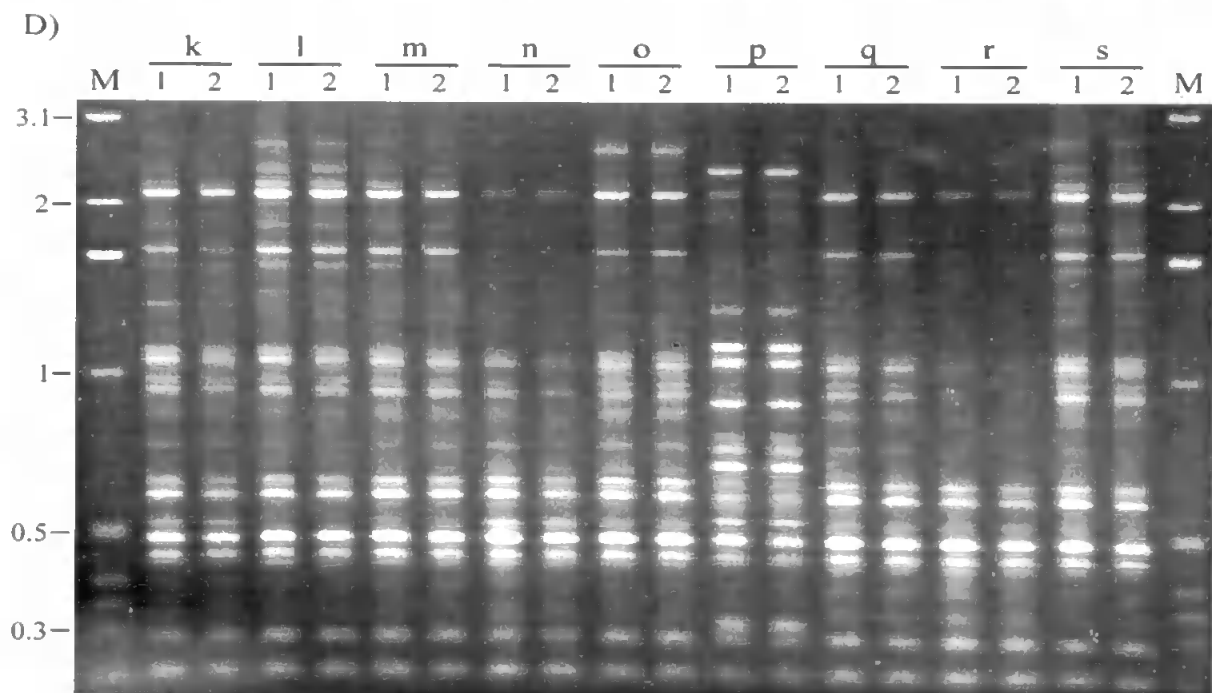
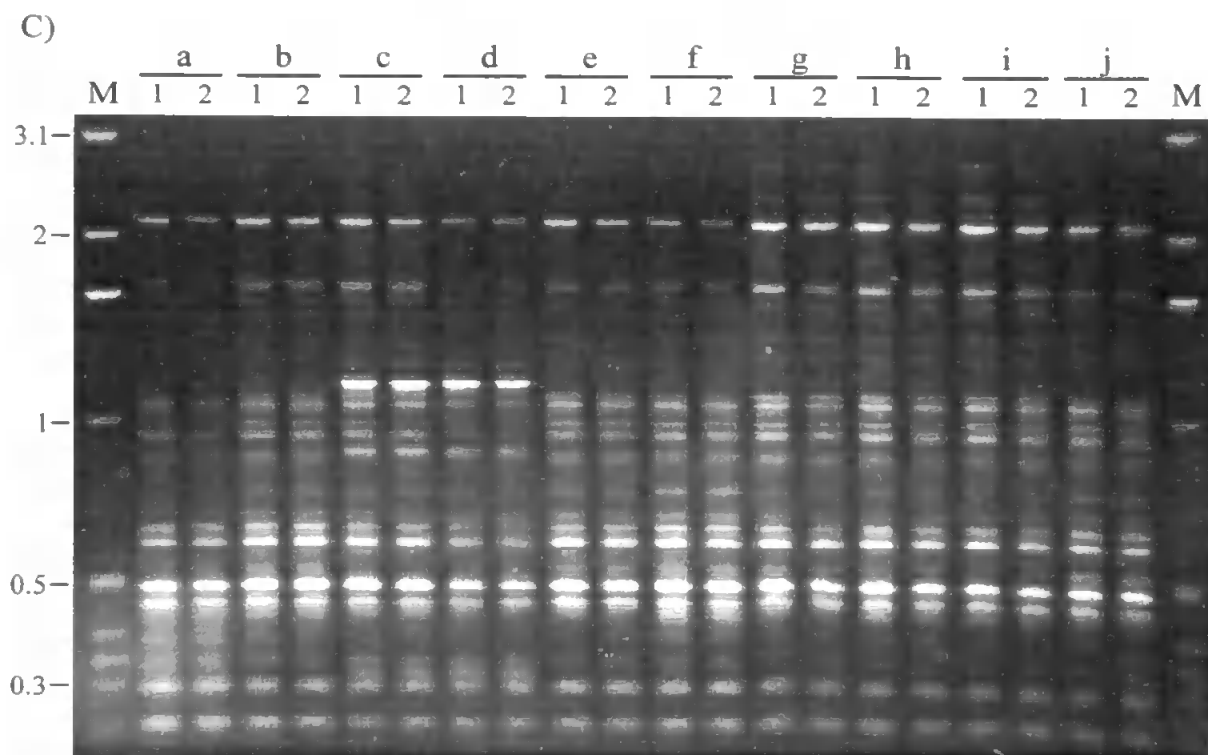


Figure 9.9 RAPD profiles of 19 isolates of *Renibacterium salmoninarum* from a variety of geographic areas and host species (salmon). RAPD patterns were generated using primers OPA9 (A, C, and D), and OPB1 (B). M: 1Kb DNA ladder (BRL); The molecular sizes (Kb) of selected bands are indicated on the left of each gel. - = no DNA control. Lanes a-s: each lane represents an isolate; a: DR384; b: DRI28; c: ATCC 33209; d:



Round Butte; e: W2; f: W6; g: Little Goose; h: MT1363; i: BA99; j: S-182- 90; k: F-273-87; l: RS-TSA; m: MT410; n: F-138-87; o: NCIMB2196; p: Marion Forks; q: MT417; r: DR143; s: MT420. The origin of each isolates is indicated at the top of gel A. Reproducibility was also checked by using 2 DNA template concentrations (lanes 1 and 2: 2.5 and 10 ng DNA, respectively) with primer OPA9 (C and D).

9.3.6 Sensitivity of the RAPD method

This experiment was carried out to evaluate the minimum level of mutated/altered genomic DNA that can be detected using the RAPD protocol. Figure 9.10 revealed that a main band generated by a single source of DNA (i.e. 100 %) could be easily detected even when the contribution of the DNA was as low as 15-25 % (e.g. bands labelled B1A, B2A, B3A, B1B, B2B). Some bands were even generated when as little as 1 ng (5 %) of a genome was mixed with 19 ng (95 %) of a different genomic DNA. But the band intensity appeared to be quite weak. It is noteworthy that for bands B2A and B3A the intensity was easily identifiable when only 3 ng (15 %) of the *D. magna* DNA was mixed with 17 ng (85 %) of *E. intestinalis* DNA, as compared with the intensity of the same bands generated with 20 ng *D. magna* (100 %).

9.3.7 Overview of the results

Table 9.2 summarises the results obtained in the present study. The results clearly revealed that random breakage obtained by sonication had much more effect on RAPD profiles than enzymatic restriction. Sonicated DNA, B(a)P adducts and pyrimidine dimers generally led to similar qualitative changes. It was also very clear that random mutations did not affect the RAPD profiles whereas a single base change in the 10-mer primer had considerable effects. Finally, the presented results unambiguously showed that genetic diversity (including mutation) in *R. salmoninarum* was easily detected by the RAPD technology.

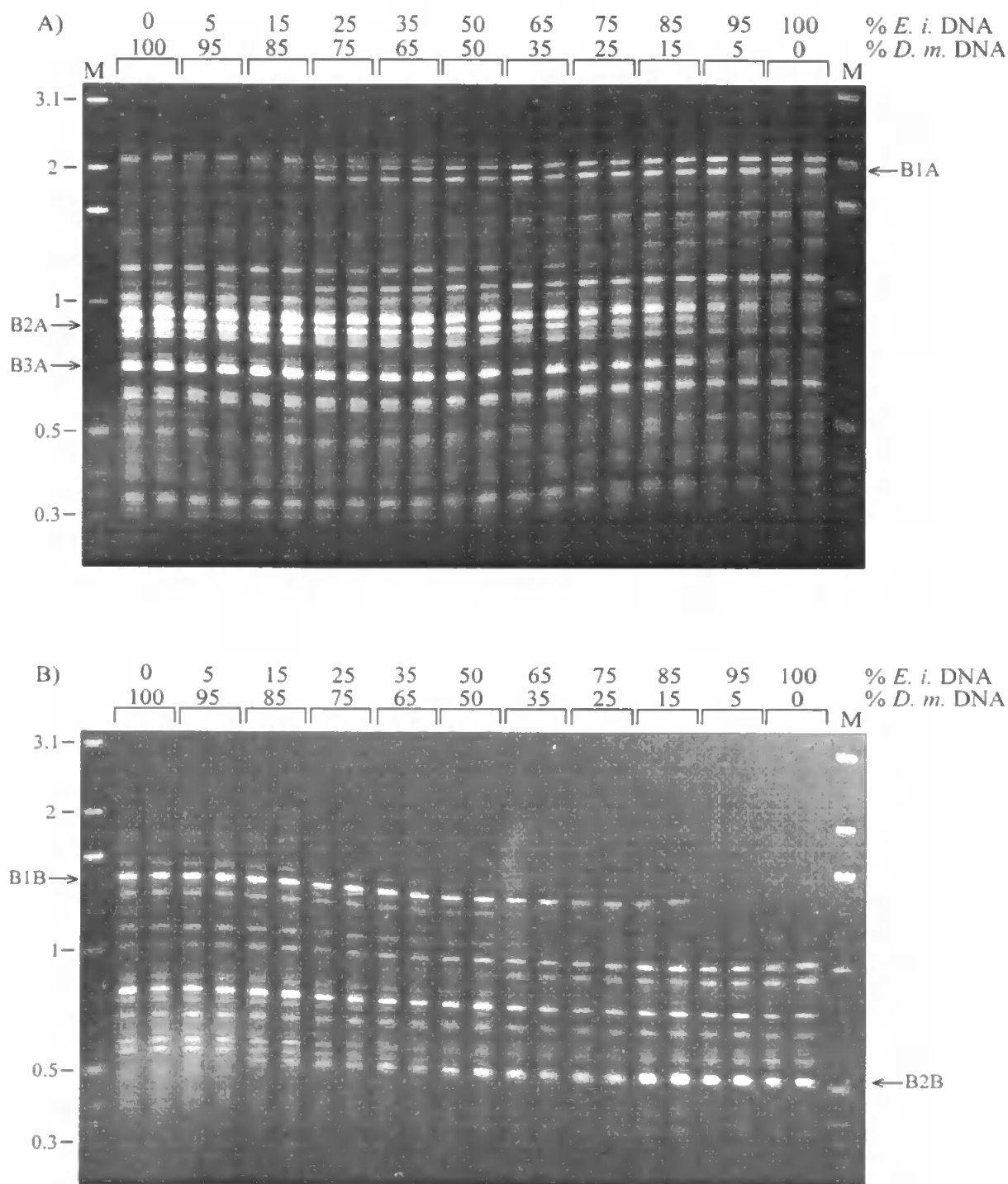


Figure 9.10 Evaluation of the sensitivity of the RAPD technique. PCR reactions were performed with the appropriate mixture of genomic DNA extracted from *D. magna* (*D. m.*) and *E. intestinalis* (*E. i.*) as indicated at the top of each gel. M = 1 Kb DNA ladder (BRL). The molecular sizes (Kb) of selected bands are indicated on the left of each gel. PCR reactions were performed using primers OPB5 (A) and OPA9 (B). PCR reactions were run in duplicate.

Table 9.2 A summary of the effects of diverse types of DNA effects on RAPD profiles.

DNA effects	Changes in RAPD profile			
	Disappearance of bands	Appearance of bands	Decrease in band intensity	Increase in band intensity
Non random DNA breakage (restriction)	Major event	No	No	No
Random DNA breakages (sonication)	Major event (high molecular weight bands)	Minor event	Major event	Minor event
Benzo(a)pyrene induced adducts	Major event (mainly high molecular weight bands)	Minor event	Major event	Minor event
Pyrimidine dimers	Major event (mainly high molecular weight bands)	No	Major event	Minor event
Random mutations	No	No	No	No
Change in primer sequence	New RAPD profile			
Genetic Diversity	Yes	Yes	Yes	Yes

9.4 Discussion

The RAPD patterns which were generated using genomic DNA restricted by diverse restriction enzyme did not always differ from the patterns generated using undigested control DNA. Generally the number of bands that were either absent or not affected varied according to the DNA, the primer and the enzyme used. Similar results were obtained by Khandka *et al.* (1997) except these researchers found that bands also appeared in comparison to control patterns. It seems obvious that PCR products disappeared when the restriction site was contained between 2 closely (e.g. maximum ~3 kb) opposite annealing sites. The data also indicated that the restriction enzymes which cut the genomic DNA more frequently were more likely to have an effect on RAPD profile. The results presented in this research project showed that there was a distinct difference in the amplification pattern of mechanically (sonication) and enzymatically (restriction enzymes) fragmented DNA. The sonication of template DNA had a much greater effect on RAPD profiles compared with the results obtained with restriction enzymes. Unlike restriction enzymes, sonication induces rapid random and more frequent DNA breakage. After sonication, bands of the highest molecular weight initially disappeared because mechanical shearing occurs much more readily in larger fragments. Although bands were observed to appear in RAPD profiles produced using sonicated template DNA, this event was quite rare. In these cases, DNA breakages may have induced some structural rearrangements which could facilitate the binding of the primer to the DNA compared to control DNA in which the same site may not have been accessible to the primers. The detection of DNA breakages is of particular interest because chromosome aberrations and rearrangements are thought to result from strand breakage misrepair. This could also contribute to genetic amplifications, aneuploidy, alterations in gene expression and loss of heterozygosity, which may promote neoplastic progression (Bohr *et al.*, 1995).

The main change in RAPD profiles produced using template DNA containing B(a)P induced adducts was an overall decrease in the intensity of bands. Since bulky adducts have been shown to block or reduce the processivity of the *Taq* DNA polymerase (Brown and Romano, 1991; Choi *et al.*, 1994; Christner *et al.*, 1994; Chary and Lloyd, 1995), bands of the highest molecular weight were initially affected. However, the fact that some lower molecular weight bands were much more affected than amplified products of larger size suggested that there were specific areas (hot spots) of interaction between the diol-epoxide and DNA (Boles and Hogan, 1986). Non-random binding of agents with DNA has also been demonstrated in numerous studies (Gupta, 1984; Cosman *et al.*, 1992; Vogelstein and Kinzler, 1992; Shou *et al.*, 1993). The two other minor changes in RAPD patterns were an increase in the intensity of some bands as well as the appearance of PCR products of a distinct size. Some of these bands may represent products which were amplified more efficiently because more primers were available to a site due to the structural changes induced by B(a)P. Alternatively the structural changes may allow new primer/DNA annealing at sites which were not available in control DNA.

When DNA is irradiated with UV, the two major types of damage produced are the *cis-syn* cyclobutane pyrimidine dimer and pyrimidine (6-4)-pyrimidione lesions (Varghese and Wang, 1968), formed in about a 3:1 ratio (Mitchell and Nairn, 1989). Thus to simplify the discussion any changes in profiles will be attributed to the direct and indirect effects of pyrimidine dimers. The T4 endonuclease V has been used in this study because this restriction enzyme cuts at every pyrimidine dimer; Smith and Taylor (1993) reported that the T4 endonuclease V did not cleave the (6-4) or Dewar product. UV-induced pyrimidine dimers are also known to block or reduce the processivity of the PCR enzyme (Rabkin *et al.*, 1983; Taylor and Oday, 1990; Donahue *et al.*, 1994; Nelson *et al.*, 1996a). Consequently, the highest molecular weight bands disappeared (main event) as a result of a high frequency of pyrimidine dimers which increased as UV-C exposure was raised. It is

possible that the *Taq* DNA polymerase did not dissociate from the pyrimidine dimers. Donahue *et al.* (1994) reported that the complex cyclobutane pyrimidine/RNA/RNA polymerase II was stable and that neither the polymerase nor the RNA product dissociated from the DNA template. In addition, similarly to B(a)P-induced DNA adducts, pyrimidine dimers unwind the DNA and thus produce some distortion in DNA structure (Ciarrochi and Pedrini, 1982; Wang and Taylor, 1991). For instance, the *trans-syn* thymine dimer bends DNA by about 22 degrees and unwinds DNA by about 15 degrees (Wang and Taylor, 1993). Hanawalt (1998) reported that the 6-4 pyrimidine-pyrimidone photoproducts is the more distorting lesion, bending the DNA about 44 degrees, while the cyclobutane pyrimidine dimers bend the DNA about 9 degrees. These alterations are likely to induce changes in RAPD profiles (see above). Finally, the fact that pyrimidine dimers represent the major UV-induced lesions does not necessarily mean that changes in RAPD profiles are all due to pyrimidine dimers. Ganguly and Duker (1992) reported that thymine hydrates were formed in UV-irradiated DNA and partially inhibited polymerase activity during DNA synthesis. This could be potentially lethal if unrepaired. Finally, the detection of DNA damage such as B(a)P adducts and pyrimidine dimers is of primary importance because such lesions have been shown to produce mutations after DNA replication and DNA repair events.

In this chapter it was also attempted to better understand the effects of mutations on RAPD profiles. In the first study, *E. coli* strains SMR 346 and SMR 387 probably developed a high rate of mutation during replication as the mismatch repair gene was deficient in both strains. Indeed, Schaaper and Dunn (1987) reported that *E. coli* displayed elevated spontaneous mutations rates because of a deficiency in the process of post-replicative mismatch correction. The spectra consisted of base substitutions (75 %) and single-base deletions (25 %). In addition, strains deficient in mismatch repair have been used to study adaptive mutation in non-growing cells (Schaaper and Dunn, 1987;

Longerich *et al.*, 1995). In the present study, after the latent phase these strains needed approximately 3.5 hours to reach an optical density (260 nm) of 2 which meant that replication of the genomic DNA occurred 10 times (*E. coli* doubles the amount of DNA every 20 minutes). This suggests that the mutations probably accumulated over time. RAPD profiles performed on DNA extracted from both strains over a time course revealed that no readily detectable changes arose in comparison to the parental strain. However, mutations which may have accumulated in both strains would have occurred randomly and consequently mutations among the same population of bacterial cells (SMR 346 or SMR 387) would have been different. To reiterate, the RAPD method did not reveal any changes in RAPD profiles because there were no hot spot mutations in both mismatch repair deficient strains. In the second study, results showed that a single base change in the sequence of the 10-mer had a tremendous effect on RAPD profiles. Only in one case did a single base change in the sequence of the primer produced very similar patterns (Figure 9.8C; primer OPB5 and OPB5-1) although the intensity of the amplified bands was quite different. It is likely that a perfect annealing between the primer and the DNA produces an intense band if the site is easily accessed by the primer and if the extension reaction proceeds without impediment. When the products of PCR reactions generated by the original primers, OPB5 or OPB7, were subjected to reamplification by the single base substituted primers, profiles were obtained in all cases. Thus, in presence of high concentration of PCR products and very low concentration of genomic DNA template perfect pairing between PCR product and primers were not required. The amplification was significantly reduced (compared to the pattern produced by the original primers) when the 4th base from the 5' end of the original oligonucleotide was changed (Figure 9.8 and appendix III). This suggested that the 4th base of the 10-mer primer required a perfect annealing to allow an efficient reamplification. This may emphasise the importance of structural effect even for short primers. Caetano-Anolles *et al.* (1992) also stressed the

significance of structural effects in DNA amplification fingerprinting. Caetano-Anolles *et al.* (1992) proposed a model in which a single primer preferentially amplifies certain products due to competition for annealing sites between primer and terminal hairpin loop structures of the template. In addition, as some of the patterns were different from those obtained from the original patterns it could be argued that in absence of genomic DNA (or in presence of very little amount of genomic DNA compared to PCR product) artefacts or false amplification were generated. However, this was certainly not the case as the reproducibility was generally satisfactory for most bands when 2 different concentrations of PCR products were used. The ability for an oligonucleotide to serve as a primer is dependent on several factors, including (i) the kinetics of association and dissociation of primer-template duplexes at the annealing and extension temperatures, (ii) the effects on duplex stability of mismatched bases and their location, and (iii) the efficiency with which the polymerase can recognise and extend a mismatch duplex (Kwok *et al.*, 1990; Caetano-Anolles *et al.*, 1992). Sommer and Tautz (1989) reported that primers (17-20 nucleotides) needed at least three homologous nucleotides at their 3'-end for successful priming and that the remainder of the primer needed only very little homology with the target sequence. However it must be borne in mind that when PCR are performed at high annealing temperature mismatches are less stable and therefore less tolerated (Caetano-Anolles *et al.*, 1992). For instance, Caetano-Anolles *et al.* (1992) reported that at an annealing temperature of 55°C, only the perfectly matched primer (8-10 base long nucleotide primers) was successful in amplifying the synthetic template. As in the present study an annealing temperature of 50°C for 10-mer primers was used, we believe that most of the amplified PCR products are the result of perfect pairing between DNA and primer sequence. This was confirmed by the fact that a single base change in the sequence of the 10-mer primer resulted in tremendous changes in RAPD profiles. This was an important outcome which suggested that a single mutation in the genomic DNA could be easily detected under high

stringency conditions. In the third study, the RAPD analysis was evaluated to determine the genetic diversity in *R. salmoninarum*, a highly conserved genospecies. The genetic diversity includes fixed mutations such as deletion, insertion, and larger rearrangements which may have occurred as a consequence of the evolution and possibly other processes. The results obtained with primers OPA9 and OPB1 plus those produced by a set of six other primers (analysis performed by Dr Thomas Grayson, University of Plymouth, UK) revealed that none of the isolates produced identical RAPD patterns. In addition, the molecular variation of the 16S-23S ribosomal RNA intergenic spacer sequence of *R. salmoninarum* isolates from widely separated environments was extremely limited (Grayson *et al.*, 1999). Similarly, RAPD analysis of strains of *Bacillus cereus* revealed a remarkable diversity which was not divulged by ribosomal RNA or transfer RNA intergenic spacer target PCR (Daffonchio *et al.*, 1998). Numerous studies have demonstrated that RAPD constitute a reliable and reproducible method for molecular typing and genetic characterisation of a variety of micro-organisms (e.g. Hansen *et al.*, 1998; Wagner *et al.*, 1998; Wang *et al.*, 1998). Thus, the RAPD method was particularly useful for examining the genomic diversity among strains of bacteria which were indistinguishable by other molecular methods such as traditional subtyping techniques. This study clearly showed that the RAPD method which has been optimised in this research project can detect non-random mutations in bacterial strains leading to genomic diversity as demonstrated by other studies (e.g. Williams *et al.*, 1993).

While this study clearly demonstrates the potential of the RAPD method to detect DNA damage and mutations, it was crucial to assess the sensitivity of the assay. In other words, the objective was to determine the minimum or basal level of mutated/altered genomic DNA that can be detected using the RAPD assay. When PCR was performed using a mixture of two different genomic DNAs, a band unique to one of the templates was detectable even when the source of that template was as low as 5-25 % depending on the

brightness of the band in the original pattern, that is when RAPD was performed using 100 % of this genome. Thus, by extension, for the successful detection of mutation in *D. magna* and *E. intestinalis* using the RAPD technique, the same mutations (hot spot mutation) must prevail in at least 5-25 % of all the cells of these organisms. In this context, a band will disappear if the mutation which results in the loss of the pairing between primer and DNA occurs in 75-95 % of the cells. Alternatively, a band will appear, due to the creation of a new annealing site, if the mutation occurs in 5-25 % of the cells. Thus the appearance of new PCR products as a result of mutation is more likely to occur rather than the disappearance of bands. Under certain circumstances, it is possible that mutations occurring in 2-3 % of the cells might be detected. However, the sensitivity of detection of mutations by RAPD is dependent upon the localisation of the mutations in relation to the primer binding sites. For instance, a single base substitution which occurs outside or even within a priming site which lies more than 4 Kb from the next opposite priming site will go undetected even if the mutation arises in 25% of the cells. However, this is probably only partly true because mutations outside the annealing site can induce structural changes which might lead to change(s) in the RAPD pattern (Bowditch *et al.*, 1993). Since DNA damage such as DNA adducts can lead to mutations after DNA replication, this suggests that only a small proportion of the cells (e.g. 10-20 %) needs to be damaged at specific chromosomal loci in order to have an effect on RAPD profiles. Interestingly, local 'hot spots' mutation have been detected mostly in mammalian systems following exposures to both single chemicals (Vogelstein and Kinzler, 1992; Coller and Thilly, 1994; Lambert *et al.*, 1994), and complex chemical mixtures (Demarini, 1994). It is also known that DNA damage do arise at specific loci (Boles and Hogan, 1984; Gupta; 1984). Several studies have even shown that the frequency of inclusion of specific chromosomes in micronuclei is not random (Hando *et al.*, 1994; Nath *et al.*, 1995). In this context, it appears that the RAPD provides a very useful means to detect diverse kinds of DNA lesions and mutations

in mammalian systems. Although much less is known about hot spot interactions between genotoxins and invertebrate genomic DNA, this research project suggests that hot spot interactions occur at least for a number of agents such as B(a)P, UV radiation, copper, 17- β oestradiol, 4-*n*-nonylphenol, and oxygen radicals in diverse invertebrate species. In this context, this would suggest not only that specific interactions between genotoxins and DNA arise in any species but also that RAPD has the potential to detect genotoxin-induced DNA effects in any species.

In conclusion, this study clearly shows that the RAPD method is a sensitive method for detecting DNA damage, namely DNA breakages, B(a)P-induced adducts, pyrimidine dimers, as well as mutations. The structural changes induced by DNA damage (DNA adducts, DNA breakage) and mutations are likely to have a significant effect on RAPD profiles. The RAPD technique can potentially detect all classes of mutations (substitutions, insertions, deletions) and, more importantly, mutations are not necessarily required in the sequence of the primer binding site to be detected (Bowditch *et al.*, 1993). The results presented in this chapter also suggest that only 10-20 % of the cells need to be damaged/mutated at specific genomic loci to possibly detect a visible effect in RAPD profiles.

Chapter 10

**THE RAPD ASSAY AS A TOOL TO DETECT
THE KINETICS OF DNA EFFECTS, DNA REPAIR
AND TRANSGENERATIONAL EFFECTS IN
B(A)P-EXPOSED *DAPHNIA MAGNA***

10.1 Introduction

Since the RAPD assay shows great potential for detecting DNA damage and mutations, it was challenging to develop a strategy which would differentiate between these two types of DNA effects. Mutations which occur in germ cells are transmitted to the next generation unlike damage to the DNA arising in germ and somatic cells. In this context, any changes arising in neonatal RAPD profiles generated from the exposed populations can be attributed to heritable mutations. In addition, a powerful approach would be to look at the kinetics of DNA effects in samples collected over a time course rather than at a single point time. In the same way, DNA repair could be qualitatively evaluated by exposing a species to a genotoxin and by allowing the species to recover. This chapter evaluates the potential of the RAPD assay to qualitatively assess the kinetics of DNA effects, DNA repair and transgenerational effects.

DNA repair is a crucial defence against genotoxins which may constitute an important factor affecting the carcinogenic potential of DNA damaging agents (Braithwaite *et al.*, 1999). Recently, PCR has been successively applied to evaluate the extent of DNA damage and repair in specific genes (Wei *et al.*, 1995; Bingham *et al.*, 1996; Brandt and Ali-Osman, 1997; Ploskonosova *et al.*, 1999; Braithwaite *et al.*, 1999). Although B(a)P adducts have been shown to be efficiently repaired (Feldman *et al.*, 1980; Yang *et al.*, 1980), it has been reported that the repair of B(a)P adducts in a specific gene, hypoxanthine phosphoribosyltransferase (*hprt*), occurred preferentially on the transcribed strand (Chen *et al.*, 1990) and that inefficient DNA repair played an important role in the formation of mutation hotspots (Wei *et al.*, 1995). However, most of the studies which have evaluated the DNA repair process have been performed on mammalian systems (e.g. Bodell and Banerjee, 1976; Mirsalis *et al.*, 1982; Burke *et al.*, 1990; Gao *et al.*, 1994; Hanawalt, 1998; Cui *et al.*, 1999). Much less is known about DNA repair in invertebrates. Zahn *et al.* (1983)

reported that DNA repair in sponges was efficient for one type of B(a)P-induced DNA damage but that another type of DNA damage induced by the same agent was persistent. Zahn *et al.* (1981) also indicated that DNA damage caused by PAHs and DNA repair in sponges seemed to differ from that of most eucaryotes. Harvey and Parry (1998) reported that the removal of 4-nitroquinoline 1-oxide -induced DNA adducts in the common mussel appeared to be biphasic in nature, with the rapid removal of a large proportion of adducts occurring within 48 h of the cessation of the exposure, followed by a slow rate of adduct removal for 56 d. In another study, James *et al* (1992) showed that B(a)P metabolites could bind to the hepatopancreatic DNA in lobsters, but the adducts were not persistent suggesting that the DNA repair process worked efficiently. Because persistent adducts can lead to the generation of mutation following replication of damaged bases (Livneh *et al.*, 1993), there is a growing need to develop sensitive methods for detecting mutations. Of particular importance are mutations which occur in germ cells as they will be transmitted to the next generations (Douglas *et al.*, 1995). It has been reported that the frequency of germline mutations significantly increased in human as a result of radioactive contamination in the Chernobyl region (Dubrova *et al.*, 1996). In another study, the AP-PCR was successively used for studying environmental germ-line mutagenesis (Shimada and Shima, 1998). Yauk (1998) reported that minisatellite DNA mutations may be sensitive biomarkers for induced heritable mutations in populations exposed to radioactive and non-radioactive contamination. In addition, powerful new PCR approaches have also been developed for the analysis of *de novo* mutations arising in pedigrees (Jeffreys *et al.*, 1990) and for the analysis of sperm samples (Jeffreys *et al.*, 1994).

In this chapter *D. magna* were exposed to different concentrations of B(a)P in order to evaluate the potential of the RAPD method (i) to detect DNA effects in samples collected over a time course during exposure and recovery and, (ii) to determine whether any observed DNA effects are transmitted to the next generations.

10.2 Materials and methods

10.2.1 Culture of *Daphnia magna*

D. magna (clone 5) was cultured in M7 medium at $20 \pm 2^{\circ}\text{C}$ as described in section 3.1.1.

10.2.2 Kinetic of exposure

10.2.2.1 Experimental design

Freshly born neonates (less than 48 h post hatch) were exposed in groups of 20 to 25 and $50 \mu\text{gL}^{-1}$ B(a)P; the animals were exposed for 7 d (experiment 1), and 6 d (experiment 2), respectively. It was decided to continue both experiments which were not carried out at the same time until *D. magna* were considerably affected. Test solutions were prepared from stock solutions of B(a)P at a concentration of 2 gL^{-1} in DMF, then stored at 4°C prior to use. The B(a)P solutions were added to the *D. magna* culture medium using a volume of 100 μl of DMF in 1 L of medium thereby ensuring a level below the 0.05 % maximum percentage of the solvent recommended by ASTM (1975). For each experiment, 2 control groups containing 20 animals were used. The controls consisted of a non-exposed group and a group exposed to 100 μl DMF alone in 1 L of medium. A total of 180 (9x20) animals were required for each experiment including control, solvent control, and B(a)P treated groups. Animals were fed during the experiments and the medium was changed three times a week as described in section 3.1.1. Surviving animals were recorded and samples were placed in 1.5 ml microcentrifuge tubes and stored at -80°C on a daily basis for 7 d (experiment 1) and 6 d (experiment 2). The whole population was sampled (i.e. maximum 20 animals) and when the number of survivors fell below 6 animals, surviving *D. magna* were used from other beakers to get a minimal number of 6-8 animals per tube. This explains why the starting number of *D. magna* in both experiments was higher than

120 (6x20, experiment 1) or 140 (7x20, experiment 2) for the B(a)P exposed groups.

10.2.2.2 Generation of RAPD profiles

At the end of both experiments, genomic DNA from *D. magna* was extracted and purified using a conventional phenol/chloroform method (see section 3.2.1.1). Extracted DNA was then electrophoresed on 1.2 % agarose gels at 100 V for 2 h and the DNA concentration of unknown samples was determined by comparison with known amounts of Lambda phage DNA (see section 3.3.1). RAPD profiles were generated using 10-mer primers OPA9, OPB1, OPB5, OPB6, OPB7, OPB8, OPB10 and OPB17 (see section 3.4.2) under the optimised conditions as described in section 3.4.1. PCR products were then electrophoresed on 1.2 % agarose gel at 90 V for 6 h, stained with ethidium bromide and visualised under UV light. A photograph was also taken and the image of the gel was saved in the computer for analysis when necessary (see section 3.5).

10.2.3 Recovery experiments and transgenerational effects

10.2.3.1 Experimental design

Freshly born neonates (less than 48 h post-hatch) were exposed in groups of 20 to 50 $\mu\text{g L}^{-1}$ B(a)P. Two control groups consisted of non-exposed *D. magna* and also animals exposed to 100 μl DMF in 1 L of M7 medium. In total 80 (4x20) and 160 (8x20) animals were used for both control groups and the B(a)P exposed *D. magna*, respectively (Figure 10.1). For the preparation of the test solution and volume used, please refer to section 10.2.2.1. Animals were fed during the experiments and the medium was changed three times a week as described in section 3.1.1. After 3 d exposure, control animals were pooled and 60 healthy (actively swimming) animals were placed in 3 beakers containing 1 L of M7 medium (recovery 1). This was done separately for each control group. B(a)P exposed animals contained in 5 beakers (BP1-BP5; Figure 10.1) were pooled and 60 healthy (actively swimming) *D. magna* were also placed in 3 beakers containing 1 L of M7

20 *Daphnia* per beaker*

C1 C2 C3 C4
(Control *Daphnia*)

S1 S2 S3 S4
(Solvent control *Daphnia*)

BP1 BP2 BP3 BP4
BP5

(*Daphnia* exposed to
50 µg L⁻¹ B(a)P)

BP6 BP7 BP8

3 day exposure

Recovery 1

pooled
Daphnia⁺

C1 C2
C3 C4

S1 S2
S3 S4

BP1 BP2 BP3
BP4 BP5

5 *Daphnia*
sampled⁺
(5 weak B(a)P
exposed *Daphnia*
were also sampled)

Recovery 2

6 day exposure

pooled *Daphnia*

5 swimming *Daphnia* + 5
weak *Daphnia* were sampled

Recovery (*Daphnia* in M7 medium)

1 2 3

(20 *Daphnia* per beaker)
Each day 5 *Daphnia*
were sampled for 12
days (1, 2, ..., 11, 12⁺)

4 5

(4 and 5: 20 and 15
Daphnia, respectively)
Each day 4 or 5
Daphnia were sampled
until day 5' and at day 8'
(1', 2', 3', 4', 5', 8')

Details of sampling (recovery 1 and 2)

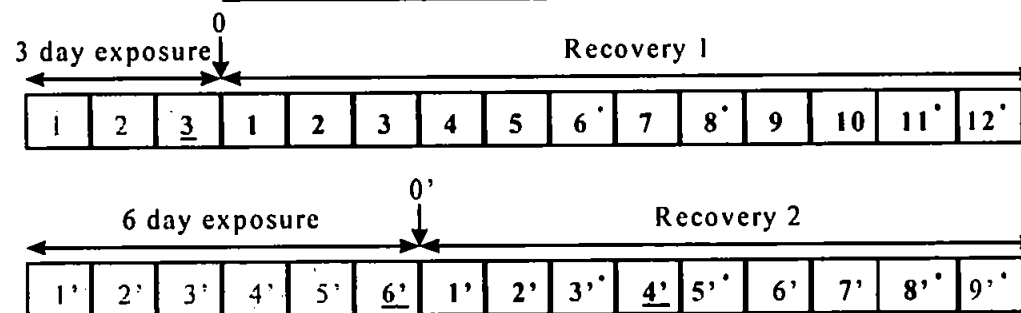


Figure 10.1 Summary of the protocol adopted in the recovery experiments of *Daphnia magna* exposed to 50 µgL⁻¹ B(a)P. The meaning of the symbols can be explained as follow: +: comment applied to each group (control, solvent control, and B(a)P exposed *D. magna*), bold number: RAPD performed on 5 active animals (except for recovery experiment 2 for which a mixture of active and weak animals was used), underlined bold number: RAPD performed on 5 active and weak animals separately, \square : *Daphnia* exposed to 50 µgL⁻¹ B(a)P, \square : RAPD performed on the offspring, and *: number of neonates determined. To avoid any confusion each day in the second recovery experiment was labelled « day' ». O and O' refer to the end of the exposure for recovery 1 and 2, respectively.

medium (Figure 10.1). At this stage, five active *D. magna* were sampled from each group and were placed in separate 1.5 ml microcentrifuge tubes and stored at -80°C. Five 'weak' animals (unable to swim properly) were also sampled from the B(a)P treated group at this time. The remaining animals from the pooled samples were discarded. After a recovery period of 1 d, five healthy animals were sampled for each group and this operation was repeated on a daily basis for 12 d (Figure 10.1). In both recovery experiments, healthy and non healthy animals were sampled to compare the profiles generated by these animals.

In the second recovery experiment (recovery 2), the B(a)P exposed animals present in the 3 remaining beakers (BP6-BP8; Figure 10.1) were exposed for another 3 d (i.e. 6 d in total). To avoid any confusion with recovery experiment 1, the days were labelled « day' ». The 35 survivors which did not look healthy (at least for most of them) were split into 2 groups consisting of 20 and 15 animals and were placed in 2 beakers containing 1 L of M7 medium. At this stage, 5 weak and 5 active (just able to swim) animals were placed into separate 1.5 ml microcentrifuge tubes and stored at -80°C (Figure 10.1). After a recovery period of 1' d, five or four animals which consisted of a mixture of active and weak *D. magna* were sampled from the previously exposed population and this operation was repeated on a daily basis (except for the 6th, 7th, and 9th d) for 9 d (Figure 10.1).

For both recovery experiments, the number of surviving and healthy animals were recorded. The number of ovigerous females was also determined just before *D. magna* released the first batch of neonates and the number of offspring were counted on d 6, 8, 11, and 12 (recovery 1) as well as d 3', 5', 8', and 9' (recovery 2) (Figure 10.1). New born neonates were stored in 1.5 ml microcentrifuge tubes and placed at -80°C. The controls which have been used in recovery 2 originated from recovery 1. The non-exposed control were maintained in M7 medium throughout. However, with regard to the solvent control, the animals were only exposed to DMF for 3 d and not for 6 d. However, this should not affect the outcome as 0.01 % DMF does not induce DNA effects in *D. magna* (chapter 5).

10.2.3.2 Generation of RAPD reactions

Genomic DNA from parental and neonatal *D. magna* was extracted and purified using a conventional phenol/chloroform method (see section 3.2.1.1). The extracted DNA was quantified as described in section 10.2.2.2. RAPD profiles, electrophoresis, and analysis of the results were performed as described in section 10.2.2.2.

10.3 Results

10.3.1 Kinetics of exposure

Figure 10.2 displays the number of surviving animals in both experiments (i.e. *D. magna* exposed to 25 and 50 μgL^{-1} B(a)P for 7 and 6 d, respectively). As expected, the results revealed that the mortality in controls from both experiments was limited and that the control animals were healthy until the end of the experiments. The highest proportion of dead animals amongst controls was 10 % (Figure 10.2A). In contrast the number of surviving animals was much more reduced due to the B(a)P treatments. *D. magna* exposed to 25 μgL^{-1} B(a)P were weak from the fifth day of exposure and only 10 % of the animals survived at the end of the experiment (Figure 10.2A). When the water flea was exposed to 50 μgL^{-1} B(a)P, the animals were weakened from d 2 until the end of the experiment. The number of surviving *D. magna* decreased gradually to a low level; only 20 % of this group survived at d 6 (Figure 10.2B).

The RAPD profiles generated by the population of *D. magna* exposed to 25 and 50 μgL^{-1} B(a)P, including controls, are presented in Figure 10.3 and 10.4, respectively, and in appendix III. The profiles obtained from the population exposed to 25 μgL^{-1} B(a)P revealed that the intensity of some bands (e.g. 9-1, 9-2, 5-2, 5-3, 7-1, 7-2, 7-3) decreased mainly for the last 2 or 3 d of the experiment compared to control profiles (Figure 10.3). On the other hand, band 9-3 (Figure 10.3A) was more intense from d 2 until d 7. Finally, primer OPB10

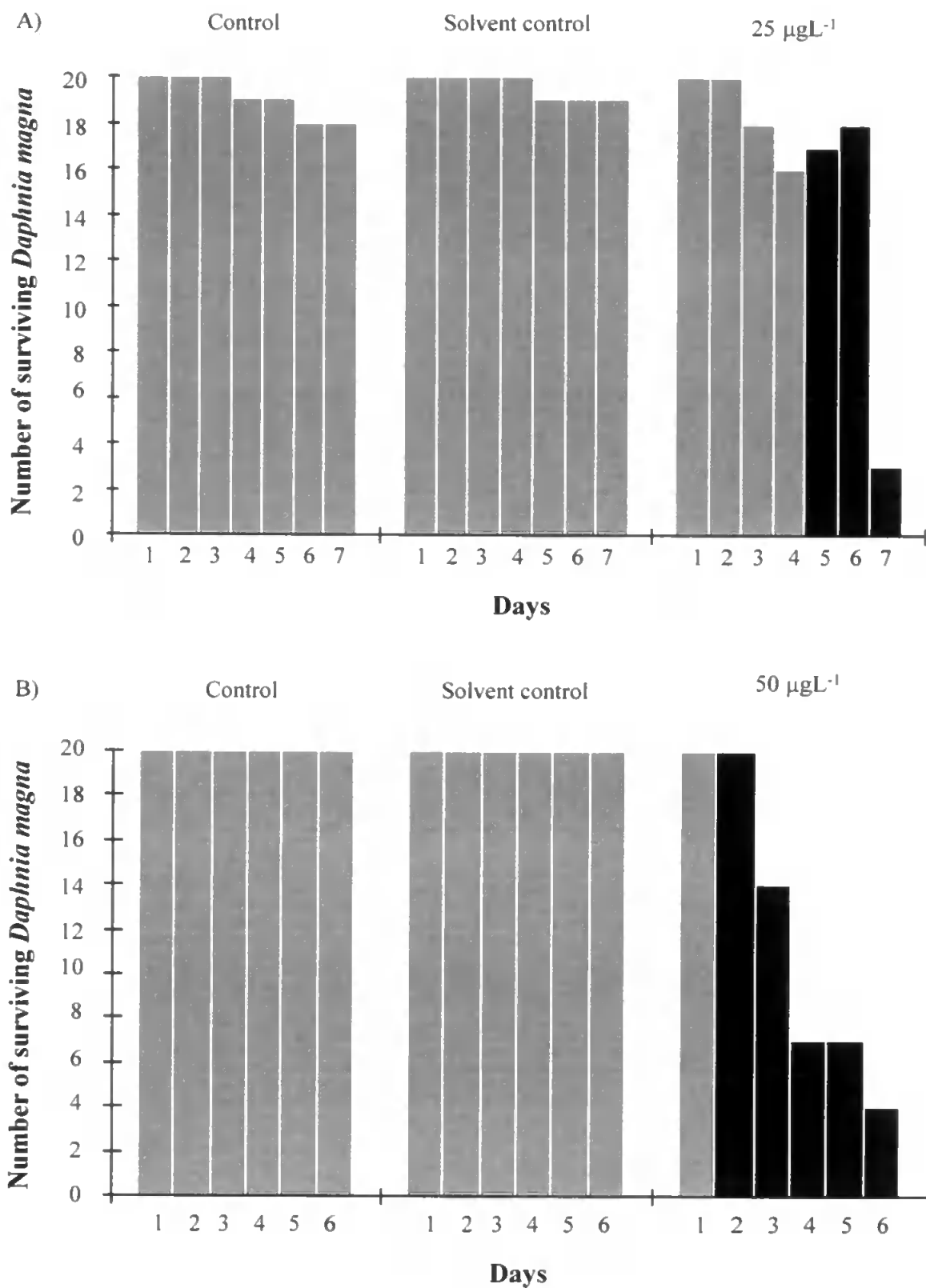


Figure 10.2 Daily number of surviving *Daphnia magna* in controls and populations exposed to 25 (A) and 50 (B) μgL^{-1} B(a)P. ■ and ■ indicate that the populations were healthy (actively swimming) and weak (unable to swim), respectively. Originally, each beaker contained 20 *D. magna*.

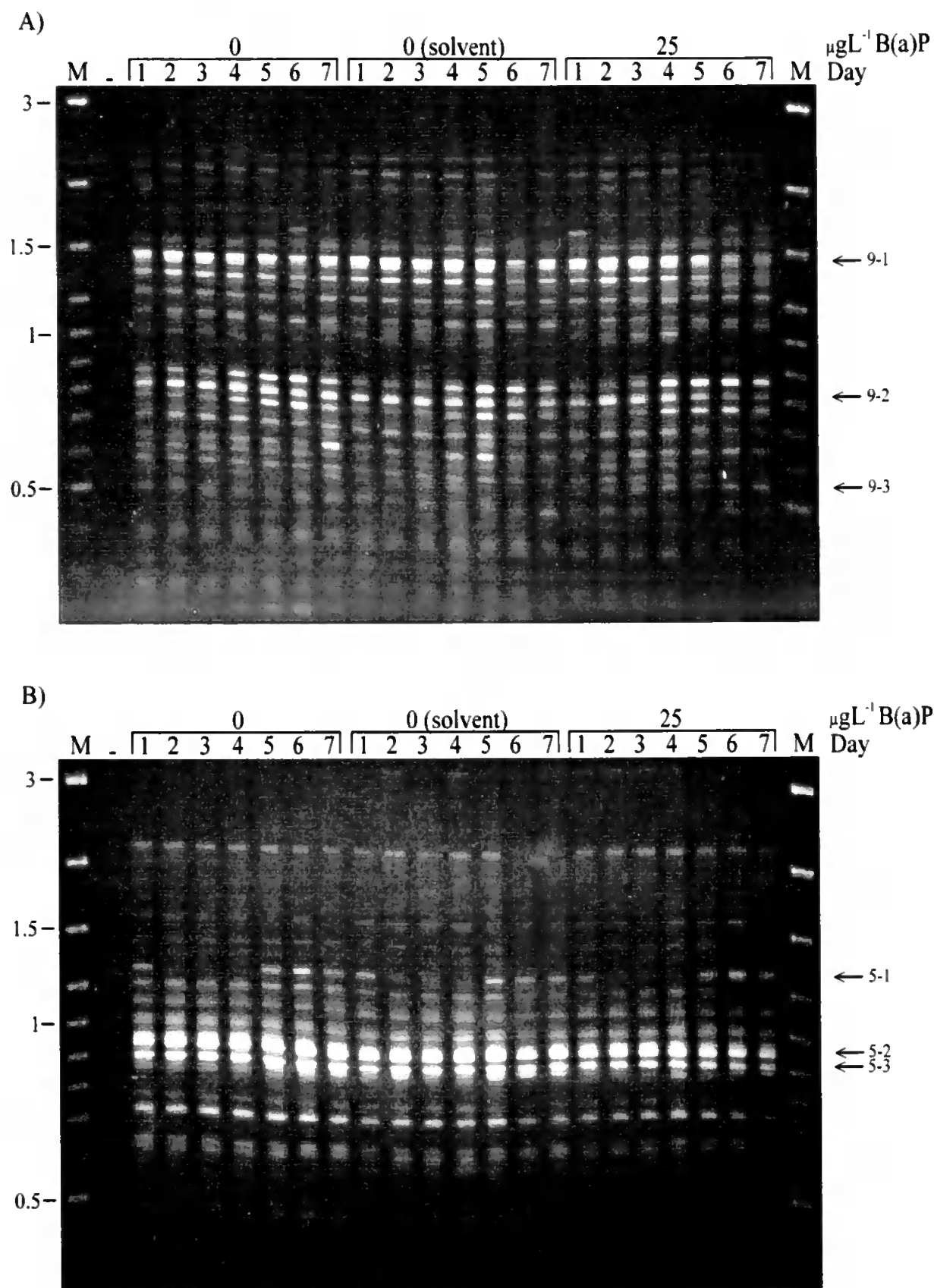
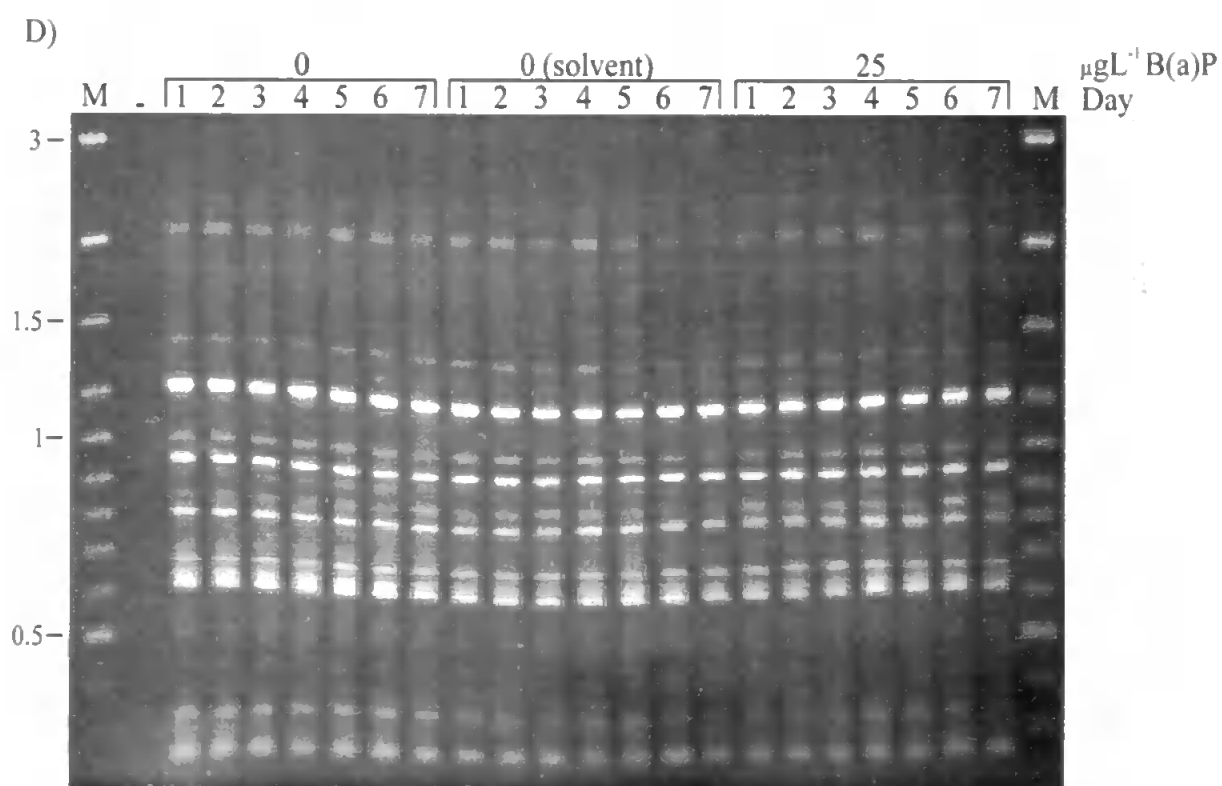
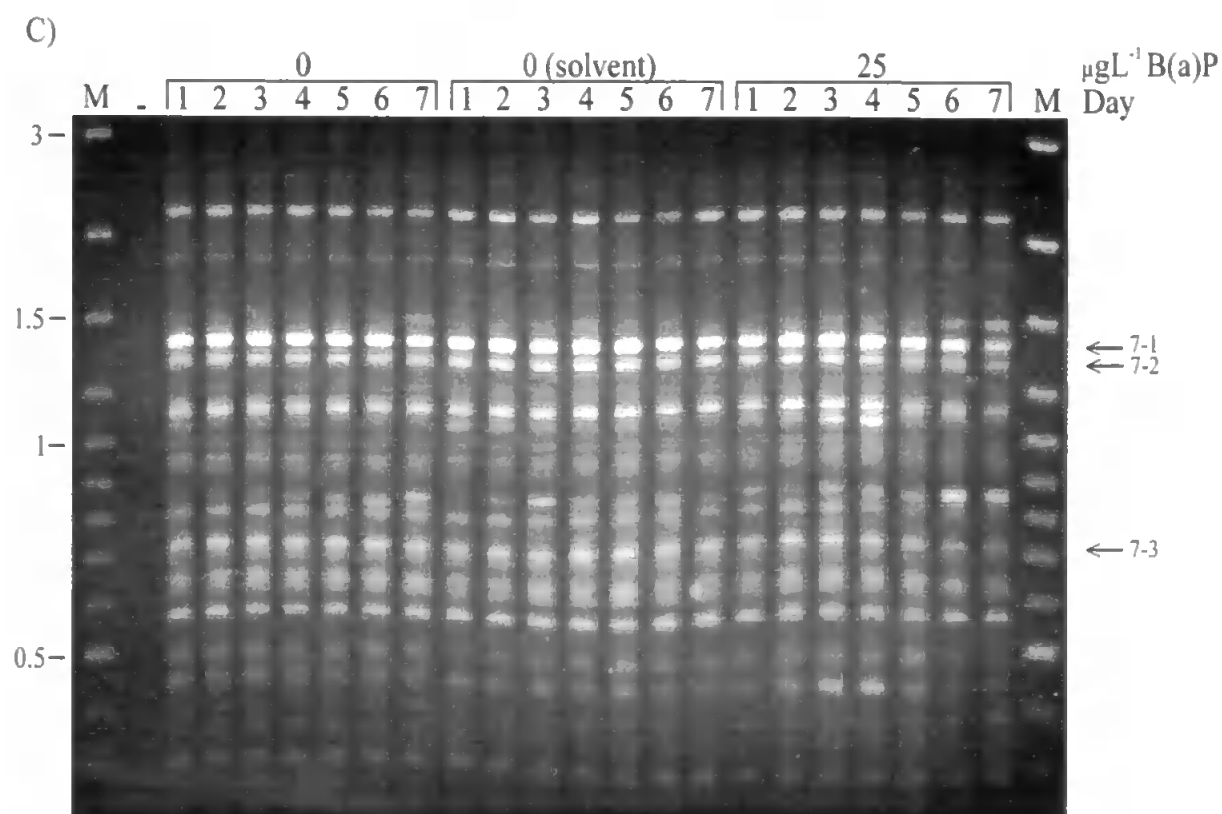


Figure 10.3 Kinetics of RAPD profiles of *Daphnia magna* exposed to $25 \mu\text{g L}^{-1}$ B(a)P.

The patterns were obtained using 10-mer primers OPA9 (A), OPB5 (B), OPB7 (C), and OPB10 (D). M = GeneRuler™ DNA ladder plus (Igi). The molecular sizes (Kb) of



selected bands are shown on the left of each gel. - = no DNA control. Selected changes are indicated by arrows in comparison to control patterns.

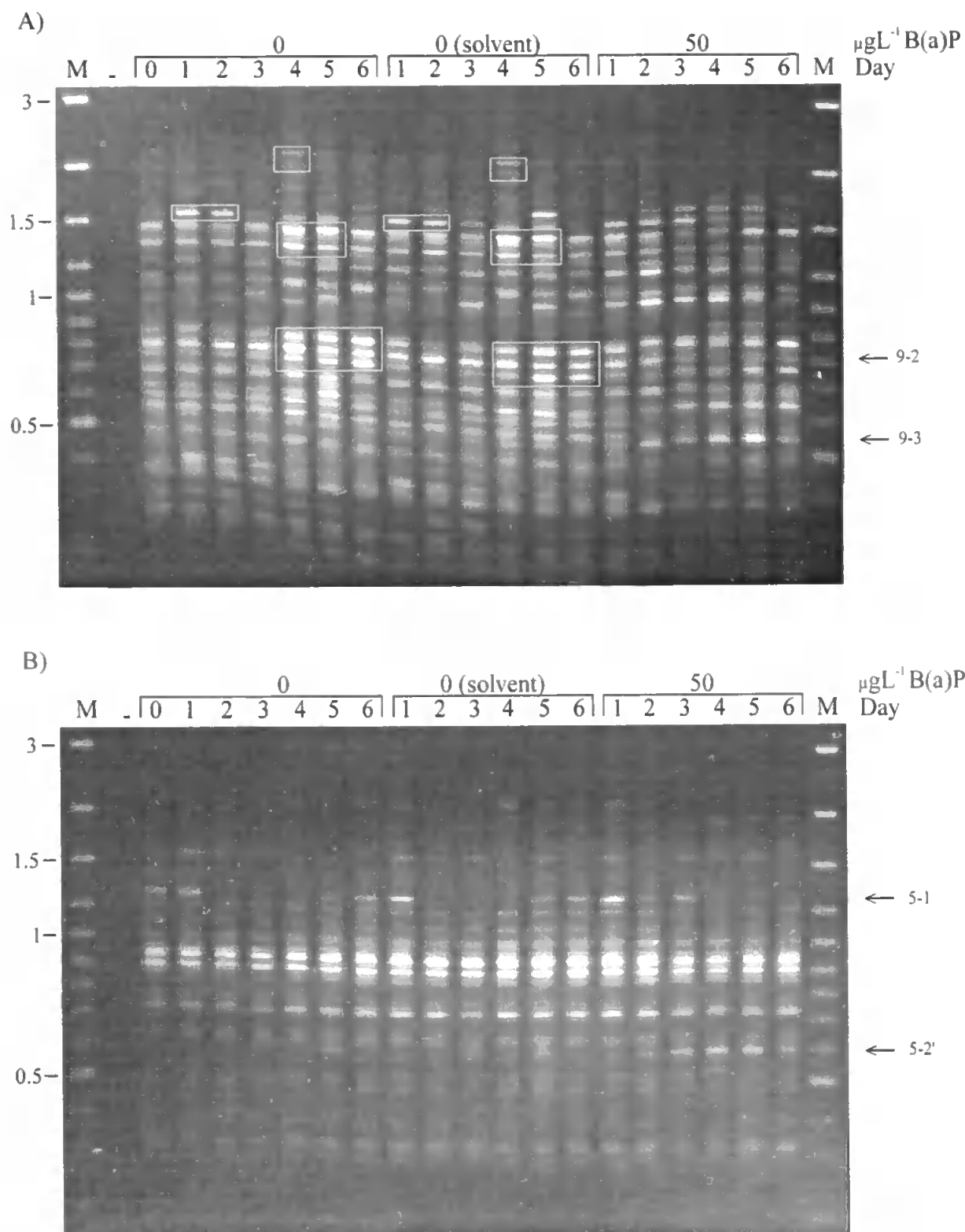
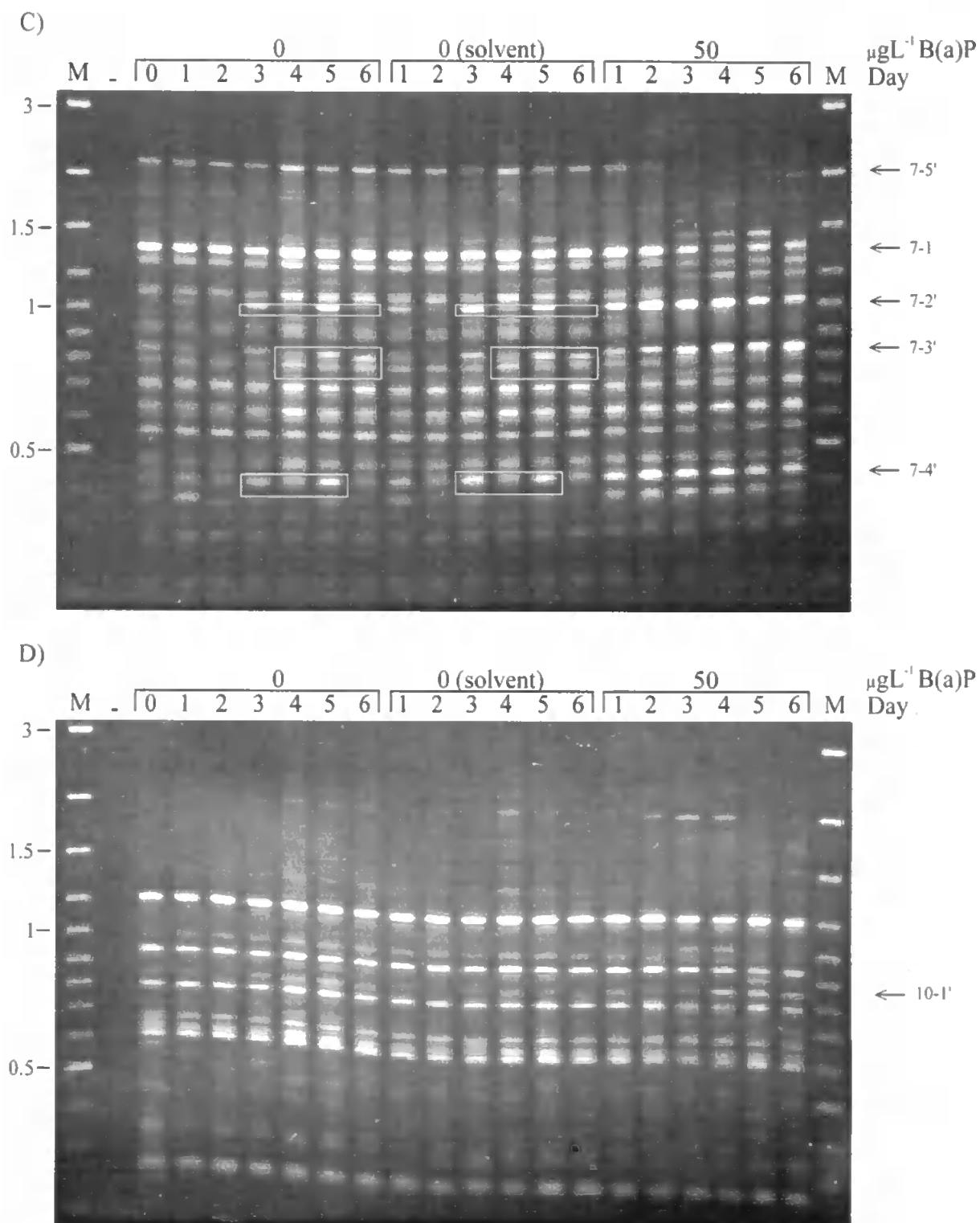


Figure 10.4 Kinetics of RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{g L}^{-1}$ B(a)P.

The patterns were obtained using 10-mer primers OPA9 (A), OPB5 (B), OPB7 (C), and OPB10 (D). M = GeneRuler™ DNA ladder plus (Igi). The molecular sizes (Kb) of selected bands are shown on the left of each gel. - = no DNA control. Selected changes



are indicated by arrows in comparison to control patterns. Bands which are different from those selected in Figure 10.3 are indicated by '. The frames indicate that the same variations occur in profiles generated by both controls.

revealed no obvious differences among profiles (Figure 10.3D). The patterns which were obtained from the population exposed to 50 μgL^{-1} B(a)P divulged that the intensity of some bands decreased (e.g. 9-2, 7-1) while others increased (e.g. 9-3, 7-2', 7-3', 7-4', 10-1'). Furthermore, band 5-2' appeared from d 2 until d 6 whereas band 7-5' clearly disappeared after 2 d of exposure and onwards (Figure 10.4C). However, the major effect of exposure to 50 μgL^{-1} B(a)P generally increased band intensity whereas at 25 μgL^{-1} B(a)P the main change in profiles was a decrease in band intensity in comparison to control patterns. The presence of some bands (e.g. 5-1, Figure 10.3B and 10.4B) was not constant even in control patterns which did not facilitate the analysis. However, it was also clear that band 5-1 followed a specific pattern of variation in both non-exposed and solvent control groups which was perturbed when the *D. magna* were exposed to 50 but not to 25 μgL^{-1} B(a)P (Table 10.1). This phenomenon was also observed for a number of other bands (Figure 10.4A and 10.4C; changes indicated by a frame) and the extent of variation seemed to depend on the choice of primer. For some primers the variation in RAPD profile observed in both controls was identical (band 5-1 Figure 10.3B versus Figure 10.4B) whereas for other primers no variation occurred between both profile controls (Figure 10.3D and 10.4D). In addition, changes in RAPD profiles generated by the control population of *D. magna* (Figure 10.3) did not reveal as many changes (except band 5-1) when compared to control patterns presented in Figure 10.4.

Table 10.1 Presence (+) and absence (-) of band 5-1 in control, solvent control and B(a)P exposed *Daphnia magna* in both kinetic experiments. For more details please refer to Figure 10.3 and 10.4. nd stands for not determined.

	Control							Solvent control							B(a)P exposed population						
Days of exposure	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Band 5-1 Figure 10.3	+	-	-	-	+	+	+	+	-	-	-	+	+	+	+	-	-	-	+	+	+
Band 5-1 Figure 10.4	+	-	-	-	+	+	nd	+	-	-	-	+	+	nd	+	+	+	-	-	-	nd

10.3.2 Recovery experiments and transgenerational effects

10.3.2.1 Effects on population in recovery 1

In the recovery 1, after 3 d exposure to B(a)P, 77 % of the B(a)P exposed animals and almost 100 % of the control *D. magna* were swimming actively. The percentage of surviving animals was higher than 95 % in each group. The proportion of surviving and active animals was high (> 95 %) in each group until the end of the experiment (Figure 10.5). The proportion of ovigerous females after 2 d recovery was 22 % in the exposed animals, 74 % in non-exposed controls and 70 % in solvent controls (Figure 10.5). However after 4 d exposure the proportion of ovigerous females in the B(a)P exposed population had almost reached the same level as the controls (i.e. 82 % versus 98 % for non-exposed control and 92 % for solvent control). The number of offspring produced from each group in both recovery experiments is presented in Figure 10.6. The results showed that there was no delay for the release of the neonates. However less offspring were produced due to the B(a)P treatment. The total number of neonates produced was 76 % in comparison to controls (i.e. if control = 100 %). The number of neonates generated from control *D. magna* followed a similar pattern with the maximum number of offspring produced at d 6. In contrast, the highest value in the B(a)P exposed population was obtained the 8th d of recovery.

10.3.2.2 Effects on population in recovery 2

In the recovery 2, after 6 d exposure to B(a)P, 85 % of the exposed population had survived but none of these animals were able to swim properly (Figure 10.5). Indeed, only 65 % of the exposed *D. magna* could barely swim. More than 85 % of the previously exposed population survived during the recovery experiment. Some of the weak *D. magna* recovered and were able to swim properly; for instance 38 % of the *D. magna* were active after 3' and 5' d of recovery. The proportion of ovigerous female was 54 % at day 0' and 1'. The number of offspring produced from each group is presented in Figure 10.6. The

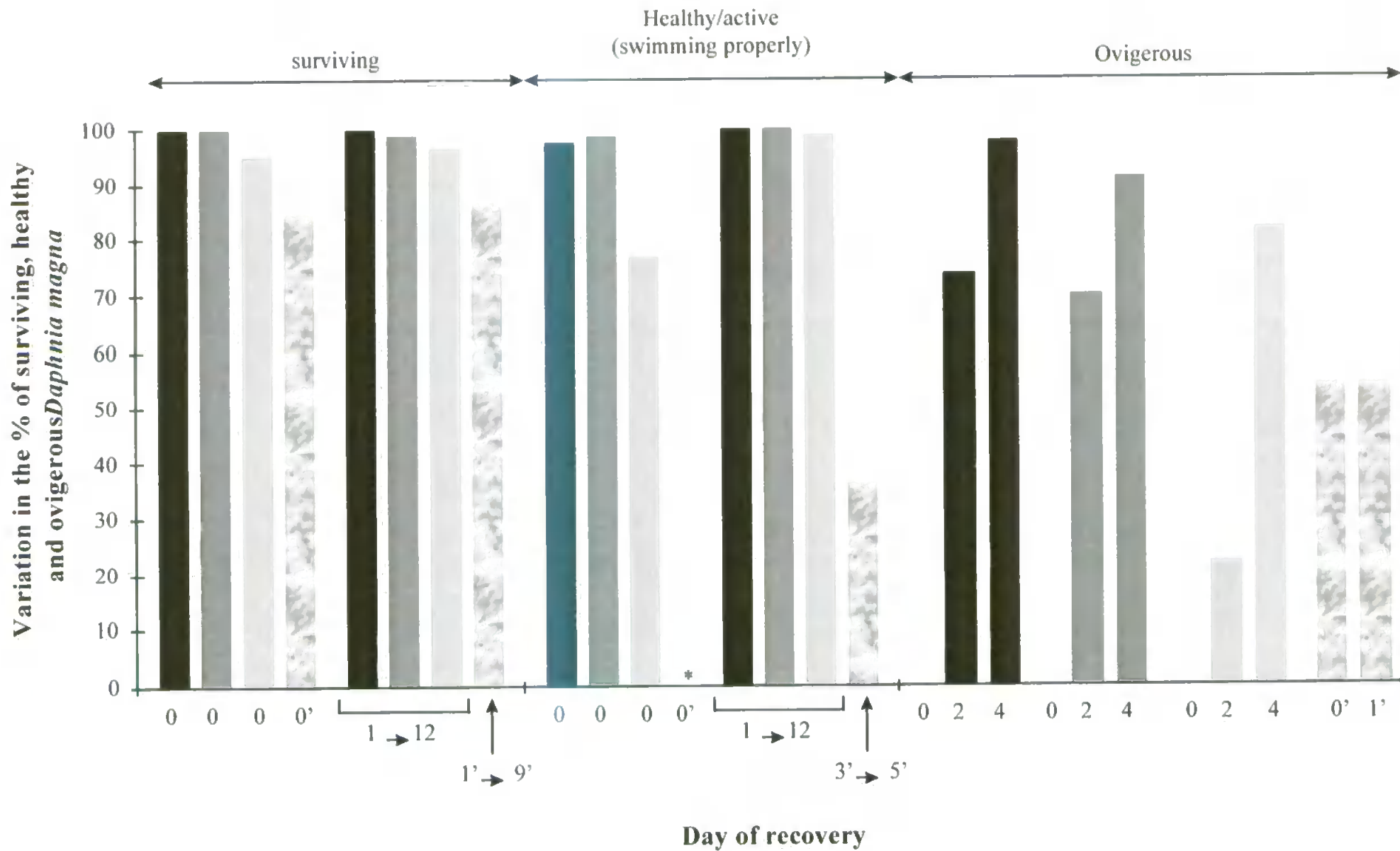


Figure 10.5 Variation in the number of surviving, healthy and ovigerous *Daphnia magna* in control (■), solvent control (■), and B(a)P exposed *Daphnia magna* (■: recovery 1; ■: recovery 2). For more detail please refer to Figure 10.1. *: 65 % of the animals were just able to swim. ' refers to recovery experiment 2. The proportion of ovigerous females was determined until the first generation of neonates was released.

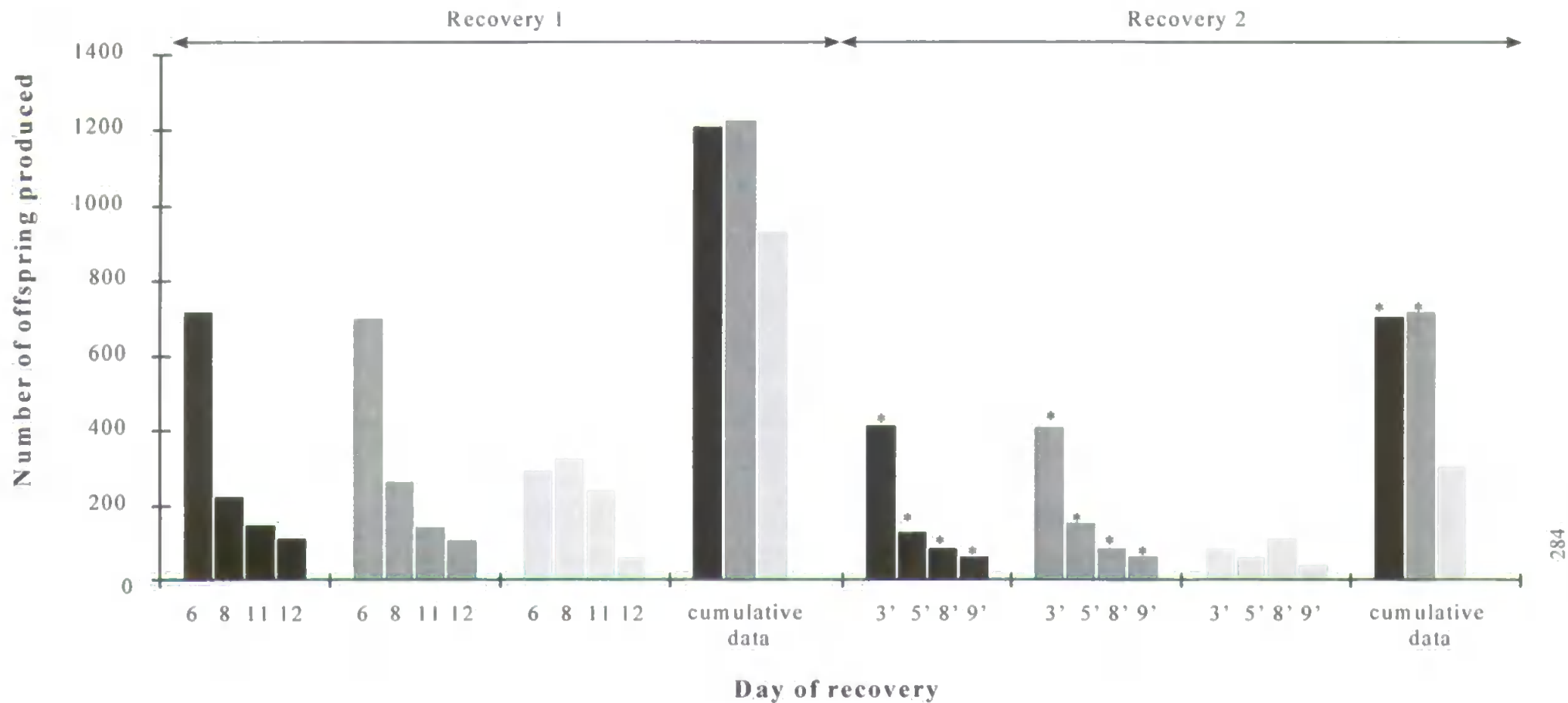


Figure 10.6 Variation in the number of offspring generated from control (■), solvent control (■), and B(a)P exposed *Daphnia magna* (■) during both recovery experiments. * indicate that the data were calculated from recovery 1 (i.e. number of offspring generated from both controls x 35/60). For more detail please refer to Figure 10.1.

number of neonates produced by the non-exposed and solvent controls was calculated using the data obtained in the first recovery experiment (please see Figure 10.6 for more details). The results showed that there was no delay for the release of the neonates as for recovery experiment 1. The total number of neonates produced was 43 % in comparison to controls (i.e. if control = 100 %). The number of neonates generated from control *D. magna* followed a similar pattern with the maximum number of offspring produced at d 3'. In contrast, the highest value in the B(a)P exposed population was obtained at d 8'.

10.3.2.3 RAPD analysis for recovery experiment 1

Figures 10.7 and 10.8 show the RAPD profiles obtained following exposure of the *D. magna* to 50 μgL^{-1} B(a)P for 3 d and recovery for 12 d using 10-mer primer OPB7, OPB8, OPB10, and OPB11. The examination of the RAPD profiles of exposed animals revealed that there was a difference between the active and weak animals which had been sampled after 3 d exposure to B(a)P. Indeed, the intensity of some bands obtained from the weak *D. magna* were more intense [e.g. 7-1, 7-2, 7-3, 8-1, 8-2, 8-3, 8-4 (Figure 10.7), 10-1, 10-2, 11-1 (Figure 10.8)] than those generated by the exposed healthy animals. At this stage it is important to note that only active animals were used for the recovery phase of the experiment. At the beginning of the recovery experiment the profiles which were obtained from the active animals that had been exposed to B(a)P for 3 days showed some differences in comparison to control patterns but the changes were not as pronounced as those which were produced by the weak *D. magna*. For instance, bands 7-2, 8-3, and 8-4 were more intense in comparison to the intensity of control amplicons (Figure 10.7).

Surprisingly, new changes occurred in the patterns produced by the exposed population during the recovery experiment; the intensity of band 7-4 increased compared to controls in the first 4 d of recovery (Figure 10.7A). After the 5th d of recovery the intensity of band 5-4 was not as intense but the band was brighter compared to controls until the 9th

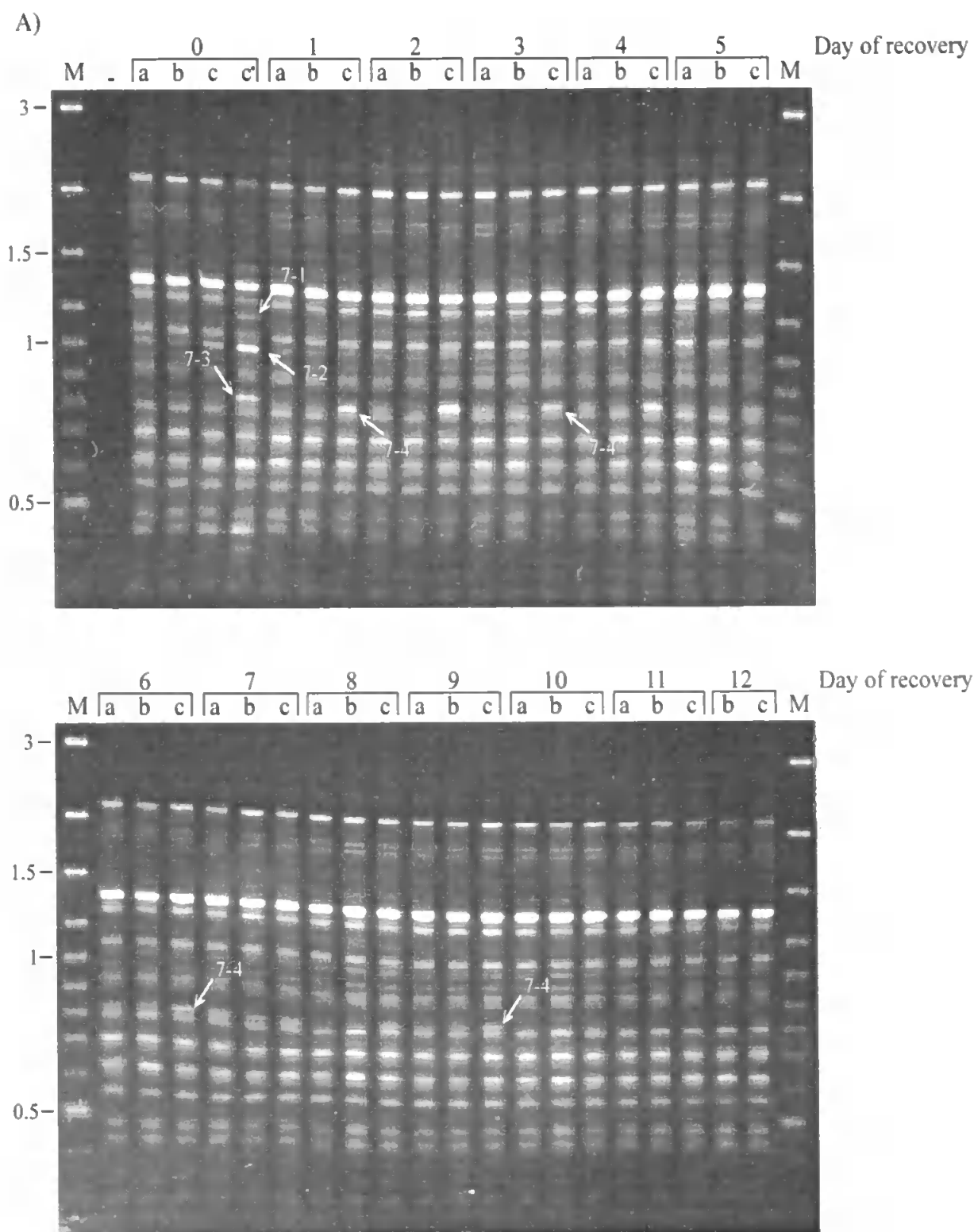
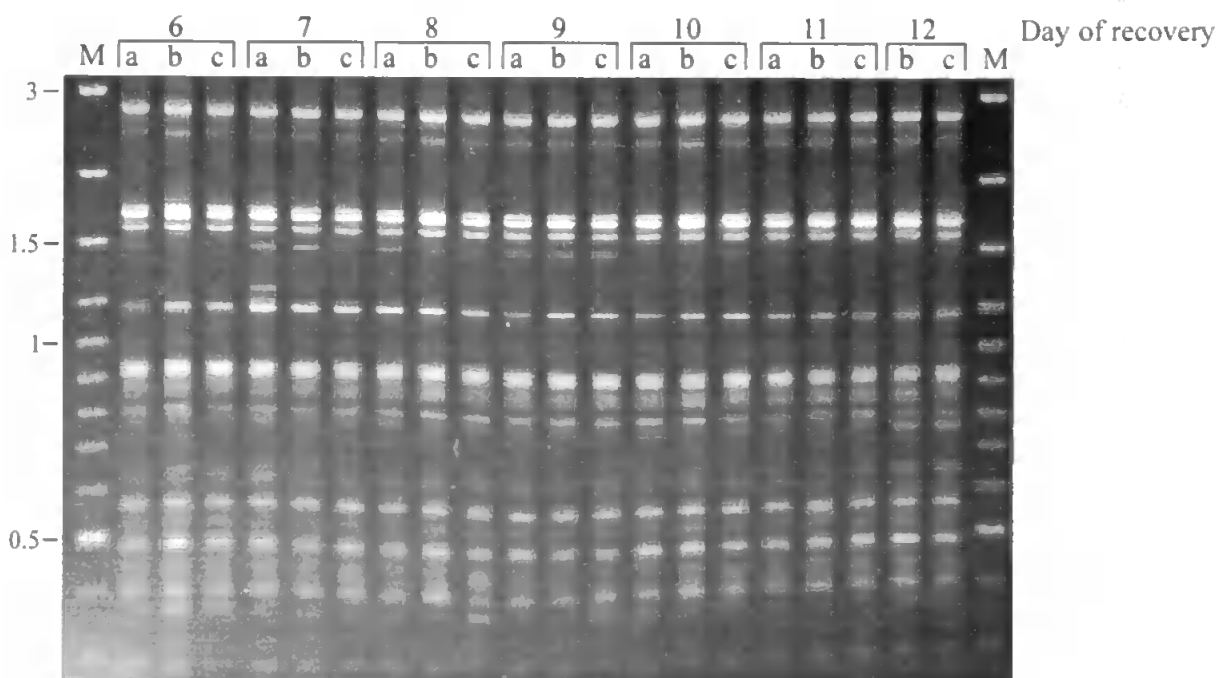
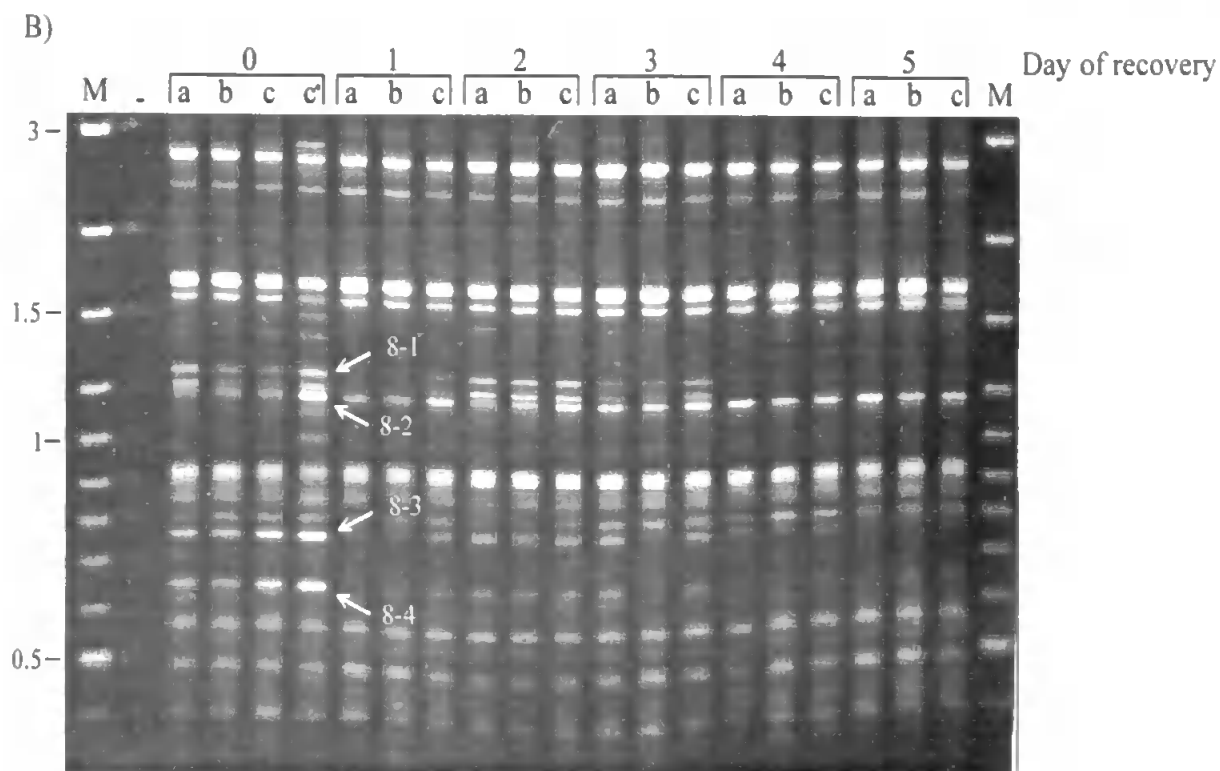


Figure 10.7 RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P for 3 d and recovered for 12 d. The patterns were obtained using 10-mer primers OPB7 (A) and OPB8 (B). a: control, b: solvent control, and c: *D. magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P. a, b, and c: active animals; c*: weak *D. magna*. For more details please refer to Figure 10.1.



M = GeneRuler™ DNA ladder plus (Igi). The molecular sizes (Kb) of selected bands are shown on the left of each gel. - = no DNA control. Selected changes are indicated by arrows in comparison to control patterns.

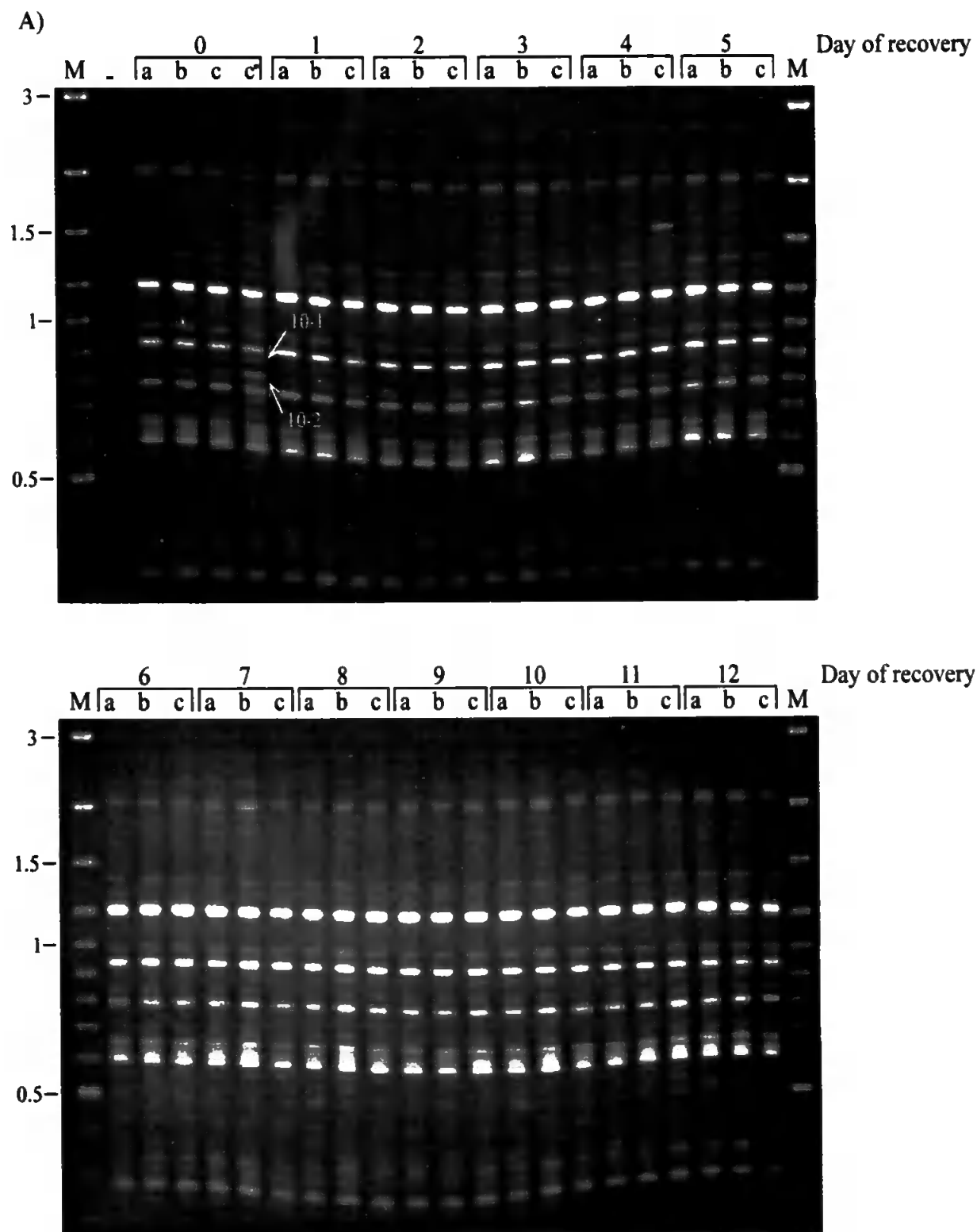
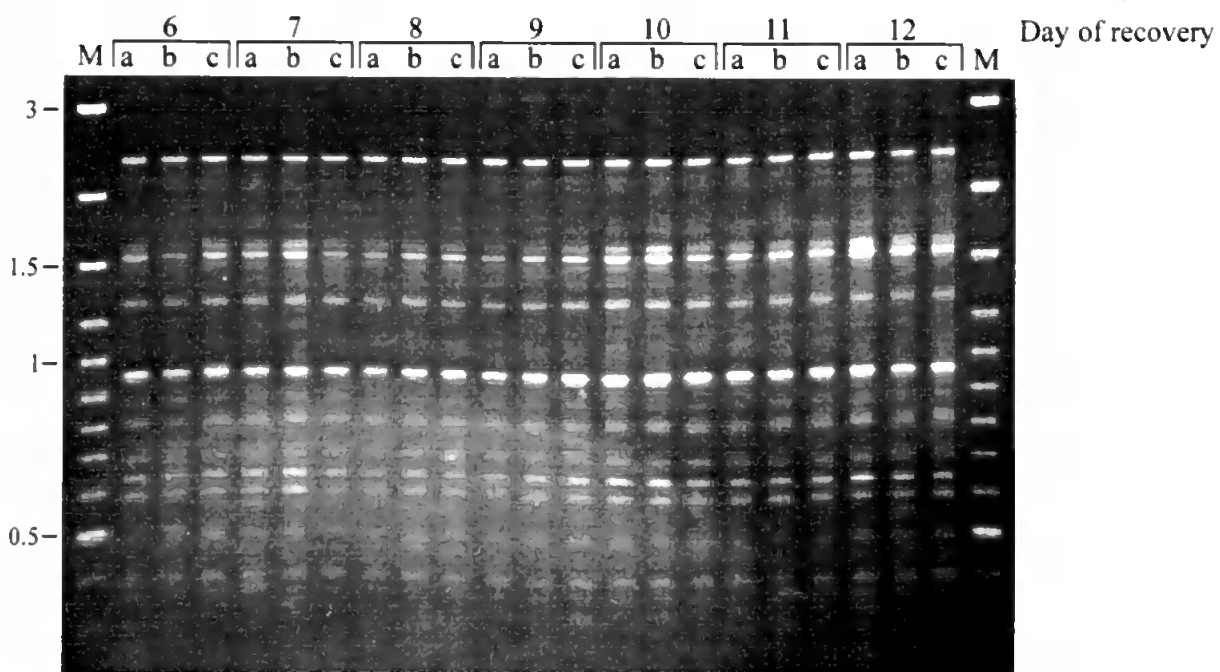
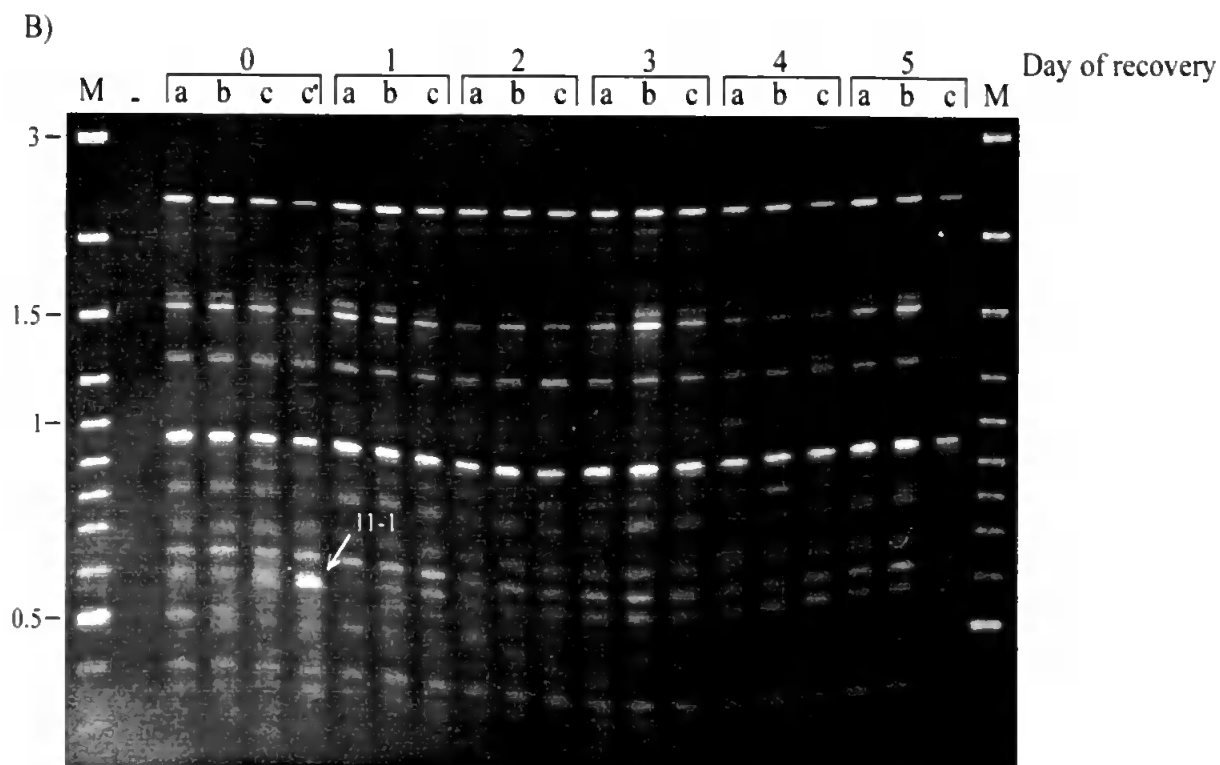


Figure 10.8 RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P for 3 d and recovered for 12 d. The patterns were obtained using 10-mer primers OPB10 (A), and OPB11 (B). a: control, b: solvent control, and c: *D. magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P. a, b, and c: active animals; c*: weak *D. magna*. For more details please refer to Figure 10.1.



M = GeneRuler™ DNA ladder plus (Igi). The molecular sizes (Kb) of selected bands are shown on the left of each gel. - = no DNA control. Selected changes are indicated by arrows in comparison to control patterns.

d of recovery. However, this band disappeared in each group from the 10th d until the end of the experiment. Finally, the results showed that after 10 d of recovery the RAPD profiles obtained from each group were very similar (Figure 10.7A). With regard to primers OPB8, OPB10, and OPB11 it seemed that, during the recovery experiment, no obvious change occurred in profiles generated by the exposed population in comparison to control patterns (Figure 10.7B and 10.8).

Figure 10.9 shows the RAPD profiles of offspring *D. magna* generated by the parental population which had been exposed to 0, 0 (solvent) and 50 µgL⁻¹ B(a)P for 3 d. The offspring were gathered at day 6, 8, and 12 following the exposure of parental animals (Figure 10.1). We considered that day 6, 8, and 12 corresponded to the first, second and third generation, respectively. No obvious changes in the RAPD profiles were observed among the different groups when primers OPB10, and OPB11 were used in the analysis (Figure 10.9). However, when primer OPB7 was used band 7-4 was brighter in the offspring generated by the B(a)P exposed parents than in control patterns after 6 and 8 d of recovery. In addition, the profiles produced by the neonates generated by the parents recovered for 12 d were identical among each group. With regard to primer OPB8, neonatal bands 8-5 and 8-6 were brighter than control amplicons after the parents had recovered for 6 and 12 d but not after 8 d of recovery (Figure 10.9A). Surprisingly, the RAPD profiles obtained from the parental *D. magna* did not display any changes for these 2 bands compared to control patterns (Figure 10.7B).

10.3.2.4 RAPD analysis for recovery experiment 2

Figure 10.10 shows the RAPD profiles obtained from control *D. magna* and animals exposed to 50 µgL⁻¹ B(a)P for 6' d and which had recovered for 9' d using 10-mer primer OPB6, OPB7, OPB8, and OPB10. The results revealed a few differences between the 'mixed' and weak exposed animals after 6' d exposure. 'Mixed' animals refers to a mixture of active and moribund animals. For instance, after 6'd exposure, bands 8-1, 8-2

Figure 10.9 RAPD profiles of offspring *Daphnia magna* generated by parental animals which had been exposed to 50 μgL^{-1} B(a)P for 3 d. The offspring were sampled during the recovery experiment 1 at day 6, 8, and 12. a, b, and c: offspring *D. magna* generated by parents exposed to 0, 0 (solvent) and 50 μgL^{-1} B(a)P, respectively. Primers as indicated at the top of each gel. For more details please refer to Figure 10.1. M = GeneRuler™ DNA ladder plus (Igi). The molecular sizes (Kb) of selected bands are shown on the left of each gel. Selected changes are indicated by arrows in comparison to control patterns.

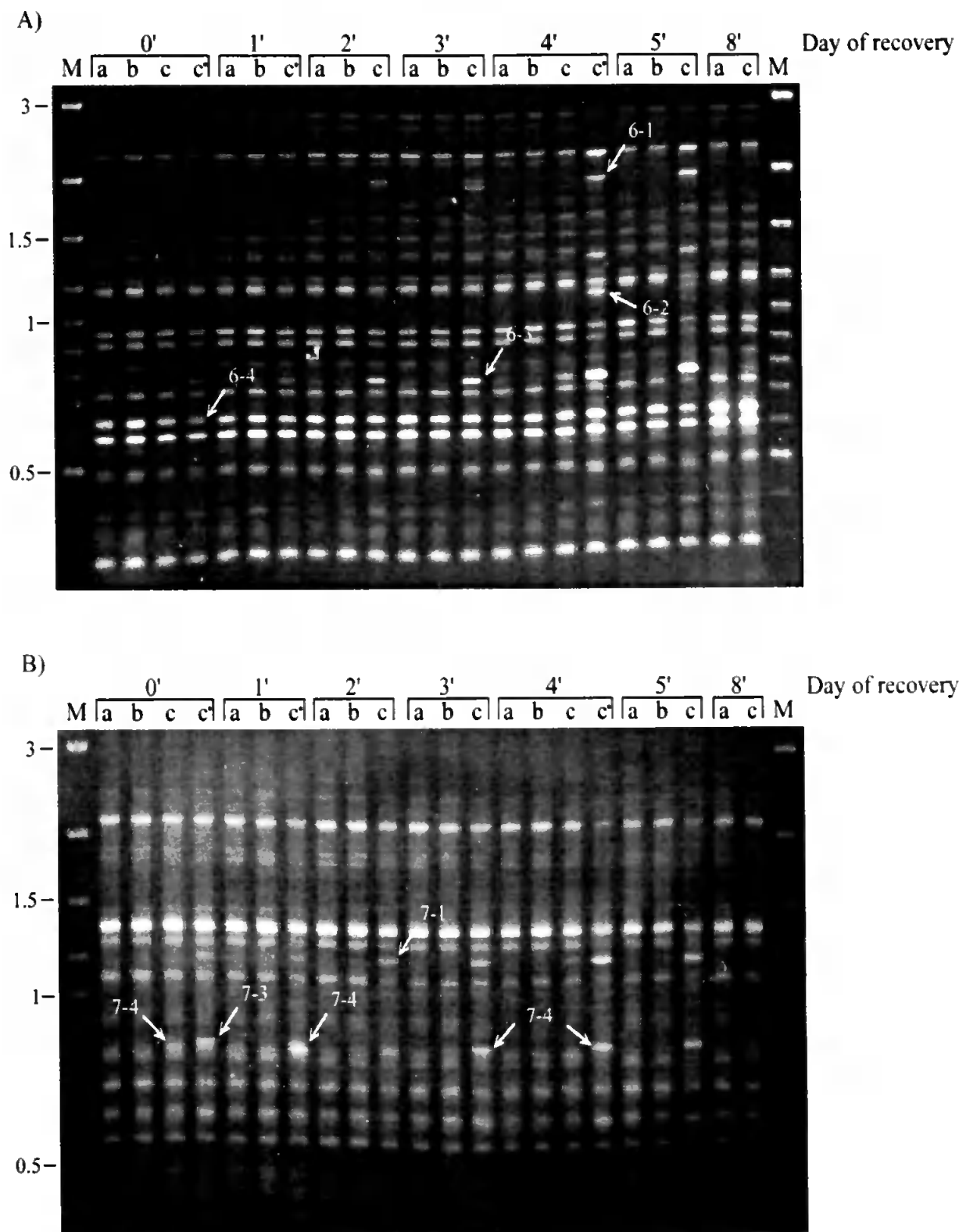
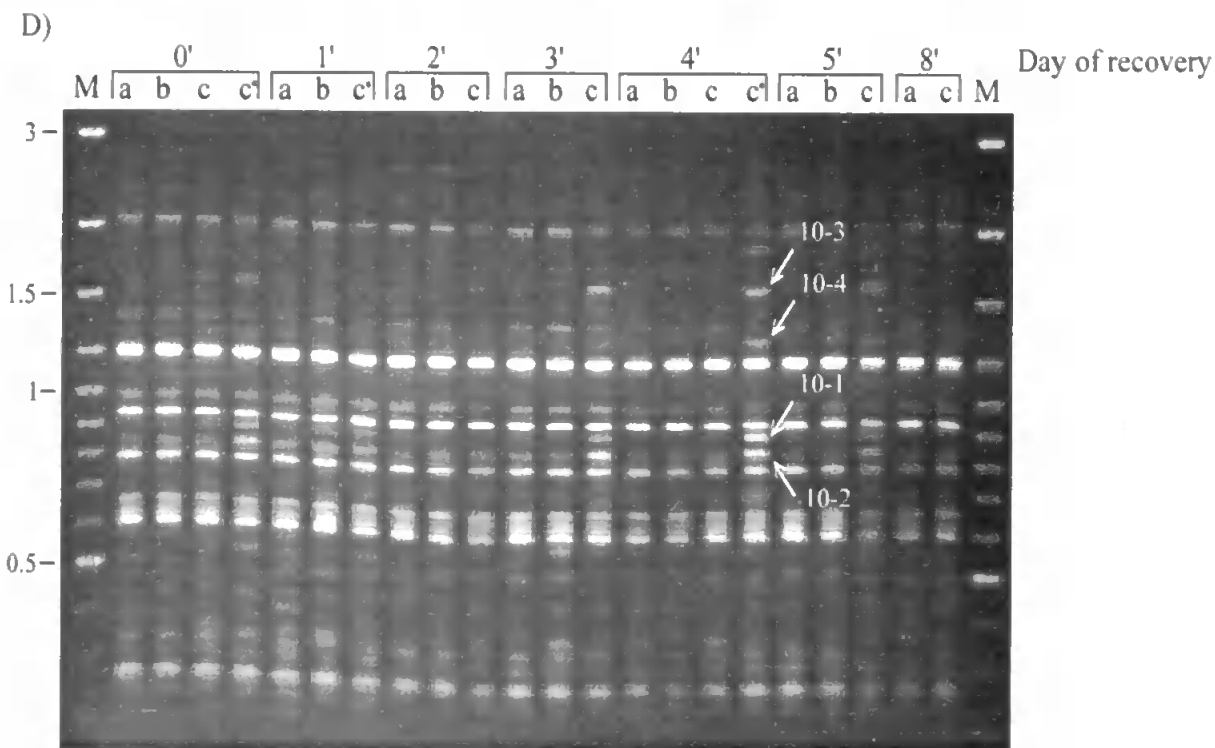
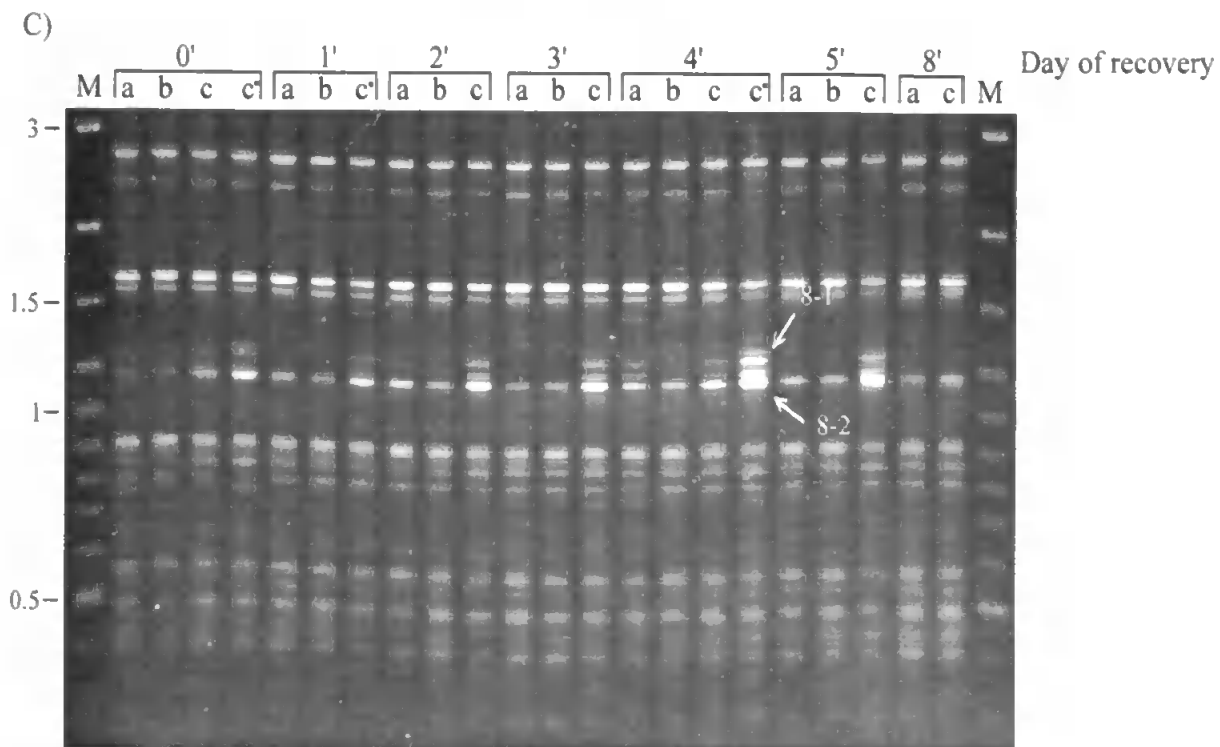


Figure 10.10 RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P for 6' d and recovered for 9' d. The patterns were obtained using 10-mer primers OPB6 (A), OPB7 (B), OPB8 (C), and OPB10 (D). a: control, b: solvent control, and c: *D. magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P. a and b: active animals, c: mixture of active and weak *D.*



magna, c*: weak animals. For more details please refer to Figure 10.1. M = GeneRuler™ DNA ladder plus (Igi). The molecular sizes (Kb) of selected bands are shown on the left of each gel. Selected changes are indicated by arrows in comparison to control patterns.

(Figure 10.10 C), 10-1 and 10-2 (Figure 10.10D) generated by the weak swimmers were more intense than those produced by the 'mixed' swimmers. After 6' d exposure the profiles obtained from the 'mixed' *D. magna* also displayed some changes compared to control patterns. The intensity of band 6-4 decreased in comparison to control profiles (Figure 10.10 A). On the other hand, some bands such as 7-4 (Figure 10.10B) and 8-2 (Figure 10.10C) were brighter than those generated by the control patterns. However the number of changes in RAPD profiles produced by the 'mixed' animals was relatively low compared to control patterns. Nevertheless, the results generated by the 4 primers revealed that the number of changes in patterns generated from the B(a)P exposed population increased after recovery compared to control profiles (Figure 10.10). The main change was an obvious increase in band intensity (e.g. band 6-3, 7-4, 8-1, 8-2, 10-1, 10-2, Figure 10.10) compared to the brightness of control amplicons arising from the 1st until the 5th d of recovery. On the other hand, extra bands (i.e. 6-1 and 10-3) also occurred in the profiles generated by exposed population during the recovery. Interestingly none of these changes were observed at the end of the recovery experiment at d 8'.

Figure 10.11 shows the RAPD profiles of the offspring *D. magna* generated by the control parents and those exposed to 50 μgL^{-1} B(a)P for 6' d and recovered for 3', 5', and 9' d. There were no obvious changes among the different groups when primers OPB1, OPB8, OPB11 and OPB17 were used in the analysis (Figure 10.11). However, the use of other primers showed that clear changes occurred in the profiles produced by the offspring originating from the B(a)P exposed population in comparison to control patterns. Some of the changes which occurred in the B(a)P treated parents (Figure 10.10) were transmitted to the offspring. These changes involved bands 6-3, 7-1, 7-4, and 10-2. With primer OPB7 (Figure 10.11C), band 7-5 generated the 5th day of recovery was brighter than the corresponding amplicon displayed by the controls. However, this band was not brighter in the patterns produced by the exposed parental group. Profiles generated by primer OPA9

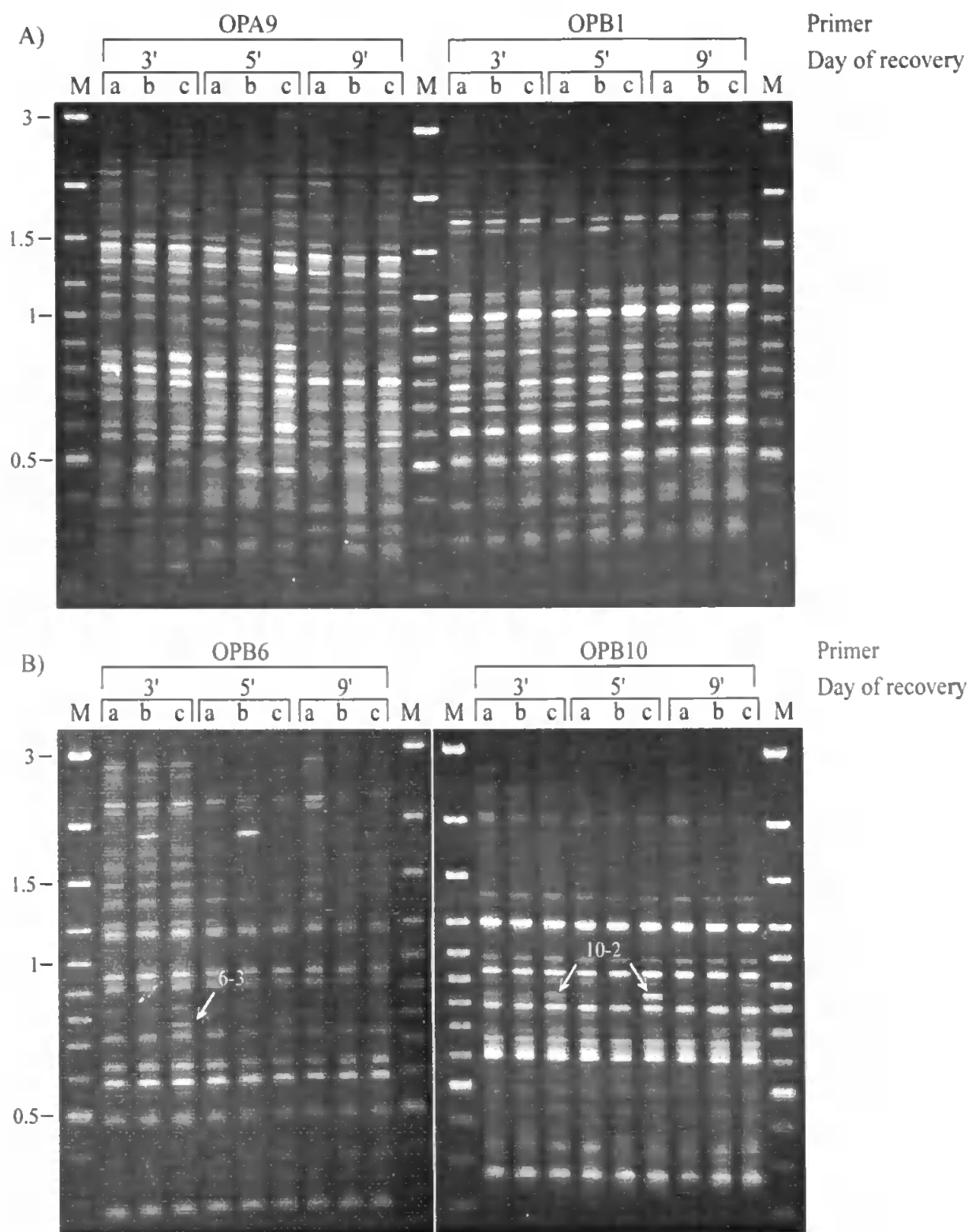
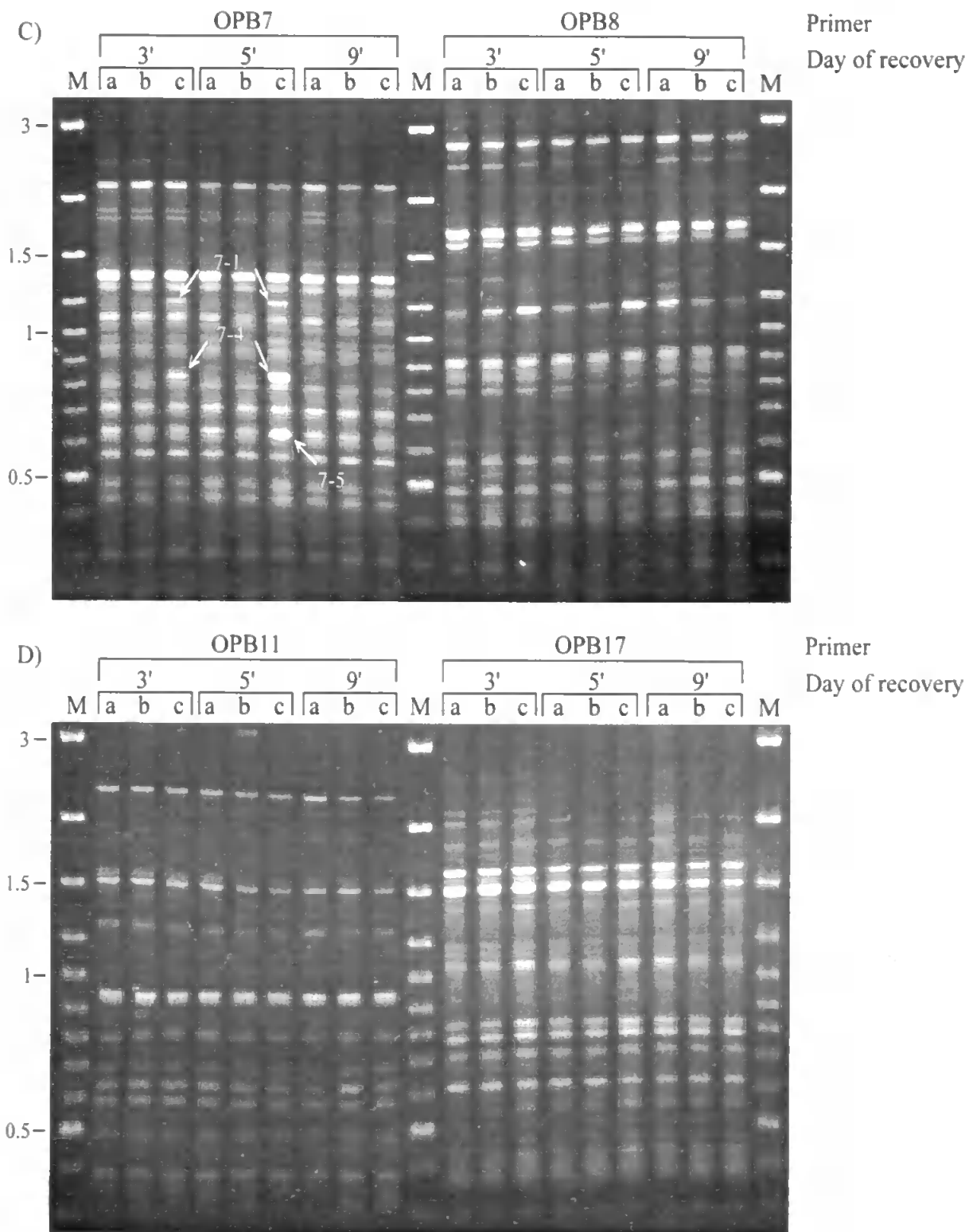


Figure 10.11 RAPD profiles of offspring *Daphnia magna* generated from parental animals exposed to $50 \mu\text{gL}^{-1}$ B(a)P for 6' d. The produced neonates were collected after the parents had recovered for 3', 5', and 9' d. a, b, and c: offspring *D. magna* generated by parents exposed to 0, 0 (solvent) and $50 \mu\text{gL}^{-1}$ B(a)P, respectively. Primers as indicated at



the top of each gel. For more details please refer to Figure 10.1. M = GeneRuler™ DNA ladder plus (Igi). The molecular sizes (Kb) of selected bands are shown on the left of each gel. Selected changes are indicated by arrows in comparison to control patterns.

are also presented in Figure 10.11. It is also clear that the neonatal patterns produced by the B(a)P exposed population displayed some differences in comparison to control profiles. Overall, all primers revealed that no changes occurred among the profiles generated by the neonates released at the 3rd generation (i.e. the 9th day of recovery) compared to control patterns.

To check the reproducibility of some of the samples, RAPD reactions were performed using 2 and 0.5 ng of template DNA (Figure 10.12). The profiles displayed good reproducibility at both DNA template concentrations and the brightness of bands 7-1, 7-4, and 10-2 was independent on the DNA concentration.

10.3.2.5 Overview of the results obtained in recovery 1 and 2

Appendix III gives an overview of the RAPD profiles obtained with all the primer tested in both recovery experiments.

10.4 Discussion

The general usefulness of DNA damage measurements will rely upon the further development of methodologies which allow the detection of a wide range of damage in the affected organisms (Baan *et al.*, 1991). Furthermore, the kinetics of formation and removal (repair) of DNA damage, as well as their probability to lead to actual mutations, need to be determined before conclusions can be made about their significance in terms of mutagenesis (Mohn and De Raat, 1993). In this chapter, an attempt was made to evaluate the potential of the RAPD technique to qualitatively assess the B(a)P-induced DNA effects in *D. magna* in kinetic experiments of exposure and recovery. In addition, we investigated whether changes in RAPD profiles were transmitted to the next generations.

The exposure of *D. magna* to 25 µg L⁻¹ B(a)P resulted in detectable DNA effects on the 6th and 7th day of exposure whereas the animals exposed to 50 µg L⁻¹ B(a)P showed

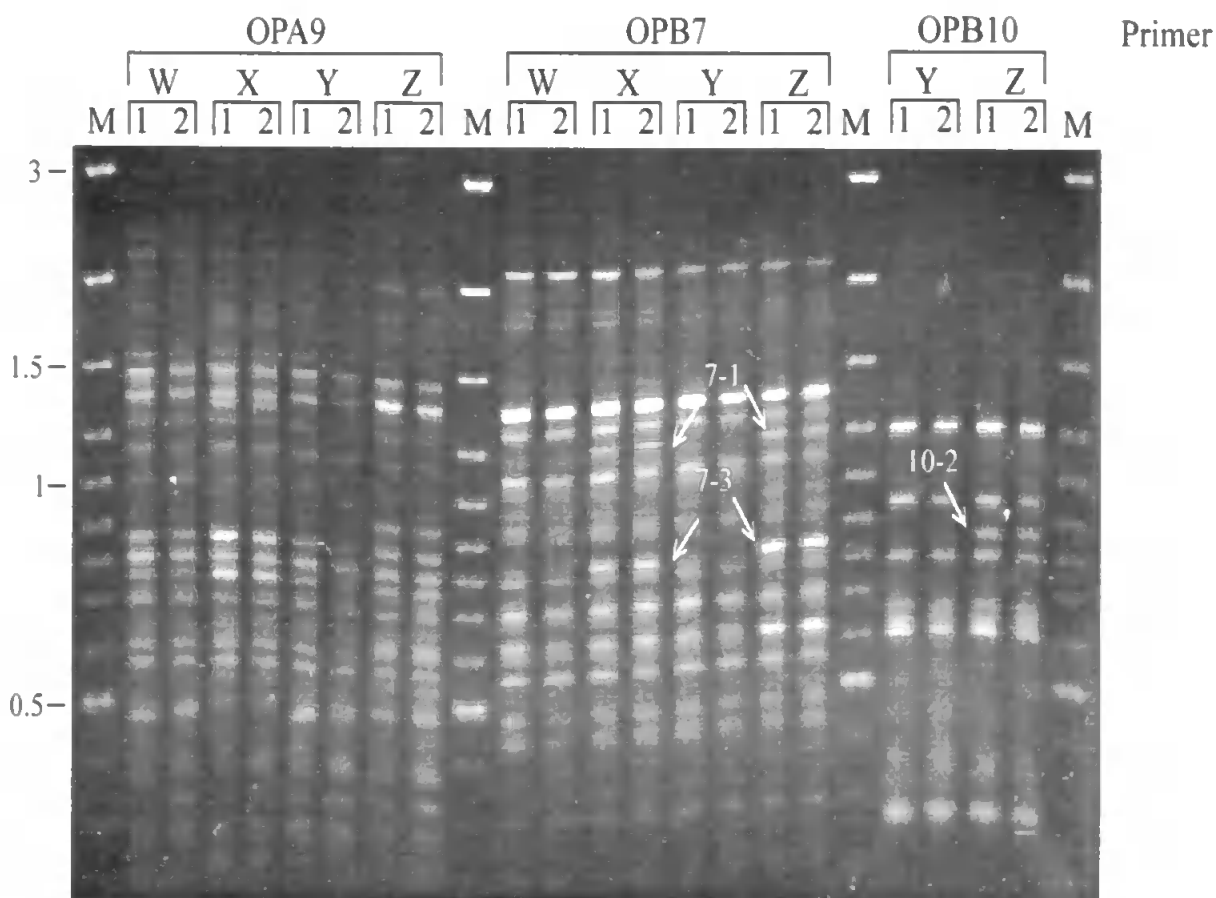


Figure 10.12 Reproducibility of RAPD profiles using 4 samples and 2 DNA template concentrations. Primers as indicated at the top of the gel. PCR reactions were performed using 2 DNA template concentrations (lanes 1 and 2: 2 and 0.5 ng genomic DNA, respectively). W and Y: neonates produced by the solvent exposed population after 6 and 8 d of recovery, respectively (recovery 1), X and Z: neonates produced by the B(a)P exposed population after 3' and 5' d of recovery, respectively (recovery 2). M = GeneRuler™ DNA ladder plus (Igi). The molecular sizes (Kb) of selected bands are shown on the left of each gel.

evidence of DNA effects from the first day of exposure. In addition the swimming capability of the population of *D. magna* exposed to 50 μgL^{-1} B(a)P was affected much more rapidly than the *D. magna* exposed to 25 μgL^{-1} B(a)P. This dose-response is not unexpected and may reflect the outcome of a variety of process. The processes which are likely to impact on the health of the animals include the rate of uptake, bioaccumulation and metabolism of B(a)P as well as the extent of DNA damage and the efficiency of DNA repair. Grinfield *et al.* (1986) found an increased incidence of micronuclei in the newt *Pleurodeles waltl*, kept for 8 days in B(a)P-containing water, that was dose dependent up to 75 μgL^{-1} B(a)P. Moreover, periods of exposure of less than 2 days did not result in a marked increase in micronuclei. In this study, the main change occurring in RAPD profiles produced by the population of *D. magna* exposed to 25 and 50 μgL^{-1} B(a)P was a decrease and increase in band intensity, respectively. This suggests that the DNA effects induced by both B(a)P concentrations were qualitatively and/or quantitatively different and may have been the results of DNA damage and/or mutations. For more details please refer to chapter 5. Interestingly, band 5-1 generated by control and solvent control followed a particular pattern (Table 10.1) and some of the changes were very reproducible between both controls (Figure 10.4A). This suggests that gene expression also may have had some underlying effects on RAPD profiles. Indeed, control *D. magna* can triple their body size within six days which probably reflects substantial changes in gene expression. Numerous studies have shown that gene expression correlates with a change in DNA structure (Westerhoff and Van Workum, 1990; Kohwi and Kohwi-Shigematsu, 1991; Wolffe and Hayes, 1999). Thus the structural changes induced in control *D. magna* may have some observable effects on RAPD profiles. Although the first step of the PCR reaction involves DNA denaturation at 94°C for 5 min, it is likely that the energy is insufficient to totally denature all the DNA in the reaction. This is especially likely in GC rich regions with strong secondary structure. The presence of such variations in control RAPD profiles may have led some researchers to

reject the RAPD method because of the non-reproducibility of some bands generated by individuals of the same species. This potential problem has been examined very often in this research project. Before concluding that false amplifications have occurred, it is necessary to test the PCR reactions using two DNA template concentrations as recommended by Welsh *et al.* (1995a). However, an important feature is that RAPD profiles between both non-exposed and solvent controls were reproducible at precise time. In this context, there was no need to use two different DNA template concentrations because specific changes occurred in patterns. Thus, the changes in RAPD profiles produced by the non-exposed and solvent exposed population of *D. magna* could be due to changes in gene expression. In addition, relative contribution of DNA damage induced by reactive oxygen species produced as a result of normal metabolic processes (which maintain steady state levels of genetic damage) may contribute to differential RAPD profile (Hanawalt, 1998). Finally, changes in RAPD profiles generated by the control population of *D. magna* (Figure 10.3) did not reveal as many changes (except band 5-1) when compared to control patterns presented in Figure 10.4. This could be due to the fact that both experiments (Figure 10.3 and 10.4) were run at two different times. As during one of the kinetic experiment the temperature in the culture room was subjected to small but unusual variation, it is likely that this external factor had an effect on metabolic process and/or gene expression, and consequently on RAPD profiles.

Recovery experiments were performed to qualitatively assess the DNA repair kinetics and to evaluate the persistence of B(a)P induced DNA effects. DNA repair rates have been successfully measured in genes containing DNA lesions using the ligation-mediated PCR (Gao *et al.*, 1994; Tornaletti and Pfeifer, 1994) and it was suggested that the efficiency of the repair process may strongly contribute to the mutation spectrum in genes which have been associated with cancer. In this chapter, *D. magna* were exposed to 50 $\mu\text{g L}^{-1}$ B(a)P for 3 and 6 days and were allowed to recover in clean medium for 12 days

(recovery 1) and 9 days (recovery 2), respectively. Although the repair of active genes has been reported to occur rapidly within a few hours (Mellon *et al.*, 1986; Gao *et al.*, 1994; Brandt and Ali-Osman, 1997), it is well established that the efficiency of DNA repair is not always optimal (i.e. slow repair) (Bohr *et al.*, 1987; Tornaletti and Pfeifer, 1994; Braithwaite *et al.*, 1999). Hence, the kinetics of DNA repair in *D. magna* was qualitatively assessed on a daily basis because the RAPD technique allows mainly the amplification of non-coding regions. Surprisingly, the appearance of new amplicons and increase in band intensity were observed in the profiles produced by the recovered population of *D. magna*. This suggested that some important effects on the DNA must have occurred from the first day of the recovery. The fact that the changes disappeared at the end of both recovery experiments suggested that the DNA effects were fully repaired and/or reversed. Nevertheless, some of the B(a)P induced changes which were detected in the parental groups were also observed in the offspring. These changes could not be the result of the direct exposure to B(a)P because the neonates were never in direct contact with the genotoxin. Only some of neonates generated in the first generation of recovery experiment 2 were present in the brood pouch of the adult *D. magna* exposed to B(a)P. However none of these neonates were released in the B(a)P solution. The transmission of modified genetic material to the offspring could be attributed to point mutations (e.g. base deletion, base insertion) and/or large genomic rearrangements and/or base methylation (epigenetic process). Each point will be separately discussed.

Previous studies have shown that non-repaired adducts could lead to mutation after DNA replication (Livneh *et al.*, 1993; Christner *et al.*, 1994). However, because the RAPD profiles of the third generation of neonates were not different to controls in recovery experiments 1 and 2 (except for primer OPB8 in recovery experiment 1, Figure 10.9A), this indicates that any detectable mutations that may have occurred must have been lost. It is well established that mutation may persist for a few or many generations (Wurgler and

Kramers, 1992). For instance, if a mutation is transmitted to the next generations and reduces reproductive fitness it is very likely that the mutation will be rapidly lost. It has been estimated that the average persistence of a mutation is five generations for autosomal dominant or X-linked mutations, and ten generations for mutations of complex inheritance (ICPEMC, 1983). However, the presence of commonly shared extra bands and increases in band intensity in parents and the first and second generation, but not the third generation, suggests that point mutations were probably not implicated in the RAPD profile changes. Indeed, each point mutation is expected to be independent from each other and thus subjected to different outcomes. Therefore, some point mutations would be fixed and other would be lost at different times.

In the second hypothesis it was proposed that rearrangement events could be responsible for the transmission of new amplicons and increases in band intensity. Chorazy (1985) reported that DNA-damaging agents contribute to the instability of the genome by introducing recombination-prone sites in genomic DNA. These agents can lead to extensive chromosomal lesions and rearrangements of genes and their regulatory sequences, and possibly to cancer (Klein, 1981; Adams *et al.*, 1983). Therman and Kuhn (1976) showed that mitomycin C is capable of enhancing the recombination frequency in Bloom's syndrome cells. Bhattacharyya *et al.* (1990) also indicated that unexcised DNA lesions stimulated intra-chromosomal homologous recombination. According to Wurgler (1992), the importance of induced recombination in genetic toxicology has been underestimated. Not only somatic cells undergo mitotic recombination and gene conversion, but premeiotic, mitotically dividing cells of the germ line show these phenomena as well (Darras and Francke, 1987). In addition, it has been reported that human chromosomes contain structures that can act as preferred substrates for mitotic recombination that may also represent hot spots for recombination (Wurgler, 1992). Finally the restoration of the former order of gene arrangements (i.e. reversion to 'wild' phenotype)

have been described suggesting that recombinational events are regulated (Chorazy, 1985). Against this background it seems likely that large rearrangements occurred in the germ cells of the *D. magna* and were transmitted to the first and second generations but not to the third because genomic reversion may have occurred at the end of each recovery experiment (i.e. before the third generation was produced).

Finally, the possibility exists that the transmission of genetically modified material could be the result of base methylation, an epigenetic mechanism (third hypothesis). Genomic methylation of CpG dinucleotides is essential for embryonic development. The methylation is performed by the DNA cytosine 5-methyltransferase which uses the cofactor S-adenosylmethionine (Zingg and Jones, 1997). Methylation of DNA in the common B forms facilitates a conformational change to the Z form (Zacharias *et al.*, 1990). CpG dinucleotides are the sites of DNA maintenance methylation in non-bacterial genomes, and are the predominant sites of aberrant hypo- or hypermethylation in cancer and other human genetic or somatic diseases (Jones, 1986; Counts and Goodman, 1994; Klein and Costa, 1997a; 1997b; Wachsman, 1997; Zingg and Jones, 1997). Key genes can become fully methylated and these silenced genes may then be transmitted as such to daughter cells since established methylation patterns are generally inherited in Mendelian fashion (Holliday, 1987; Silva and White, 1988). Interestingly, methylated cytosine arising as a result of epigenetic effects can also lead to point mutations due to deamination (Gonzalzo and Jones, 1997). Several carcinogens can affect DNA methylation either by modifying the target DNA or by inactivating the DNA methyl transferase (Boehm and Drahovsky, 1983; Wilson and Jones, 1984). In particular, B(a)P has been reported to inhibit cytosine 5-DNA methylation (Wilson and Jones, 1984). Cisplatin is the most potent inducer of DNA hypermethylation, possibly due to the conformational changes induced by cisplatin adducts which render the affected DNA a better substrate for DNA cytosine 5-methyltransferase (Nyce, 1997). Nickel is a human carcinogen that can alter gene expression by enhanced

DNA methylation and compaction, rather than by mutagenic mechanisms (Lee *et al.*, 1995). Active oxygen species can lead to genetic damage (e.g. 8-hydroxyguanine) and to epigenetic alterations in DNA methylation, without changing the DNA base sequence (Weitzman *et al.*, 1994; Cerda and Weitzman, 1997). For more detail please refer to section 2.2.2.4. Thus, the possibility exists that the patterns of cytosine methylation in germ cells of the *D. magna* were altered as the results of B(a)P exposure and were transmitted to the first and second generations but not to the third because cytosine methylation may have been reversed at the end of each recovery experiment (i.e. before the third generation was produced). The changes in RAPD profiles which were observed in the offspring may reflect this. In conclusion, the extra amplicons and increases in band intensity arising in the RAPD profiles of neonates derived from a parental population of *D. magna* which had been exposed to B(a)P are believed to be due to large rearrangements and/or DNA methylation, whilst point mutations are probably of limited importance.

It was observed that some changes in the parental *D. magna* were not transmitted to successive generations and there are two main reasons for this. Firstly, some damaged bases, in parental DNA, such as B(a)P adducts and 8-hydroxyguanine cannot be inherited by the offspring. Thus, the results of recovery experiment 2 revealed that DNA damage occurring in somatic cells persisted until the 5th day but was repaired by the 8th day. James *et al.* (1992) reported that spiny lobsters which were given 20 µg B(a)P/kg and sacrificed 28 days afterwards had less than 10 % of the DNA adducts found 3 days after the dose was administered. Secondly, a new amplicon in the parental RAPD profile may be the result of DNA effects in somatic but not in germ cells. Finally, the results also indicated that some of the changes in the profiles of the neonates did not arise in parental patterns. This could be the result of genomic instability (Hanawalt, 1998) due to epigenetic modifications. In other words, it is possible that the combination of genotoxic and epigenetic events led to a

RAPD profile which would be different from the one induced by the same epigenetic events alone.

White *et al.* (1999) investigated the survivorship of fathead minnow larvae which were two generations removed from an exposure to B(a)P. The results indicated that B(a)P concentrations as low as $1 \mu\text{gL}^{-1}$ decreased the reproductive capacity and larval survivorship compared to the solvent control possibly because of an increase in the frequency of deleterious mutations carried by the exposed parents. The results presented in this study suggest that some of the DNA effects induced by B(a)P occurred in germ cells and that these DNA effects were reversed after recovery (i.e. after 12 days and 9 days for recovery 1 and 2, respectively). It would have been very interesting to determine whether (i) the RAPD changes inherited by the two first generations were persistent, and whether (ii) population parameters such as growth and reproduction measured in the neonates (showing changes in RAPD profiles) were impaired.

DNA damage induced by environmental contaminants occurs at many levels of organisation in the genome including, the nucleotide, gene, and chromosome. Despite the availability of technologies to quantitate and characterise damage at each of these levels, it has been difficult to show a relation between anthropogenic chemicals found in nature and the induction of germline mutations (Yauk, 1998). Although DNA adducts, DNA breakages, and cytogenetic effects can be measured in genotoxin exposed organisms, these measures do not reveal direct DNA sequence changes that may be transmitted to offspring. In this context, there is a need to develop new methods and strategies to detect mutation (i.e. point mutations, and large rearrangements) in germ cells. Most molecular studies of DNA damage induced by mutagens have been done on a few selectable loci, such as the *hprt* or *aprt* of cultured mammalian cells (Meuth, 1990), *lacI* system (Wang and Taylor, 1992) or the *his* + reversion system (Shinoura *et al.*, 1983) of *Escherichia coli*. Although such methodologies proved useful for the detection of mutations, the information gained is

limited to that particular loci. In contrast, the AP-PCR or the RAPD technique is a nonselectable system. Shimada and Shima (1998) studied the molecular basis for germ-line mutations by using the AP-PCR method. The AP-PCR fingerprinting is a technique which is suitable for the detection of transmitted mutational changes including large DNA lesions without functional selection and bias, such as resistance to chemicals (Kubota *et al.*, 1992; 1995). This study shows that the RAPD method could be used in this matter despite some difficulties in the interpretation of the data. Another powerful method for the detection of germinal mutation is the minisatellite DNA screening (Jeffreys *et al.*, 1988; Dubrova *et al.*, 1996; Yauk and Quinn, 1996). Mutations are identified as novel fragments present in the DNA fingerprints of offspring that cannot be ascribed to either parent. Sommer (1995) reported that the great majority of germ-line mutations in humans result from endogenous processes, rather than exogenous mutagens. For instance, the oocytes of mammals are shielded from radiation and genotoxins behind multiple tissue layers. The present study suggests that this may not so for *D. magna*, which in any case is intimately in contact with a soluble or aquatic environment.

In conclusion, this study shows that the RAPD method can be useful to qualitatively assess the kinetics of DNA damage as well as DNA repair. In addition the RAPD technique shows its potential to detect DNA effects in germ cells. It is also clear that more research is needed to further investigate the molecular and holistic consequences of heritable induced DNA modifications.

Chapter 11

GENERAL DISCUSSION

This chapter is divided in two parts. The first part provides an overview of the possible effects of DNA damage and mutations on RAPD profiles, and attempts to identify some of the factors which may induce changes in RAPD profiles based on the results which have been obtained in this research project. The second part offers some general comments and conclusions.

11.1 Factors influencing RAPD profiles: an overview of the results obtained in the previous chapters

A summary of the possible effects of DNA adducts, DNA breakages, and mutations (point mutation and large rearrangements) on RAPD patterns is displayed in Figure 11.1. The presence of DNA damage and mutations within a primer binding site will alter the DNA fingerprint that is obtained from undamaged but otherwise identical DNA. However, such a situation is expected to arise less frequently than changes in RAPD patterns which occur as a consequence of the direct effect of DNA damage within the DNA contained between the primer binding sites (Figure 11.1B) as well as the structural (Figure 11.1A) effects of DNA lesions and mutations (see below). The main reason for this is that primer binding sites are small (10 bp) compared to the intervening DNA of the amplicon. The direct effect of DNA lesions (e.g. when the *Taq* DNA polymerase encounters a type of DNA damage) is expected to have a greater influence on the RAPD profiles rather than point mutations because they need to arise in the primer binding site to directly affect the patterns. This statement does not take into consideration the fact that the structural effects of point mutations may induce some changes in profiles (please see below). It has to be stressed that the stringency of the conditions may greatly influence the results; the higher the stringency the more likely it will be to detect a single base change within the priming site. When the *Taq* DNA polymerase encounters a DNA adduct there are a number of possible outcomes including blockage, by-pass and the possible dissociation of the

A)

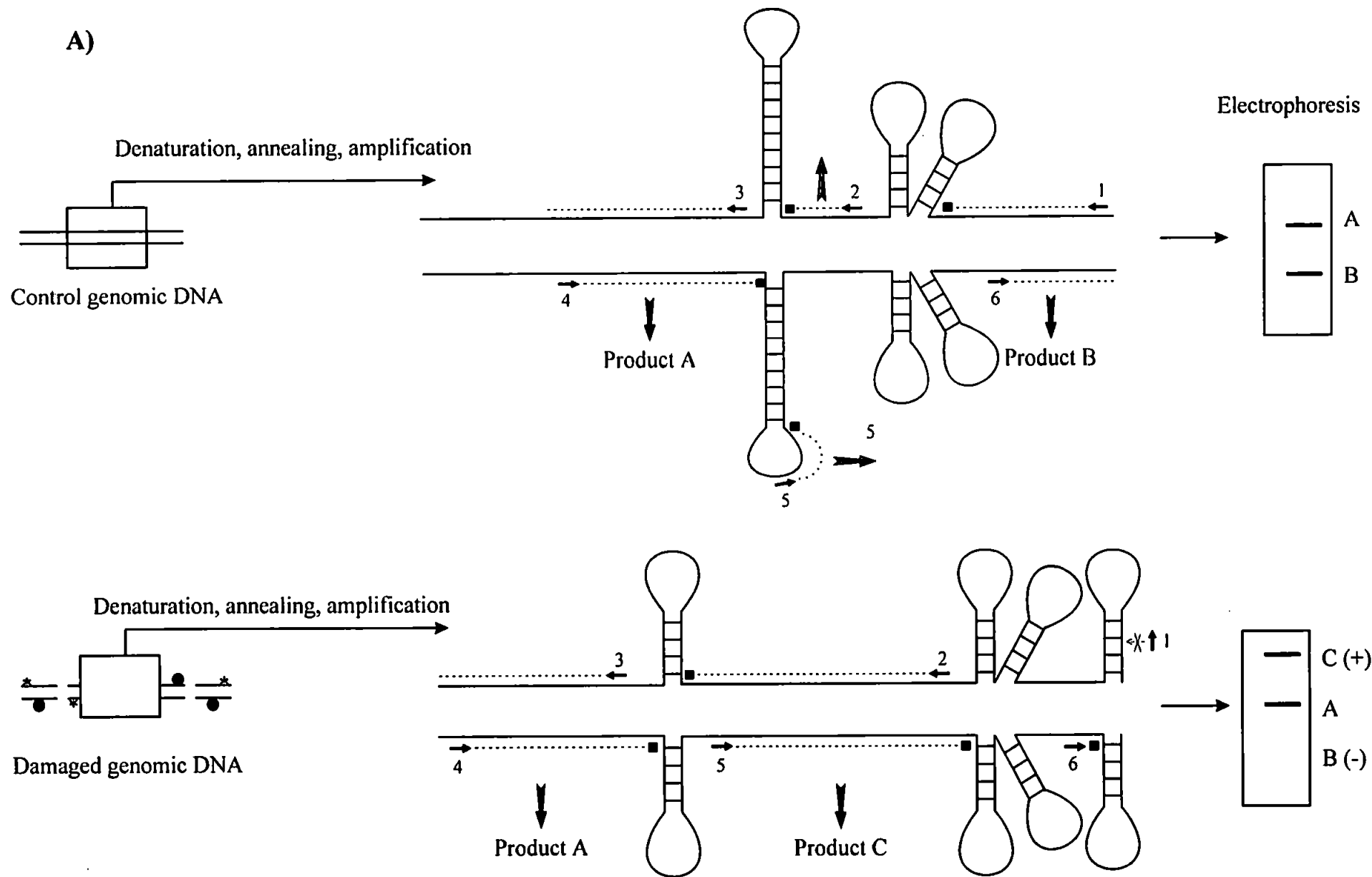
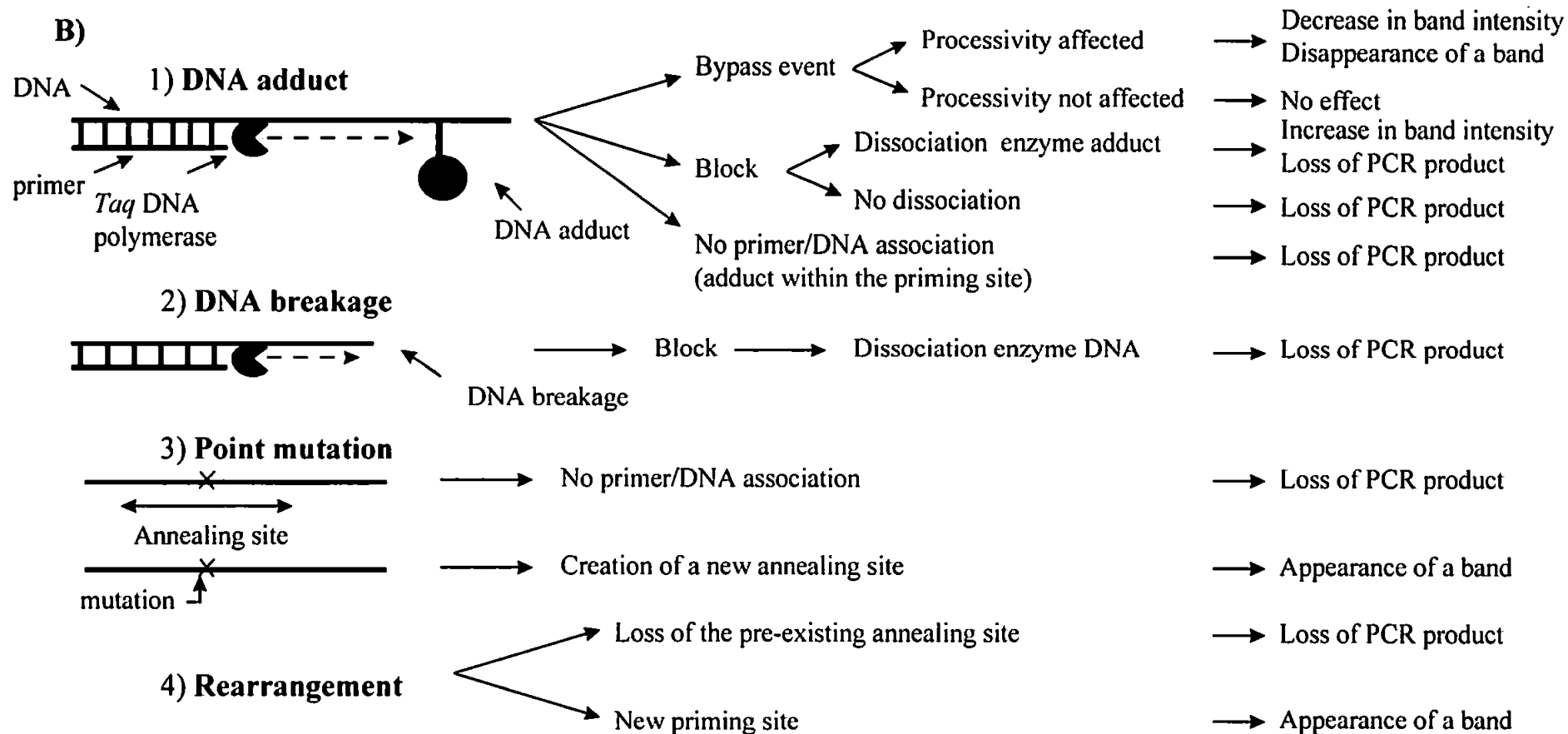


Figure 11.1 Structural (A) and direct (B) effect of DNA damage and mutation on RAPD profiles. For more details please refer to the text. A) the legend as follow: ●: DNA adduct, *: mutation, — —: DNA breakage, →: 10-mer primer, ➡➡: exponential amplification, ➡➡➡: linear amplification, ---: DNA synthesis, ■: arrest of the DNA synthesis, ⌢: hairpin loop, +: appearance of a band, and -: disappearance of a band. B) as indicated in the Figure.



enzyme/adduct complex which will cause changes in RAPD profiles (Figure 11.B). The other direct effects on RAPD profiles caused by DNA breakage, mutation, and rearrangement are also illustrated in Figure 11.1B. A breakage which occurs in the DNA template between two opposite primers may result in a loss of an amplicon whereas genetic rearrangements and point mutations may be responsible for either a loss or creation of new annealing sites which could result in the disappearance or appearance of new amplicons, respectively. In addition, DNA damage and mutation are also likely to induce structural changes in the DNA template which could lead to changes in RAPD patterns. During PCR some regions of the genomic DNA, such as GC rich sequences with strong secondary structures, may not be sufficiently denatured to allow amplification to proceed. An example of the structural effects induced by DNA damage and/or mutation is illustrated in Figure 11.1A. After RAPD amplification the control genomic DNA produces two amplicons, bands A and B. It is also shown that not all the 10-mer-primers will lead to exponential amplification (i.e. primer 2 and 5) due to the presence of strong secondary structure in the form of a hairpin loop in the control genomic DNA. The presence of DNA damage and/or mutations may lead to structural changes which have observable consequences in profiles. For example, as a result of the structural rearrangements a new product is synthesised (band C) whereas amplicon B is lost. DNA damage such as bulky adducts (Cosman *et al.*, 1992; Wang and Taylor, 1993) and mutations (Bowditch *et al.*, 1993) are known to induce structural changes. For instance, Bowditch *et al.* (1993) reported that mutations which stabilise or destabilise the secondary structure may be detectable as RAPD polymorphisms even though they do not reside within the primer binding site. In these cases, the RAPD assay potentially can detect DNA damage and mutations which affect secondary structure over a much greater area than that covered by the primer binding sites. This also suggests that the sequence of the primer binding site, which is small in comparison to the intervening DNA, is frequently unchanged in RAPD polymorphisms (Bowditch *et al.*,

1993). The successful application of the RAPD method to detect DNA alterations and mutations is related to the fact that the genotoxin-induced DNA damage does not occur randomly (Vogelstein and Kinzler, 1992). Hot spot interactions between genotoxins and genomic DNA have often been reported (Gupta, 1984; Boles and Hogan, 1984; Murray *et al.*, 1992; Kamiya *et al.*, 1993; Rodriguez and Loechler, 1993; Cairns and Murray, 1994) and in these cases the same structural changes will be induced in most of the damaged cells. This situation is very likely to allow the detection of DNA damage and mutations by the RAPD method.

The following paragraphs represent an attempt to identify some of the factors which may induce changes in RAPD profiles based on the results which have been obtained in the previous studies presented in this research project (Figure 11.2). Whilst DNA damage and mutations are expected to induce changes in RAPD profiles as discussed above, DNA repair and replication are likely to have some impact because they can induce point mutations. It is well established that DNA replication is arrested in cells which have been severely damaged in order to allow the process of DNA repair to proceed. The main reason for this is that the replication of damaged bases such as B(a)P adducts, pyrimidine dimers, cisplatin adducts and 8-hydroxyguanine can lead to mutagenesis. Originally, these results have been extrapolated from *in vitro* replication of DNA templates containing diverse DNA lesions using prokaryotic and eukaryotic polymerases. The results of these investigations have revealed that DNA damage either blocked the polymerase (Taylor and Oday, 1990; Brown and Romano, 1991; Holler *et al.*, 1992; Hruszkewycz *et al.*, 1992; Huang *et al.*, 1993; Broschard *et al.*, 1995) or that the enzyme bypassed the DNA lesion and possibly incorporated the wrong base opposite the lesion (Hruszkewycz and Dipple, 1991; Hruszkewycz *et al.*, 1991; Pillaire *et al.*, 1994; Chary and Lloyd, 1995; Hoffmann *et al.*, 1995; Nelson *et al.*, 1996a). In addition, DNA repair can also be implicated in mutagenesis. Although DNA repair is usually very efficient in deleting the DNA lesion and

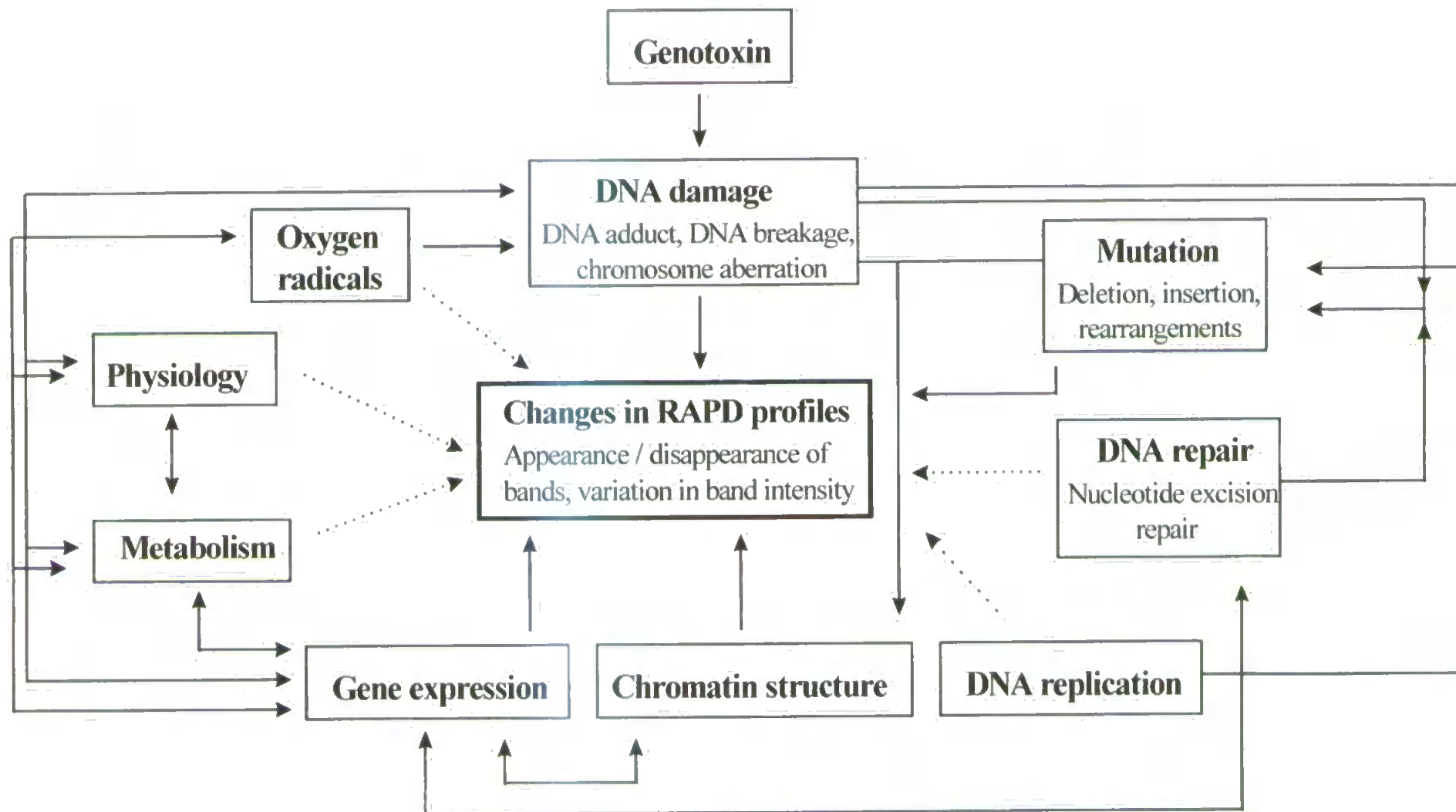


Figure 11.2 Relative influence of some processes affecting RAPD profiles following the exposure of a population to a genotoxin. This Figure is partly based on the results obtained in the previous chapters. The arrows with the full line, directing towards the central box, indicate a direct influence of the parameters on the RAPD profiles whereas the dotted line indicates indirect influence. For more details please refer to the text.

↔ is equivalent to ⇌

restoring the original sequence (Sancar and Sancar, 1988; Todo *et al.*, 1993; Britt, 1995; Sancar, 1996; Wood, 1996), it may also lead to mutagenesis, principally when two opposite DNA lesions are close to each other (Witkin, 1976). In the case of nucleotide excision repair, one lesion will be excised with the surrounding non damaged bases and the gap will be filled by using the complementary strand (containing another DNA lesion) as template (Witkin, 1976). The bypass of the lesion may potentially lead to a mutation.

In Chapter 10, it was suggested that differences in gene expression may have an effect on RAPD profiles because the genome experiences structural changes when genes are expressed (Jolly and Morimoto, 1999). In spite of the apparent stability and compaction of chromatin, complex metabolic processes involving DNA occur very efficiently *in vivo* (Wolffe and Hayes, 1999). This contrasting requirement between storage and functional utility is met through the use of specialised molecular machinery, namely the histones, that reversibly disrupt and modify the chromatin. It was previously mentioned that DNA methylation plays a significant role in gene expression and it is also well established that histone acetylation is intimately connected to transcriptional regulation (Grunstein, 1997). The phosphorylation of histone could also be implicated in this matter (Wei *et al.*, 1998). In addition, chromatin can also be disrupted by molecular processes which are driven by ATP hydrolysis, including DNA and RNA polymerases and switching mating type/sucrose non-fermenting type complexes (Varga-Weisz and Becker, 1998). Differences in gene expression are expected to have some significant effects on RAPD profiles because some of the structural changes may persist even after denaturation of the DNA during PCR (Figure 11.1A). Indeed, control *D. magna* produced specific changes during intensive growth (chapter 10). In chapter 8, the RAPD profiles of *D. magna* which had been exposed to distilled water for 24 h showed hardly any changes when compared with control patterns. It was hypothesised that either the denaturation of the genomic DNA during the first step of PCR undid the structural changes induced by the change in gene expression

(hypothesis 1) or that the animals did not induce a rapid and transient change in gene expression because the experiment lasted 24 h only (hypothesis 2). The results obtained in chapter 10 would support the second hypothesis rather than the first one.

Variations in metabolism and physiology may be expected to induce some changes in RAPD profiles. As changes in metabolic activities are often accompanied by changes in gene expression (Westerhoff and Van Workum, 1990), then metabolism may indirectly affect RAPD patterns. Furthermore, the physiology of the cells is also expected to indirectly have an influence on RAPD profiles because physiology and metabolism are closely related (Figure 11.2). For instance, cells modulate their metabolism to respond to the appropriate environmental signals. In addition, the physiology of cells is known to have a direct influence on DNA. Miralles and Serrano (1995) reported that gene expression can be altered as a result of osmotic stress. Oshimura and Barrett (1986) mentioned that the mechanisms by which aneuploidy can occur fall into several categories including alterations in cell physiology. Similarly, Carr and Hoekstra (1995) reported that cell physiology and the cell cycle can have a significant effect on the induction of DNA damage. It has also been shown that differences in directional nucleotide substitution among lineages of mammals may be explained by changes in metabolic physiology (Martin, 1995). This process may have been mediated by the effect of oxygen radicals because these toxic compounds are among the products of aerobic metabolism and are known mutagens. Unfortunately the mechanisms implicated in the studies previously mentioned are not known.

The results presented in chapter 8 suggested that the changes occurring in the RAPD profiles of any treated populations should not only be attributed to the direct action of the genotoxin used in the experiment. Cavalieri and Rogan (1995) have revealed that radical cations of PAHs play a major role in both the metabolism and metabolic activation leading to formation of DNA adducts. Furthermore, Pritsos and Sartorelli (1986) showed

that reactive oxygen radicals were produced after the reactivation of mitomycin. Thus, as oxygen radicals play an important role in the production of DNA damage after exposure of any population to genotoxins, it is very likely that they significantly induce changes in RAPD profiles. Oxygen radicals are known to produce oxidative damage and modified bases such as 8-hydroxyguanine which can affect the methylation of CpG islands and consequently alter gene expression (Weitzman *et al.*, 1994; Cerda and Weitzman, 1997).

In conclusion, there is a complex interaction between many different processes which directly and indirectly affect the integrity of the genome and therefore interpretation of the data is not always easy. According to the results presented in this research project it seems that DNA damage and mutations (including point mutations and large rearrangements) have a very significant influence on RAPD patterns. Gene expression can induce changes in profiles but it is proposed that it will have less impact compared to DNA damage and mutations. This statement is based on the fact that the detection of changes in gene expression depends on the sequence of the primer (chapter 10) whereas extensive DNA damage and mutations are expected to induce changes in RAPD profile with most of the primers if not all (chapter 9). It is obvious that at this stage extensive experiments are required to understand the relative contribution of each parameter on RAPD profiles.

11.2 General discussion and conclusion

RAPD has been used in many areas of research (chapter 2). Only recently, the RAPD assay has been successfully applied to detect DNA damage and mutations (Savva, 1996; 1998; Atienzar *et al.*, 1999; 2000a; Becerill *et al.*, 1999). In addition, the arbitrarily primed polymerase chain reaction (AP-PCR), a closely related methodology, has also been used to detect DNA effects (Kubota *et al.*, 1992; 1995; Peinado *et al.*, 1992; Ionov *et al.*, 1993; Kohno *et al.*, 1994; Arribas *et al.*, 1997; Lopez *et al.*, 1999). The main objective of this research project was to evaluate the potential of the RAPD method to detect genotoxin-

induced DNA effects under *in vivo* conditions in aquatic organisms. However, before doing so it was essential to optimise each of the RAPD parameters and also to understand the effect of the variation of each parameter on RAPD profiles. The results presented in this research project unambiguously revealed that, under optimised conditions, the RAPD method provided a robust assay with good reproducibility as already reported by numerous researchers (Rothuizen and Wolferen, 1994; Benter *et al.*, 1995; Bielawski *et al.*, 1995). However, while the existing literature suggests an increasing use of this technique for multiple applications, many studies have also criticised the technique on the basis that it lacks reproducibility (Ellsworth *et al.*, 1993; Khanda *et al.*, 1997), and Mendelian inheritance (Scott *et al.*, 1992; Riedy *et al.*, 1992; Ayliffe *et al.*, 1994) because of the low stringency conditions employed (i.e. low annealing temperature) which could lead to spurious amplification. Most of the studies which experienced some problems of reproducibility claimed that the results were due to artefacts, contamination or mutation in genomic DNA (e.g. Scott *et al.*, 1992; Riedy *et al.*, 1992). Since many factors (reviewed in the first section of this chapter) may induce some changes in RAPD profiles, it is likely that other factors than those previously mentioned could be the culprits (e.g. variation in gene expression). When RAPD profiles are not reproducible, researchers generally claim that the variation is due to artefacts although no evidence of such event is usually presented. On the other hand, Ayliffe *et al.* (1994) clearly demonstrated that heteroduplex molecules formed between allelic RAPD products are a potential source of artifactual polymorphism that can arise during RAPD analysis.

As low stringency conditions (i.e. low annealing temperature) may allow spurious amplification to form, it was attempted to generate RAPD profiles at an annealing temperature which was sufficiently high to circumvent these problems. Atienzar *et al.* (2000b) reported that reproducible profiles could be generated using 10-mer primers in RAPD reactions at an annealing temperature of 50°C with template DNA extracted from

phylogenetically different groups of organisms such as bacteria, plants and animals. These results are in agreement with other studies using high annealing temperature (Bentley and Bassam, 1996; Caetano-Anolles, 1998). In addition it has been often stated that the Mg^{++} concentration and annealing temperature should be optimised for each species and primer which are studied (Wolff *et al.*, 1993). The results presented in this research project do not support this statement.

It has been reported that RAPD reactions often incur spurious amplification products in negative control reactions that do not contain template DNA. This situation arises as a consequence of primer dimers and other artefacts (Ruano *et al.*, 1989; Mullis, 1991) which consume the stock of primers and enzyme and consequently reduce the yield of the appropriate products. Bottger (1990) who also reported the presence of patterns in negative controls claimed that the *Taq* DNA polymerase was naturally contaminated with DNA. Although methods such as the Booster PCR (Ruano *et al.*, al., 1991) and the Hot-StartTM PCR (Erlich *et al.*, 1991) have been developed to tackle these problems, these procedures are not convenient when PCR reactions are performed with a large number of samples. A more suitable approach consists of using higher dNTP concentrations in comparison to conventional protocols (e.g. 0.22 mM dNTP). Atienzar *et al.* (1998a) reported that a dNTP concentration of 0.33 mM either partially or completely reduced the spurious amplifications in reactions without genomic DNA templates (negative controls).

When RAPD analysis is performed three key points need to be carefully considered. Firstly, if RAPD profiles from the same genomic template or from different individuals of the same species are found to vary, then the reproducibility of the assay should be confirmed by repeating the PCR using two DNA template concentrations that differ by at least two-fold (Welsh *et al.*, 1995a). Any differences between individuals that do not occur at both genomic DNA concentrations are rejected (Welsh *et al.*, 1995a). Unfortunately, this safeguard is routinely ignored by researchers. Consequently, some studies (e.g. Ellsworth

et al., 1993; Khandka *et al.*, 1997) have rejected RAPD on the basis of the presence of artefacts even though no evidence was presented that convincingly demonstrated the presence of artefacts using two different DNA concentrations. However, if the same changes in RAPD are shared by some of the samples, then there is no reason to doubt the integrity of the PCR reactions. Secondly, if only a few products are efficiently amplified by a particular primer, then the presence or absence of a polymorphic product can affect the amplification of other products in the same reaction. In other words, the more bands that are visible in the fingerprint, the less likely it is that the banding pattern will be influenced by the presence or absence of a particular product (Welsh *et al.*, 1995a). Consequently, our objectives were to generate reproducible and clearly discriminatory DNA profiles with maximum number of bands, good product yield and clarity. Finally, the effects of different thermostable DNA polymerases and thermal cyclers on RAPD profiles have been well studied. For instance, it has been reported that the outcome of a RAPD fingerprint pattern may depend on the type of polymerase used (Schierwater and Ender, 1993). Reproducibility is usually excellent when the thermal cycler is equipped with the best temperature regulation, but suffers when this is not the case (Meunier and Grimont, 1993). Thus, the comparison of RAPD profiles among different laboratories should take into account the PCR conditions as well as the make of the thermal cycler and the source of *Taq* DNA polymerase.

RAPD analysis has provided a reliable and reproducible method for molecular typing and genetic characterisation of a variety of species of micro-organisms which have undergone genetic rearrangement due to evolutionary processes (Akopyanz *et al.*, 1992; Bentley and Bassam, 1996; Grayson *et al.*, 1999; 2000; Hansen *et al.*, 1998; Wagner *et al.*, 1998; Wang *et al.*, 1998). However, the detection of genetic diversity using the RAPD method is not limited to bacteria as the technique has been successively applied to populations belonging to either the animal or plant kingdoms (Koller *et al.*, 1993; Tinker *et*

al., 1993; Williams *et al.*, 1993; Kemp and Teale, 1994; Tylerwalters and Hawkins, 1995; Liu *et al.*, 1999). Thus, in the absence of xenobiotic-induced DNA damage, there is no doubt that RAPD represents a powerful tool for detecting genetic diversity.

There is a great concern about the effect of environmental contaminants on the genetics of natural populations. One class of genetic effects includes alterations to the structure and function of DNA including DNA adducts, DNA breakage, and mutation as a result of chemical exposure (genotoxic effects). However, indirect genetic effects can also arise as a consequence of the interactions of genotoxic agents with DNA. That is, the effects of chemical exposure on the genetic composition of the population, in terms of genetic variability or the distribution of allele frequencies (Theodorakis *et al.*, 1998). These effects can lead to an increase in mortality, an impairment in reproduction and, possibly, to genetic bottlenecks or the introduction of novel selective pressure in a heterogenous population (Nevo *et al.*, 1986; Guttman, 1994). Consequently, this could result in an alteration in genetic variability or allele frequencies (Anderson *et al.*, 1994b; Bickman and Smolen, 1994). Very recently, the RAPD method has also been used to detect genetic diversity among populations which had been exposed to environmental contaminants, including well-known genotoxins (Nadig *et al.*, 1998; Theodorakis *et al.*, 1998; Krane *et al.*, 1999; Liu *et al.*, 1999; Theodorakis *et al.*, 1999). Surprisingly, all these studies concluded that the observed changes in RAPD profiles which occurred among the exposed population were not the result of DNA damage or mutations. For instance, Theodorakis *et al.* (1999) gave a number of explanations such as the low frequency of genotoxin-induced mutations, and the low probability of the RAPD method to detect rare mutations. In addition, Theodorakis *et al.* (1999) stated that a band could be amplified even if many of the primer binding sites were damaged. The results presented in this research project are not necessarily in agreement with the findings of Theodorakis *et al.* (1999). In conclusion it seems likely that, in these studies, the RAPD technique detected genetic diversity as well as direct DNA effects.

Recently, the RAPD technique has been successfully used to detect DNA damage in aquatic organisms or cells exposed to mitomycin C (Becerril *et al.*; 1999), benzo(a)pyrene (chapter 5; Atienzar *et al.*, 1999), ultraviolet radiation (chapter 6; Atienzar *et al.*, 2000a), copper (chapter 7), 4-*n*-nonylphenol and 17- β -oestradiol (chapter 8). These studies show that RAPD represents a useful means of detecting genotoxic effects of environmental chemicals. RAPD offers the possibility of a biomarker assay that may be used as an early warning assay since it is capable of detecting structural changes to DNA which may not be retained as permanent damage or mutations. Results presented in this research project clearly suggested that the presence of DNA damage such as DNA adducts, DNA breakages and mutations (e.g. insertion, deletion, rearrangements) could lead to changes in RAPD profiles (chapter 9). Shimada and Shima (1998) also claimed that broad spectra of genetic changes such as point mutations, large deletions, or gross chromosomal rearrangements can be easily detected using the AP-PCR technique as reported by numerous studies (Kubota *et al.*, 1992; Peinado *et al.*, 1992; Ionov *et al.*, 1993; Kohno *et al.*, 1994). Savva (1998) concluded that changes observed in the fingerprints of control and exposed animals may be due to the presence of DNA adducts, mutations or DNA strand breaks. However, the successful application of the RAPD method to detect DNA alteration and mutation is related to the fact that the genotoxin-induced DNA damage do not occur randomly (Vogelstein and Kinzler, 1992).

When the RAPD method is used as a means to detect genotoxic effects there are a number of points which need to be carefully considered. Firstly, the RAPD analysis should focus on the change in profiles generated by the exposed populations which are reproducible between samples. Secondly, all changes in RAPD profiles should not always be attributed to DNA damage and/or mutations. Thirdly, a variation in control patterns is not necessarily associated with the presence of artefacts. Fourthly, as the exposure of a species to a genotoxin can potentially lead to oxidative damage, oxygen radicals are likely

to play an important role with regard to changes in RAPD profiles. Different strategies can be applied to measure the effects of induced DNA alteration and mutation. If the RAPD patterns generated by individuals of a species are reproducible, then the profiles obtained from an exposed population of the same species can be directly compared to control patterns. On the other hand, if individuals produce truly polymorphic profiles, then a non-destructive method must be used (Savva, 1996). For instance, it may be possible to sample haemolymph or blood from an organism before and after exposure to environmental contaminants. In this case, RAPD profiles generated can be compared and the differences can be attributed to the direct or indirect effects of the contaminant. Unfortunately such a situation is not necessarily true as changes in RAPD profiles may occur in control populations (chapter 9). In addition, the size of the organism and the tissues which can be sampled non-destructively can be limiting factors. Although the precise reasons for the changes occurring in RAPD profiles can only be speculated upon, there are different strategies which can be accomplished to tackle this problem. For instance, it is possible to distinguish between DNA damage and mutations or base methylation. The strategy relies upon exposing an organism to a genotoxin and then performing RAPD analysis upon the parents and their offspring. Any changes in patterns which are present in parent but not in offspring could reflect DNA damage such as DNA adducts, 8-hydroxyguanine on the assumption that somatic and germ cells contain the same genotoxin-induced DNA effects. On the other hand, mutations and the extent of the DNA methylation in germ cells will be transmitted to the next generations. Another strategy to possibly distinguish between a damaged base (e.g. DNA adduct) and mutation is to let the exposed population recover. After a significant period of recovery, most of the damaged bases would be repaired whereas any fixed mutation would be still retained. Hence persistent changes in RAPD profiles can be attributed to mutation and/or to non-repaired bases (e.g. persistent adduct). In addition, strategies may be developed to estimate the contribution of each of the factors

which can induce changes in RAPD profiles (Figure 11.2). For instance, it is possible to specifically study the effect of oxygen radicals on RAPD patterns. This could be accomplished by either transfecting cultured cells with a plasmid containing genes coding for oxygen radicals scavengers using inducible promoters to control expression or by pre-treating the cells (or animals) with radical scavengers. The contribution of DNA repair to changes in RAPD profiles could also be studied by either 'knocking out' a gene involved in DNA repair mechanisms or by using DNA repair inhibitors. Recently, Lopez *et al.* (1999) studied the contribution of the mus-201 gene (required in the excision repair process) to the stability of the *Drosophila melanogaster* genome by using the AP-PCR assay. It seems that RAPD and AP-PCR have a promising future in studies assessing the contribution of a particular gene to the stability of a genome.

How could the RAPD assay developed in this research project be used in the field possibly after a pollution event such as an oil spill? A powerful strategy would be to look at DNA effects in diverse aquatic invertebrates (e.g. mussels, crabs) and vertebrates (e.g. fish) collected from uncontaminated sites. Non-invasive biological material (haemolymph, blood) could be individually collected from each of the species before introducing them in polluted and control sites. Acute and long term DNA effects could be evaluated by sampling biological material from these species at intervals or as per the requirement to generate RAPD profiles; a quantitative assay could also be used.

The RAPD methodology presents numerous advantages over conventional methods such as hybridisation based protocols. No prior knowledge of the genome under investigation is required. RAPD requires very little source material and under certain circumstances, the analysis can also be performed non-destructively which can be useful for the screening of rare or valuable samples. In addition, a single random oligonucleotide primer is used which means that by employing different primers, banding profiles can be rapidly generated that differ in complexity. Another remarkable advantage is the high level

of the overall sensitivity of the technique. And last but not least the RAPD method has the potential to detect a wide range of DNA damage (e.g. DNA adducts, DNA breakage) as well as mutations (point mutations and large rearrangements). The method also possesses some disadvantages, such as the lack of reproducibility and the problem of non-parental bands. However, some of these problems can be tackled by performing the PCR under high stringency conditions. Nevertheless, the major challenge of identifying the precise reasons for the changes which may be induced in the RAPD profiles may remain unresolved. Indeed, different types of DNA lesions and mutations can induce the same type of alterations in RAPD profiles (chapter 9). Another potential obstacle is the variation in RAPD profile which may occur in non-exposed organisms (chapter 10). Moreover, the use of RAPD for comparative purposes relies on the assumption that similarity of fragment size is a dependable indicator of homology (Rieseberg, 1996). Another drawback is that RAPD bands are inherited as dominant traits (Clark and Lanigan, 1993; Lynch and Milligan, 1994) which diminishes its potential in heterozygote studies. Furthermore, the fact that a particular primer does not reveal any differences among RAPD profiles does not mean that there are no DNA effects. However, this problem can be easily tackled by using different primers. Finally, it is important for all who use the technique to appreciate that the RAPD method generates qualitative rather than quantitative data.

The assessment of exposure levels of genotoxic agents and the genetic consequences requires the application of a set of inter-related techniques capable of providing both qualitative and quantitative information (Jones and Parry, 1992). However, when little or no reliable information is available on the mode of action of a potential genotoxin, the RAPD can be particularly advantageous in comparison to quantitative methods. For instance, earlier studies have failed to prove the presence of oestrogen-DNA adducts (Epe and Metzler, 1985). In addition, oestrogens were found to be negative in short-term assays for the induction of gene mutations, irrespective of whether this was

measured in prokaryotic (Glatt *et al.*, 1979) or eucaryotic (Drevon *et al.*, 1981; Barrett *et al.*, 1983) cells. The results presented in chapter 8 clearly revealed that DNA effects were generated by oestrogens. It is now well established that a wide range of DNA damage and mutations are induced by oestrogens (Roy and Liehr, 1999). In addition, RAPD may be especially useful for gathering evidence of genotoxicity in the field particularly when no chemical studies have been performed on a site, and to assist in the recognition of clean and polluted areas. In the field of ecotoxicology, methods such as ^{32}P -postlabelling (Gupta *et al.*, 1982; Gupta, 1984) and the comet assay (Singh *et al.*, 1991; Klaude *et al.*, 1996; Mitchelmore and Chipman, 1998a; 1998b) are widely used. Does the RAPD method compare favourably with these methods? The ^{32}P -post labelling technique is very sensitive and enables the detection of DNA adducts at concentrations as low as one adduct in 10^9 to 10^{10} normal nucleotides (Randerath and Randerath, 1994; La and Swenberg, 1996). Another advantage is that the method is applicable to any extracted DNA and has been successfully used in many experiments (e.g. Gupta *et al.*, 1982; Shimomura *et al.*, 1992; Canova *et al.*, 1998). However, there are a few disadvantages associated with this technique. Firstly, between 1 and 10 μg of genomic DNA are required to perform the assay (Randerath and Randerath, 1994) whereas as little as 1 ng of genomic DNA is sufficient to perform RAPD reactions. Consequently, the size of the exposed-population can be a limiting factor. Secondly, the recovery of certain adducts is structure dependent. The amounts and types of adducts present in a DNA preparation determine the selection of an optimal procedure such as nuclease or butanol procedure (Randerath and Randerath, 1994). Thirdly, optimal adduct resolution depends on the choice of the chromatographic conditions (Randerath and Randerath, 1994). Fourthly, it uses radioactivity which brings the problem of possible exposure to radioactive material and disposal after use. Finally, the ^{32}P -postlabelling is not artefact free. Scates *et al.* (1995) reported that adduct-like spots were obtained when bile acids were incubated with DNA which had not been precipitated

from high salt before it was postlabelled. With regard to the comet assay or single cell gel electrophoresis, it is a rapid simple and sensitive assay for measuring alkali-labile sites and strand breaks (Singh *et al.*, 1988; Olive *et al.*, 1990). If required, only extremely small samples containing from 1 to 10,000 cells can be used and results can be obtained in a single day (McKelvey *et al.*, 1991). As few as 1 DNA break per 10^{10} Daltons can be detected in the assay (McKelvey *et al.*, 1991). Consequently, this technique has been successfully applied in many studies (e.g. Fairbairn *et al.*, 1995; Monteith and Vanstone, 1995; Singh *et al.*, 1995; Mitchelmore *et al.*, 1998a; 1998b; Mitchelmore and Chipman, 1998a; 1998b). However, contrary to the RAPD methodology, the main disadvantage is that the procedure for the isolation of cells needs to be readjusted and optimised depending on the species or tissue used. In addition, Speit *et al.* (1999) revealed that increased temperature during alkaline treatment and electrophoresis strongly enhanced DNA migration under otherwise identical test conditions. Thus quantitative comparison between studies can only be accomplished if the comet assay has been performed under the same conditions. In conclusion, the main advantages of the RAPD method lies in its sensitivity and speed plus the fact that a wide range of DNA damage and mutations can be detected in many species without any adjustment of the method. Its main disadvantage compared to other biochemical and molecular techniques is related to the interpretation of the data. Since the ^{32}P -postlabelling method and the comet assay detect only DNA adducts and DNA breakage, respectively, the RAPD technique seems to be the preferred method. However, quantitative and specific data are also needed to better understand the effect of pollutants on genomic DNA. In the field of genotoxicology, a powerful strategy would be firstly, to perform the RAPD method to have an overview of the DNA effects and, secondly, to use more specific methods such as the ^{32}P -postlabelling method and/or comet assay. Becerrill *et al.* (1999) claimed that the RAPD technique is very useful for studying genetic alterations in fish cells because most accepted genotoxic assays cannot be performed due to

the large number and small size of fish chromosomes, as well as the long cell cycle associated with most fish species. The weight of evidence supports this statement but RAPD should not be regarded as a substitute method to be used only when well-established tests cannot be performed.

The development of molecular techniques has led to a better understanding of numerous cellular processes including the mechanism of DNA damage and DNA repair. However, little has been done to explore the consequent effects of genotoxic responses on reproduction and development in the individual and population (Anderson *et al.*, 1994b; Anderson and Wild, 1994; Depledge, 1994a; Jha, 1998). A particularly useful outcome of ecotoxicological studies would be the ability to understand and predict the significance of genotoxin-induced DNA damage at higher levels of biological organisation. For instance, there is a need to understand how toxicant-induced changes in genomes and gene pools might affect the long-term survival of populations (Anderson and Wild, 1994). Ecotoxicology is concerned with understanding the impact that pollutants may have on the integrity of ecosystems, a dynamic process which depends on the survival and reproduction of their constituents (Wurgler and Kramers, 1992). In geno-ecotoxicology, the ultimate goal should be to determine the effects of DNA damage and mutations at the population and community levels. Research should focus on methods which allow the differentiation between somatic and heritable effects in natural populations (Bickmam and Smolen, 1994). At higher levels of biological organisation the problem of correlating a particular biological response with exposure to a genotoxin becomes complicated because the final outcome may be temporally and spatially distant from the initial events of chemical-biological interactions (Jha, 1998). In this research project, it was attempted to better understand the effects of benzo(a)pyrene (Atienzar *et al.*, 1999) and copper -induced DNA effects on development, reproduction, and mortality of *D. magna*. In another study, ultraviolet-induced genotoxicity in *P. palmata* was measured using RAPD along with chlorophyll

fluorescence and growth (Atienzar *et al.*, 2000a). The data presented in this research project suggested that high levels of DNA damage led irreversibly to death. Before reaching this stage, each species showed a reduction in reproduction and growth, probably as a consequence of the diversion of resources to allow the repair of the DNA to take place before replication proceeds. The results also revealed that DNA damage did not always have an impact which could be observed at higher levels of biological organisation and, therefore, DNA effects as indicated by changes in RAPD profiles was a more sensitive indicator of toxicity than fitness parameters and growth. However, in the case of the marine algae *P. palmata* it is noteworthy that chlorophyll fluorescence which reflects perturbation at molecular level was as sensitive as genomic template stability for the evaluation of toxic/genotoxic effects. Not surprisingly the least sensitive fitness parameter was l_x (age-specific survival), whereas m_x (age-specific fecundity) and R_0 (net reproductive rate) were the most sensitive fitness parameters (chapters 5 and 7).

Future research could focus on diverse aspects such as:

- * the understanding of the events responsible for some of the changes occurring in RAPD profiles generated by the pollutant-exposed population. This would implicate the analysis of bands by using molecular techniques such as cloning, sequencing and probing.
- * the further investigation of the effect of DNA damage on RAPD profiles under *in vitro* conditions. This could be accomplished by using genomic DNA containing well characterised and specific kind of DNA lesions. Of particular interest would be to determine the effect of 8-hydroxyguanine, one of the major products formed by the reactive oxygen species which are generated in living cells (Lepage *et al.*, 1999).
- * the comparison of the RAPD method with quantitative assays measuring micronuclei, chromosome aberrations (cytogenetic techniques), DNA adducts (^{32}P -postlabelling technique), and DNA breakages (comet assay).

- * the evaluation of the effect of environmental contaminants on the RAPD assay in conjunction with the measure of effects at population levels *in vivo*.

- * the better understanding of gene expression, physiology and metabolism on RAPD profiles.

The reverse transcriptase PCR technique (Liang and Pardee, 1992; Brandt and Ali-Osman, 1997) could be used to investigate the expression of genes (differential display) in populations exposed to an environmental contaminant and to compare the messenger RNA patterns with the RAPD profiles at different time (during and after exposure). In addition, a set of experiments could be performed to induce different levels of metabolic activity to measure the DNA effects using the RAPD method.

- * the development of computer software to model the influence of structural effects on RAPD patterns. For instance, can the profiles be predictable when the sequence of an entire genome is known? Alternatively, this would allow studies of the effects of different stringency conditions on the binding of the primer.

- * the development of the RAPD to allow the amplification of longer fragments (e.g. maximum size of 10 Kb instead of 3 Kb) because they are more likely to possess regions which are prone to DNA damage. This should increase the sensitivity of the assay.

- * the development on a new assay based on the multiple amplification of genes implicated in cancer (e.g. anti-oncogenes, oncogenes). As the nucleotide sequences of these genes are highly conserved this should allow screening of a range of species which would yield quantitative data on the extent of DNA damage in specific genes. The multiple enzymatic restriction of the PCR products could allow the detection of mutations (Jenkis *et al.*, 1999).

In conclusion, the results presented in this research project show the potential of the RAPD assay as a useful method for the qualitative assessment of DNA effects including genotoxicity and changes in gene expression. Nevertheless, a great deal of further experimentation and validation are required in order to assess the applicability of the technique to a variety of other species, pollutants and locations, particularly under field conditions.

APPENDIX I

**Formulation, preparation of reagents, solutions,
growth media, technical details, and DNA ladders**

Elendt's medium for the culture of *Daphnia magna*

The preparation of Elendt M7 medium initially involves setting up a series of individual substance (trace element and macro-nutrient) and multi-substance (vitamin) stock solutions by dissolving appropriate amounts of reagent grade chemicals in double distilled water (ddH₂O). For more details please refer to Elendt and Bias (1990). The final concentrations of each chemical is given in Table I.1.

Table I.1: final concentrations of each chemical in M7 medium.

Component	Final concentration (mgL ⁻¹)
NaHCO ₃	64.8
CaCl ₂ .2H ₂ O	293.8
MgSO ₄ .7H ₂ O	123.3
KCl	5.8
Na ₂ SiO ₃ .5H ₂ O	7.466
NaNO ₃	0.274
KH ₂ PO ₄	0.143
K ₂ HPO ₄ .3H ₂ O	0.241
Thiamine hydrochloride	0.075
Vitamin B ₁₂	0.001
Biotin	0.00075
B	0.5
Fe	0.2
Mn	0.1
Li	0.05
Rb	0.05
Sr	0.05
Mo	0.025
Br	0.0125
Cu	0.002
Zn	0.0063
Co	0.0025
I	0.0025
Se	0.001
V	0.0003
C ₁₀ H ₁₄ N ₂ O ₈ Na ₂ .2H ₂ O	2.5

All the solutions were kept in a cold room (10°C) in the dark except the mixture of vitamin solutions which were kept at -80°C.

Chlorella vulgaris: culture and medium

Culture: *Chlorella vulgaris*, strain CCAP (culture collection algae and protozoa, Cumbria, UK) 211/11 was cultured in round bottom wide neck glass of 5 L capacity, at 20 ± 2°C under constant light. The culture was aerated using laboratory air supply. The algae was cultured with special care to avoid bacterial, algal and protozoan contamination. Initially, the culture was initiated by using 1-5 ml of CCAP 211/11 taken from the stock solution and inoculated in a 5 L autoclaved medium (3.5 L distilled water plus 1.5 L of tap water). After 13-15 d, the air supply was dismantled and the algae was allowed to settle down.

After 1-2 d, approximately 4 L of the supernatant were discarded and the pellet was resuspended in the 1 L left. The number of cells were counted using a haemocytometer and the concentration adjusted to 1.2×10^8 cells ml^{-1} . Algal stock solutions were refrigerated for up to 2 weeks in the dark. A new culture was immediately started using 10-20 ml of the stock solutions.

Medium: To 5 L (3.5 L distilled water plus 1.5 L of tap water) 20 ml of solution A, 10 ml of solution B, 5 ml of solution C, and 5 ml of solution D were added. Solution A, B, and C were kept under refrigeration. Add each solution aseptically to water previously autoclaved via sterilising filter (0.2 μm).

Solution A:

per 500 ml of ddH₂O:

CaCl ₂ .2H ₂ O	0.5 g
KNO ₃	68.75 g
MgSO ₄ .7H ₂ O	12.5 g
KH ₂ PO ₄	5 g

Solution B:

per 500 ml of ddH₂O:

FeNaEDTA	3.475 g
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Solution C:

per 500 ml of ddH₂O:

Na ₂ MoO ₄ .2H ₂ O	0.067 g
H ₃ BO ₃	0.715 g
MnCl ₂ .4 H ₂ O	0.615 g
ZnCl ₂	0.025 g
Cu(NO ₃) ₂ .3 H ₂ O	0.019 g
Co(NO ₃) ₂ .6H ₂ O	0.125 g

Solution D:

per 100 ml of ddH₂O, add:

1 ml of vitamin S1	(S1: 0.05 g vitamin B ₁₂ in 500 ml ddH ₂ O)
0.75 ml of vitamin S2	(S2: 0.05 g biotin in 500 ml ddH ₂ O)
10 ml of vitamin S3	(S3: 0.0375 g thiamine hydrochloride in 500 ml ddH ₂ O)

All the solutions were kept in a cold room (10°C) in the dark.

General data about the medium and bacteria used in the study

* Medium for the culture of bacteria (Luria-Bertani medium):

per litre of ddH₂O:

Tryptone	10 g
Yeast extract	5 g
Sodium chloride	10 g
Sterilised by autoclaving.	

* LB agar:

LB medium as above, with the addition 1.5 % w/v agar.

Sterilised by autoclaving.

* Antibiotic stock solution:

Tetracycline 15 mg/ml in 50 % v/v ddH₂O/ethanol
 Kanamycin 25 mg/ml in ddH₂O
 Sterilised by filtration (0.22 µm).

* Strains C600, SMR 346, and SMR 687:

The strains C600, SMR346, and SMR687 were provided by Dr Susan Rosenberg (University of Alberta, Canada) with the details as provided in Table I.2.

Table I.2 Details of the bacterial strains C600, SMR 346, and SMR 687.

Strains	C600 AKA	SMR346 AKA C600 mutL::Tn5	SMR687 AKA C600 mutL::Tn10; AKA RSH6
		mutL211::Tn5	mutS215::Tn10
Transposon		Tn5	Tn10
Sex	F-	F-	
Parent		C600 X P1 ES1293	C600 X P1 ES1481 (#685)
Auxotrophies	thi, thr, leu	thi, thr, leu	thi, thr, leu
Frozen	1/2/1989	1/2/1989	11/6/1993
FS #	39		
etc		made at NIEHS	
Source	Appleyard 1956 via Stahl		Harris
Bkg		C600	
Suppressor	II	II	II

DNA extraction and purification of genomic DNA

* **Phenol/chloroform method and Genie DNA extraction kit**

Sperm lysis buffer:

100 mM Tris-Hcl pH 8
 500 mM NaCl
 10 mM EDTA pH 8
 1 % SDS
 2 % mercaptoethanol

RNase:

A 10 % w/v solution in ddH₂O. The solution was incubated at 95°C for 10 min to inactivate any contaminating deoxyribonuclease.

Tris-Borate-EDTA (TBE) buffer (10 X):

Tris 0.9 M
 Boric acid 0.9 M
 EDTA 20 mM

pH adjusted to 8.0. This stock solution (10X) was immediately diluted to prepare a 1X TE solution.

*** Caesium chloride preparation**

Tris-EDTA (TE) buffer (100 X):

Purchased from Sigma (Poole, UK).

Ethidium bromide solution (10 mg/ml):

Purchased from Sigma (Poole, UK).

*** Marmur genomic DNA isolation**

All solutions were made in ddH₂O.

Sucrose/Tris solution:

Sucrose 25 % w/v in 0.05 M Tris-Hcl pH 8.0

Lysozyme solution:

Lysozyme 10 mg/ml in 0.25 M Tris-Hcl pH 8.0

EDTA solution:

EDTA (sodium salt) 0.25 M solution pH 8.0

Triton lysis solution:

Triton X100	2 % w/v
Tris-Hcl	0.05 M
EDTA (sodium salt)	0.063 M
pH adjusted to 7.0	

Saline EDTA solution:

Sodium acetate	3 M
EDTA (sodium salt)	0.1 M
pH adjusted to 8.0	

Standard saline-citrate solution:

NaCl	0.15 M
Trisodium citrate	0.015 M

Dilute saline-citrate solution:

NaCl	0.05 M
Trisodium citrate	0.0015 M

Concentrated saline-citrate solution:

NaCl	1.5 M
Trisodium citrate	0.15 M

RNase:

A 2 % w/v solution in 0.15 M NaCl.

pH adjusted to 8.0. The solution was incubated at 95°C for 10 min. To inactivate any contaminating deoxyribonuclease.

Agarose gel electrophoresis of DNA

Tris-EDTA (TE) buffer (100 X):

Please see above.

Tris-Borate-EDTA (TBE) buffer (10 X):

Please see above.

Gel loading buffer (5X):

per 20 ml ddH₂O:

Bromophenol blue 0.05 g

Xylene cyanol 0.05 g

Ficoll 400 5 g

The final concentration of the gel loading buffer was adjusted to 1X.

Staining by ethidium bromide:

Per 1.5 L of TBE (1X) solution 225 µl (10 mg/ml; Sigma, Poole, UK) of ethidium bromide solution was added. After 2-3 gels were stained a top up of 100 µl ethidium bromide was added. The solution was changed every week.

PCR

PCR buffer (1 X) for *Taq* DNA polymerase supplied by Promega (Madison, USA):

Tris-HCl pH 9 at 20°C 11.2 mM

KCl 56 mM

Triton-X-100 0.112 %

PCR buffer (1 X) for *Taq* DNA polymerase supplied by Immunogen International (Sunderland, UK):

Tris-HCl pH 8.8 at 25°C 10 mM

KCl 50 mM

Nonidet P40 0.08 %

Nearly all PCR reactions described in this thesis were carried out using the *Taq* DNA polymerase supplied by Immunogen International.

Enzymatic restriction

*Eco*RI 10 X buffer:

NaCl 50 mM

Tris-HCl 100 mM

MgCl₂ 10 mM

Triton-X-100 0.025 %

pH 7.5 at 25°C

Hind III 10 X buffer:

NaCl	50 mM
Tris-HCl	10 mM
MgCl ₂	10 mM
Dithiothreitol	1 mM
pH 7.9 at 25°C	

Not I 10 X buffer:

NaCl	100 mM
Tris-HCl	50 mM
MgCl ₂	10 mM
Dithiothreitol	1 mM
pH 7.9 at 25°C	

Pme I 10 X buffer:

Potassium acetate	50 mM
Tris-acetate	20 mM
Magnesium acetate	10 mM
Dithiothreitol	1 mM
BSA	100 µg/ml
pH 7.9 at 25°C	

Table 1.3 Recognised sequence and average fragment size generated in *Escherichia coli* and mouse by 4 different restriction enzymes used in the study. Arrows indicate the restriction sites. The information was collected from New England BioLabs catalogue, 1998, appendix p 277.

Restriction enzymes	Recognised sequence	Average fragment size generated in <i>E. coli</i>	Average fragment size generated in mouse
<i>EcoR</i> I	↓ 5'...GAATTC...3' 3'...CTTAAG...5' ↑	5 000	5 000
<i>Hind</i> III	↓ 5'...AAGCTT...3' 3'...TTCGAA...5' ↑	5 000	3 000
<i>Not</i> I	↓ 5'...GCGGCCGC...3' 3'...CGCCGGCG...5' ↑	200 000	200 000
<i>Pme</i> I	↓ 5'...GTTTAAAC...3' 3'...CAAATTTG...5' ↑	40 000	80 000

DNA ladders used in the different studies

Please refer to Figure I.1.

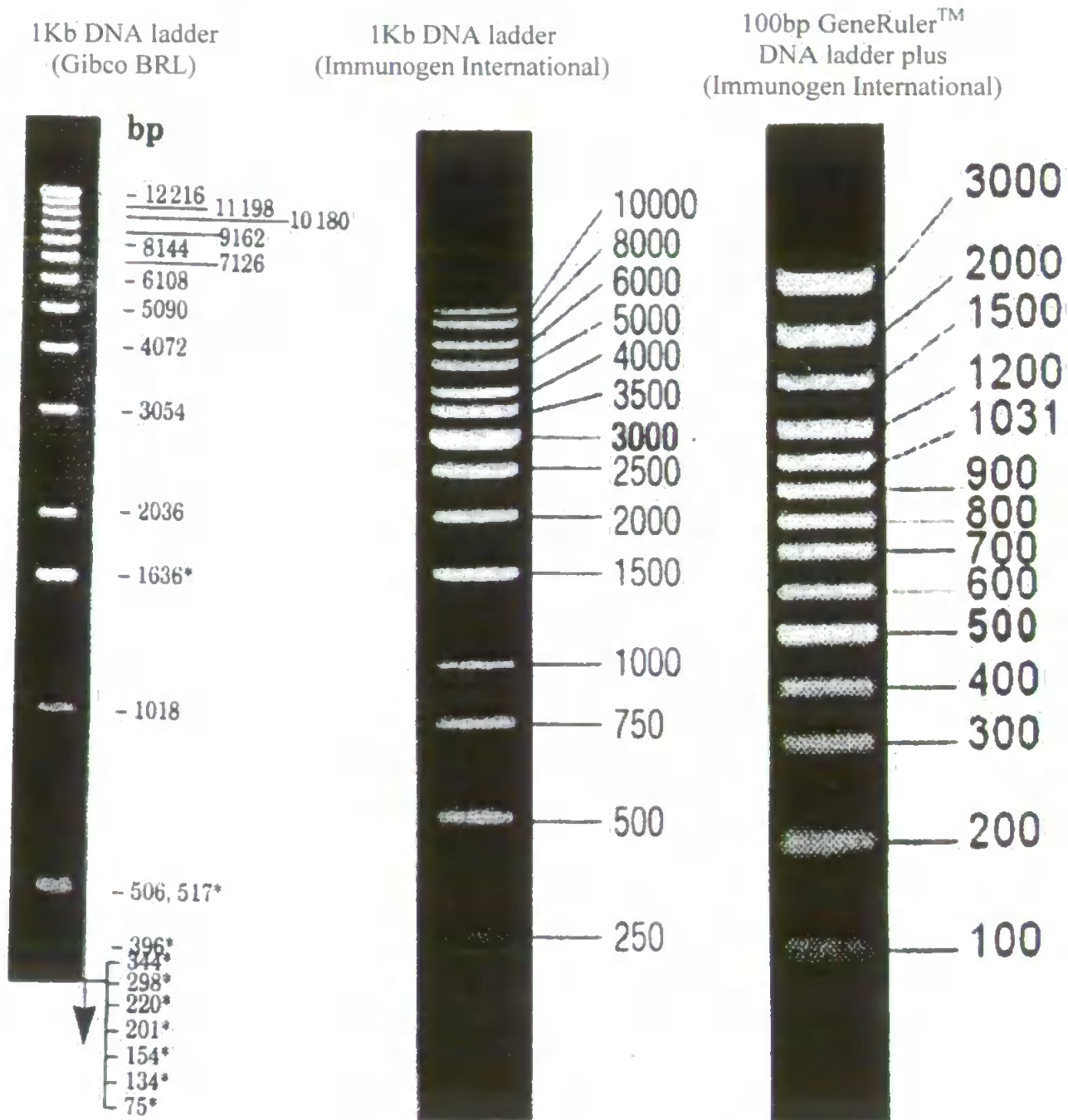


Figure I.1 DNA ladders used in the different studies. All sizes are given in bp. The name of each DNA marker is indicated at the top of each gel. Reproduced with the permission from Gibco BRL (Paisley, UK) and Immunogen International (Sunderland, UK).

APPENDIX II

Example of calculation of the genomic template stability

After exposure of an aquatic species to 0 [control (C)], X [treated 1 (T)], and Y [treated 2 (T')] μgL^{-1} environmental contaminants for a period of time, DNA is extracted and RAPD is carried out on 3 individuals originating from each group. The profiles produced by primer OPB1 is presented in Figure II.1.

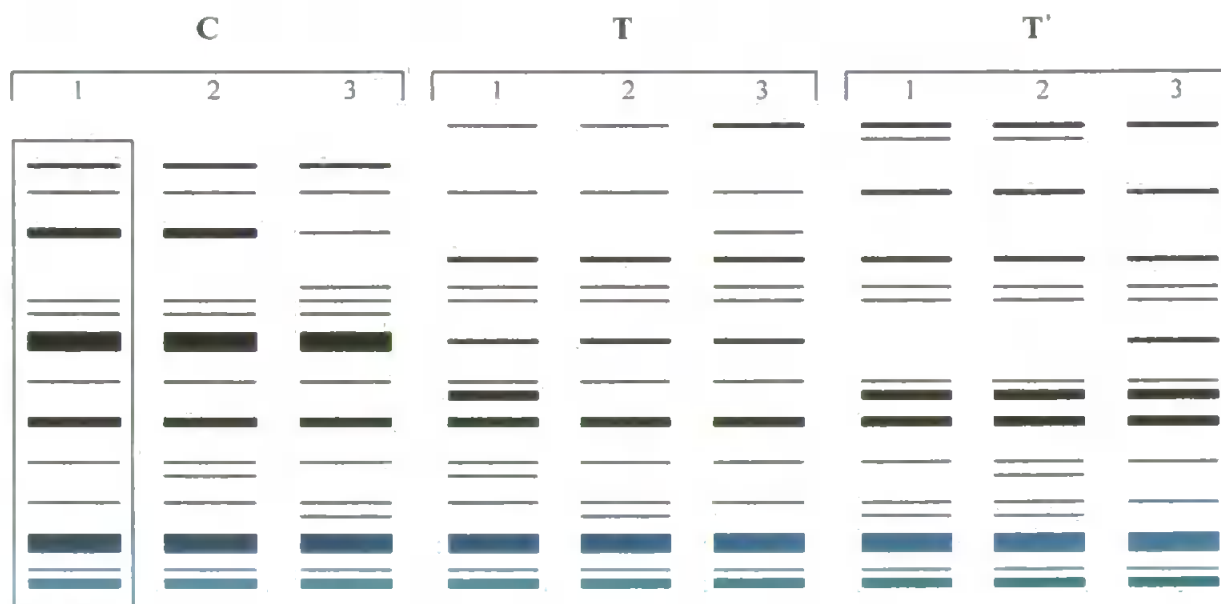


Figure II.1 Example of RAPD profiles (primer OPB1) generated by control and animals exposed to a genotoxin.

The 3 RAPD profiles produced by the 3 control individuals are not perfectly identical and consequently one of the pattern has to be chosen to compare the reference pattern with the other profiles. In this example C_1 is the reference pattern because this profile displays the highest number of bands shared with C_2 and C_3 . Each obvious variation in profiles (disappearance of bands, appearance of new bands and variation in band intensities) in comparison to pattern C_1 is given the arbitrary score of +1, and the average calculated for each experimental group of animals (Table II.1). The genomic template stability (GTS) (%) is calculated as $100 - (100a/n)$ where 'n' is the number of bands detected in control DNA profiles and 'a' the average number of changes in DNA profiles.

After the GTS is calculated for each primer, the average and standard deviation (SD) are determined (Table II.2). The data are transformed in order to calculate the average GTS (Table II.3) and to compare it with the parameters measured at population level. For instance, GTS calculated with primer OPB1 for each group was multiply by '100/92.9' (i.e. GTS in the control set to 100 %).

The next step is to calculate the average and SD of the GTS (transformed data) determined by some or all the primers used in the study (Table II.3). When the RAPD profile is too complex to study, no calculation is attempted.

It is important to understand that a GTS of 100 % in the control does not necessarily mean that no variation occurred in control profiles. Indeed to calculate the average it is necessary to transform the data (i.e. control set to 100 %). The variation occurring in the control profiles is measured by calculating the SD. * The SD of control GTS is $3.5 \times 100 / 94.5 = 3.7$

Finally, in order to compare the average GTS with the changes in fitness and Darwinian parameters, the data obtained at the population level are also transformed (i.e. control set to 100 %). An example is given in Table II.4.

Table II.1: Determination of GTS (primer OPB1).

	C ₁	C ₂	C ₃	T ₁	T ₂	T ₃	T' ₁	T' ₂	T' ₃
Total number of bands	13	14	15	-	-	-	-	-	-
Average number of bands (n)	14			-			-		
Appearance of bands	0	1	2	5	4	3	6	7	4
Disappearance of bands	0	0	0	3	3	2	4	4	3
Increase in band intensity	0	0	0	0	0	0	1	1	1
Decrease in band intensity	0	0	0	1	1	1	1	1	1
Total number of changes (a)	0	1	2	9	8	6	12	13	9
Average number of changes	1			7.6			11.3		
Calculation of the GTS (%)	$100-(100 \times 1/14) = 92.9$			$100-(100 \times 7.6/14) = 45.7$			$100-(100 \times 11.3/14) = 19.0$		
Transformation of the data (%)	100			49.2			20.4		

Table II.2: GTS generated by 5 primers before transformation.

	Control	Treated	Treated'
GTS (primer OPB1)	92.9	45.7	19.0
GTS (primer OPB2)	95.0	55.0	50.0
GTS (primer OPB3)	94.2	48.7	48.5
GTS (primer OPB4)	100.0	65.1	51.8
GTS (primer OPB5)	90.5	54.2	57.9
Average	94.5	53.7	45.4
SD	3.5	7.4	15.2

Table II.3: GTS generated by 5 primers after transformation.

	Control	Treated	Treated'
GTS (primer OPB1)	100.0	49.2	20.4
GTS (primer OPB2)	100.0	57.8	52.6
GTS (primer OPB3)	100.0	51.7	51.5
GTS (primer OPB4)	100.0	65.1	51.8
GTS (primer OPB5)	100.0	59.9	64.0
Average	100.0	56.8	48.1
SD	3.7*	6.4	16.3

Table II.4: Comparison of GTS with fitness parameters.

	Control	Treated	Treated'
GTS	100 ± 3.7	56.8 ± 6.4	48.1 ± 16.3
l_x	100 ± 4.2	80.2 ± 8.3	75.2 ± 5.6
m_x	100 ± 9.2	74.5 ± 7.2	70.5 ± 7.5
r_m	100 ± 12.5	64.2 ± 3.3	62.0 ± 5.6
R_o	100 ± 5.6	56.3 ± 6.8	50.8 ± 5.3

APPENDIX III

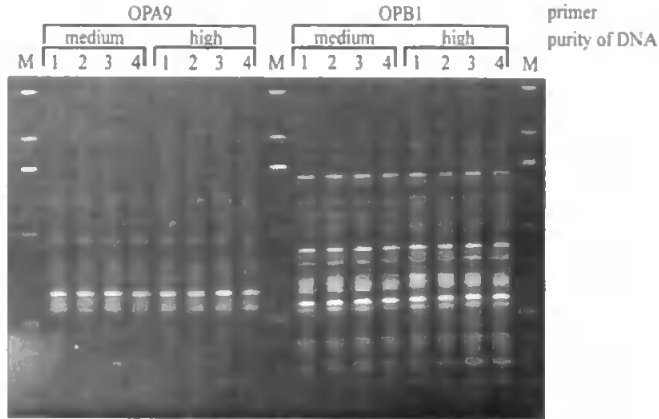
**Results not presented in the main body of the
thesis and overview of some of the data**

This appendix displays the data not presented in the main body of the thesis as well as an overview of some of the results either presented or not in the main body of the thesis.

This appendix is classified by chapters. The order of the presented figures follows the order of those appearing in the main body of the thesis. Each figure possesses a title and some information below the figures (e.g. name of the species and primer used). In addition, the figures refer to a legend presented in the main body of the thesis. For more details please refer to the legend of the figure mentioned.

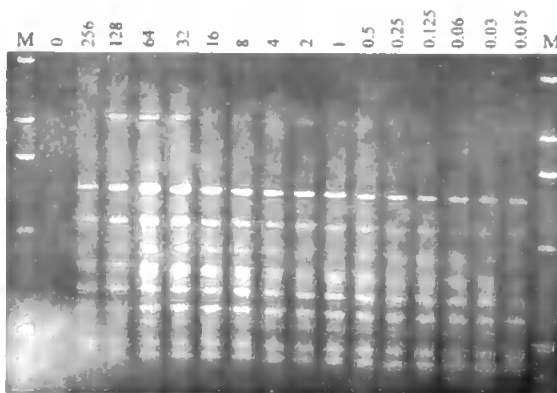
CHAPTER 4

Effect of DNA preparation on RAPD profiles

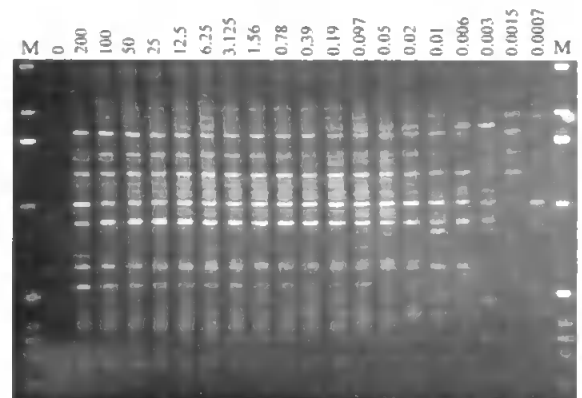


Palmaria palmata. For more details refer to legend of Figure 4.7B.

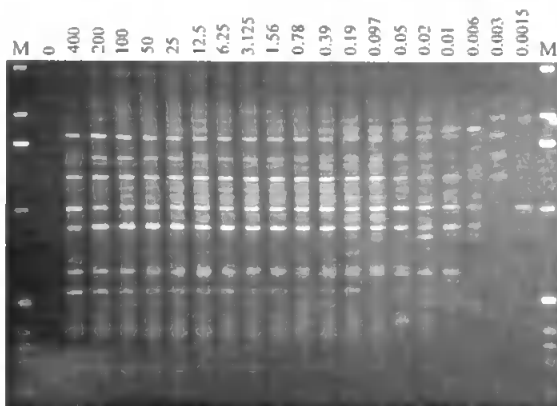
Effect of DNA concentration (ng) on RAPD profiles



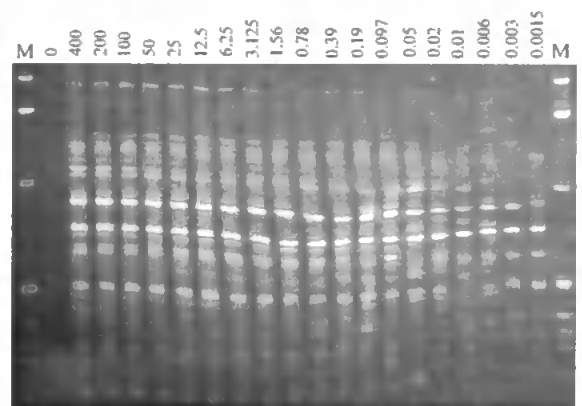
Daphnia magna, primer OPB7. For more details refer to Figure 4.8.



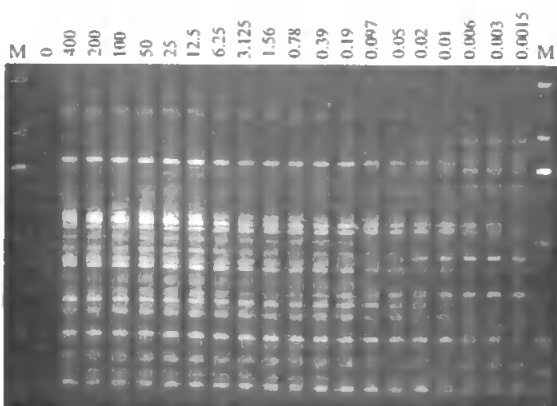
Palmaria palmata, primer OPB5. For more details refer to legend of Figure 4.8.



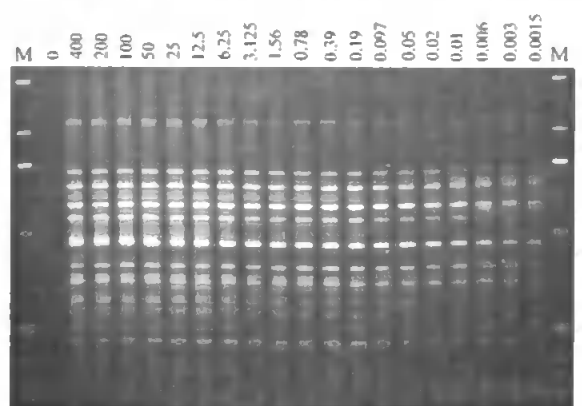
Human placenta, primer OPB5. For more details refer to Figure 4.8.



Human placenta, primer OPB8. For more details refer to Figure 4.8.



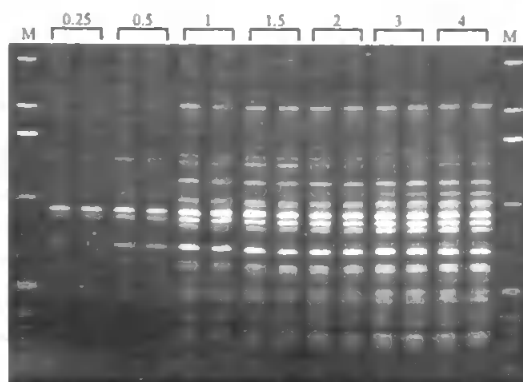
Escherichia coli, primer OPA9. For more details refer to Figure 4.8.



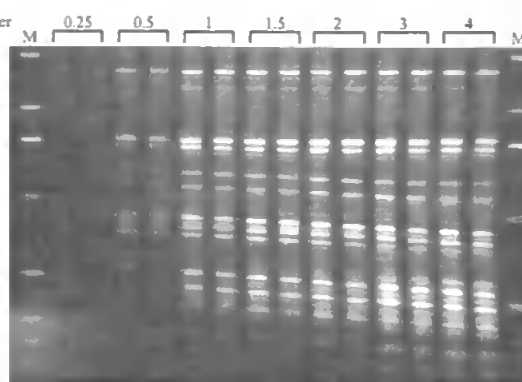
Escherichia coli, primer OPB7. For more details refer to Figure 4.8.

CHAPTER 4

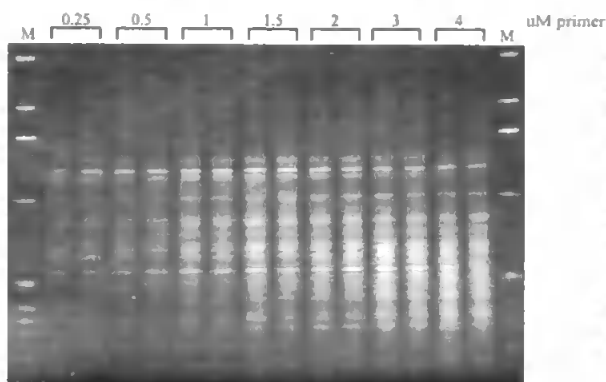
Effect of primer concentration on RAPD profiles



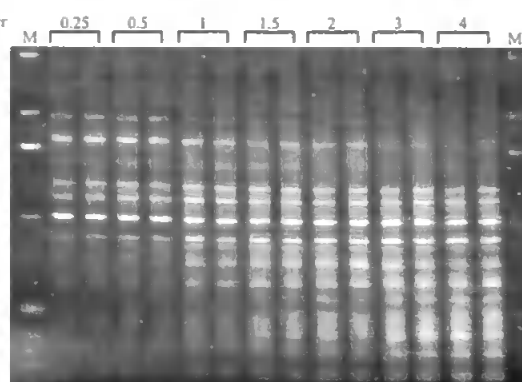
Daphnia magna, primer OPB5. For more details refer to legend of Figure 4.9.



Daphnia magna, primer OPB8. For more details refer to legend of Figure 4.9.

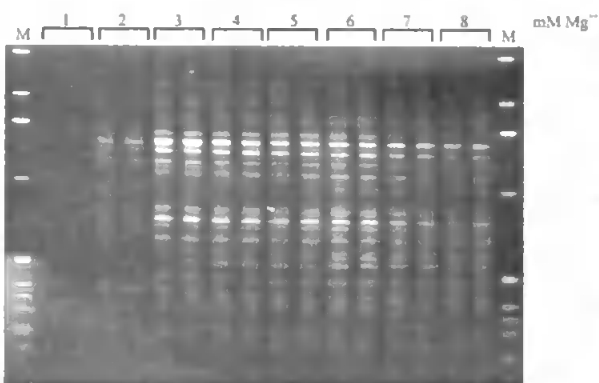


Human placenta, primer OPB5. For more details refer to legend of Figure 4.9.

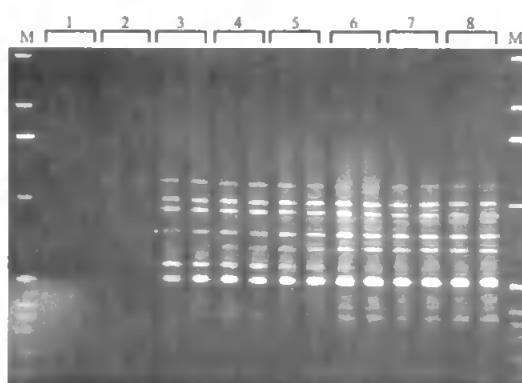


Palmaria palmata, primer OPB5. For more details refer to legend of Figure 4.9.

Effect of magnesium concentration on RAPD profiles

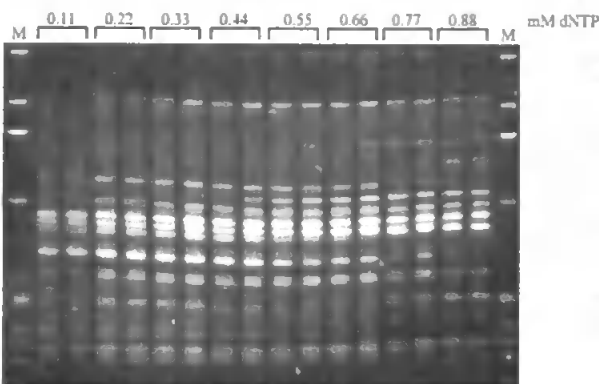


Daphnia magna, primer OPA9. For more details refer to legend of Figure 4.10.

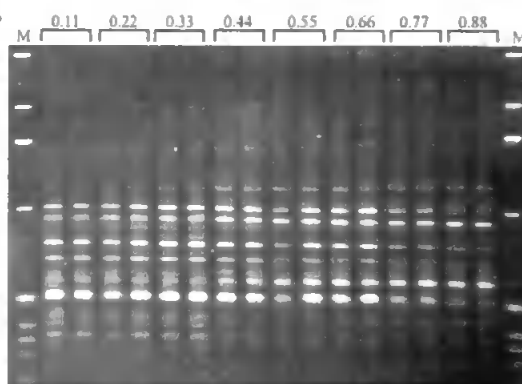


Enteromorpha intestinalis, primer OPA9. For more details refer to legend of Figure 4.10.

Effect of dNTP concentration on RAPD profiles



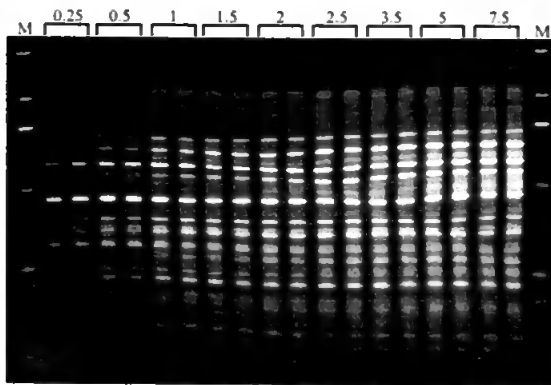
Daphnia magna, primer OPB5. For more details refer to legend of Figure 4.11.



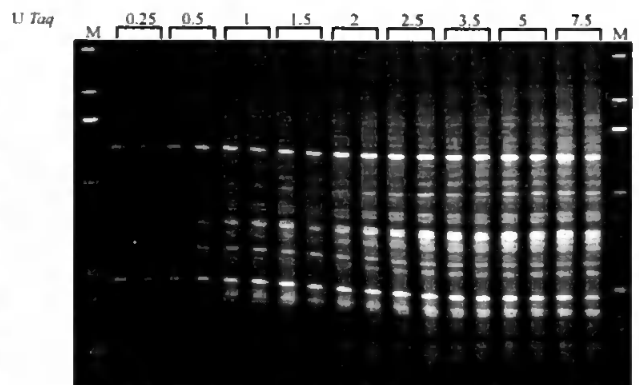
Enteromorpha intestinalis, primer OPA9. For more details refer to legend of Figure 4.11.

CHAPTER 4

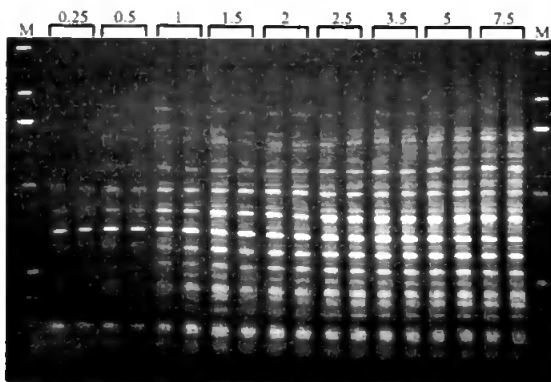
Effect of *Thermus aquaticus* DNA polymerase concentration on RAPD profiles



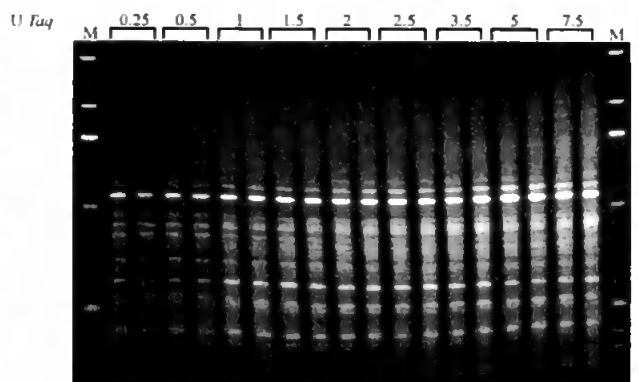
Escherichia coli, primer OPB7. For more details refer to legend of Figure 4.12.



Palmaria palmata, primer OPB8. For more details refer to legend of Figure 4.12.

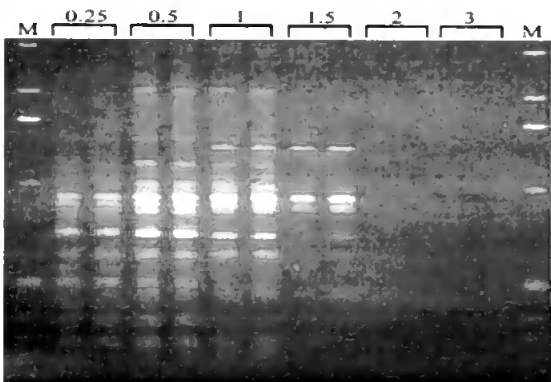


Calf thymus, primer OPB8. For more details refer to legend of Figure 4.12.

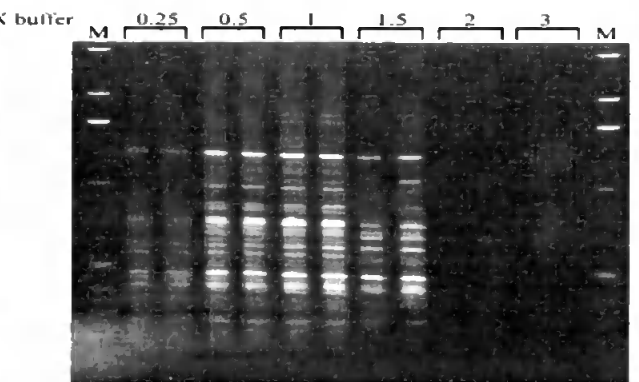


Calf thymus, primer OPB7. For more details refer to legend of Figure 4.12.

Effect of buffer concentration on RAPD profiles

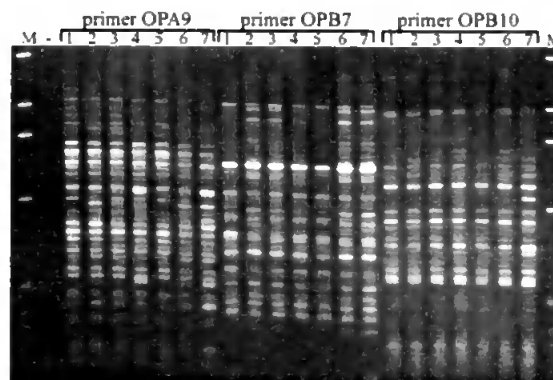


Daphnia magna, primer OPB5. For more details refer to legend of Figure 4.13.



Palmaria palmata, primer OPB8. For more details refer to legend of Figure 4.13.

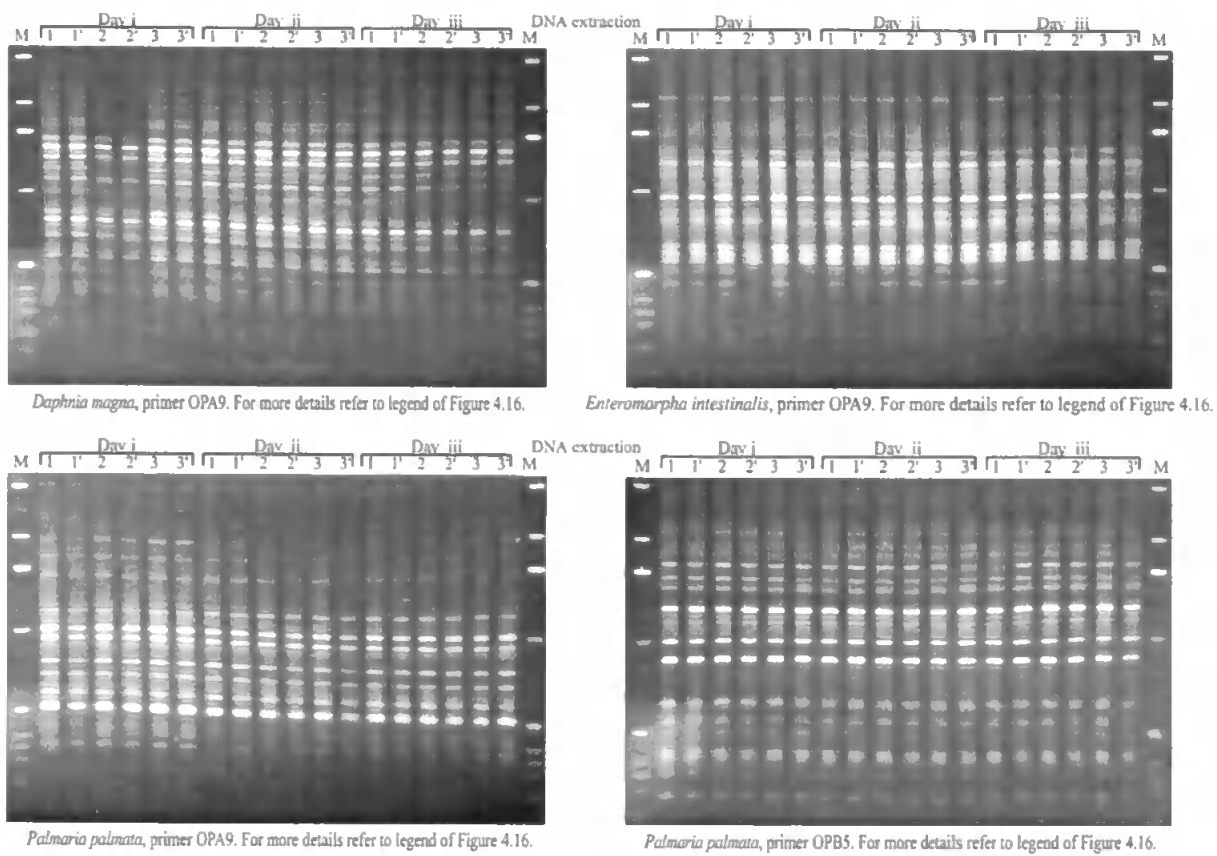
Reproducibility of RAPD profiles produced by individual *Daphnia magna*



Each number represents an individual *Daphnia magna*.
PCR performed under the optimised conditions.

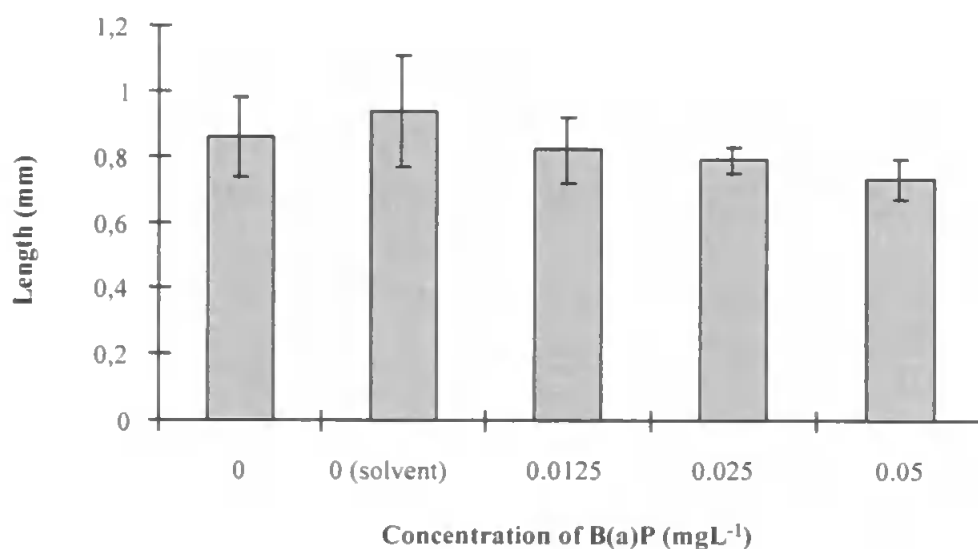
CHAPTER 4

Effect of time of DNA extraction and PCR on RAPD profiles



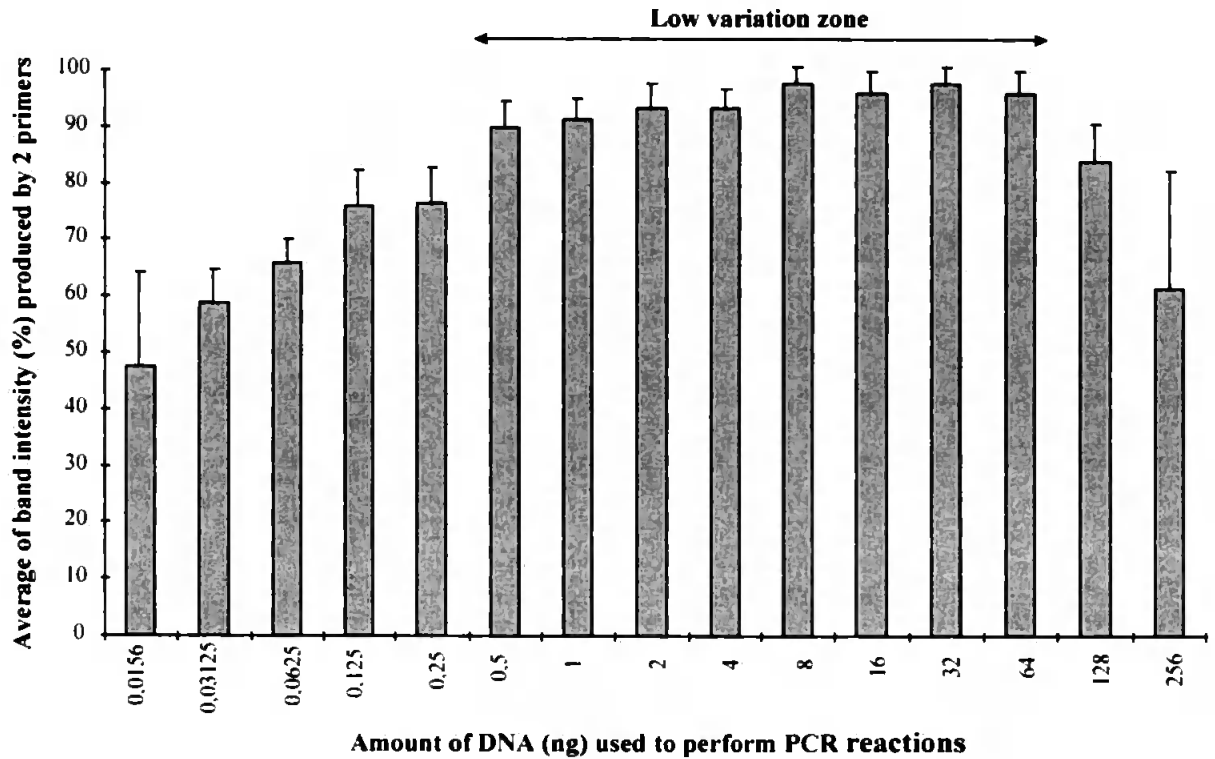
CHAPTER 5

Variation in growth of the first generation of neonates produced by populations of *Daphnia magna* exposed to B(a)P



CHAPTER 5

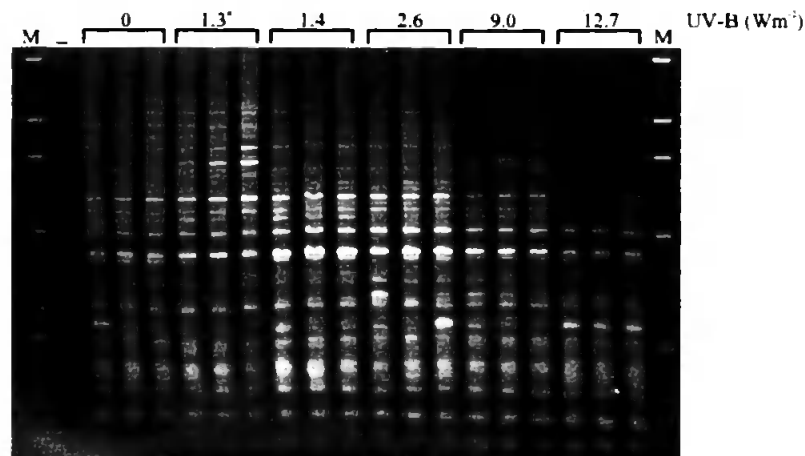
Effect of DNA concentration on band intensity



This experiment was carried using *Daphnia magna* DNA and 10-mer primers OPA9 and OPB7. Seven and four bands appearing across the width of the gels were selected for primer OPA9 and OPB7, respectively, and the average and standard deviation were calculated. This experiment is similar to the one presented in Figure 6.4 and 6.5. The data (see above) shows that band intensity varies little when the amount of DNA used to perform RAPD reactions is comprised between 0.5 and 64 ng.

CHAPTER 6

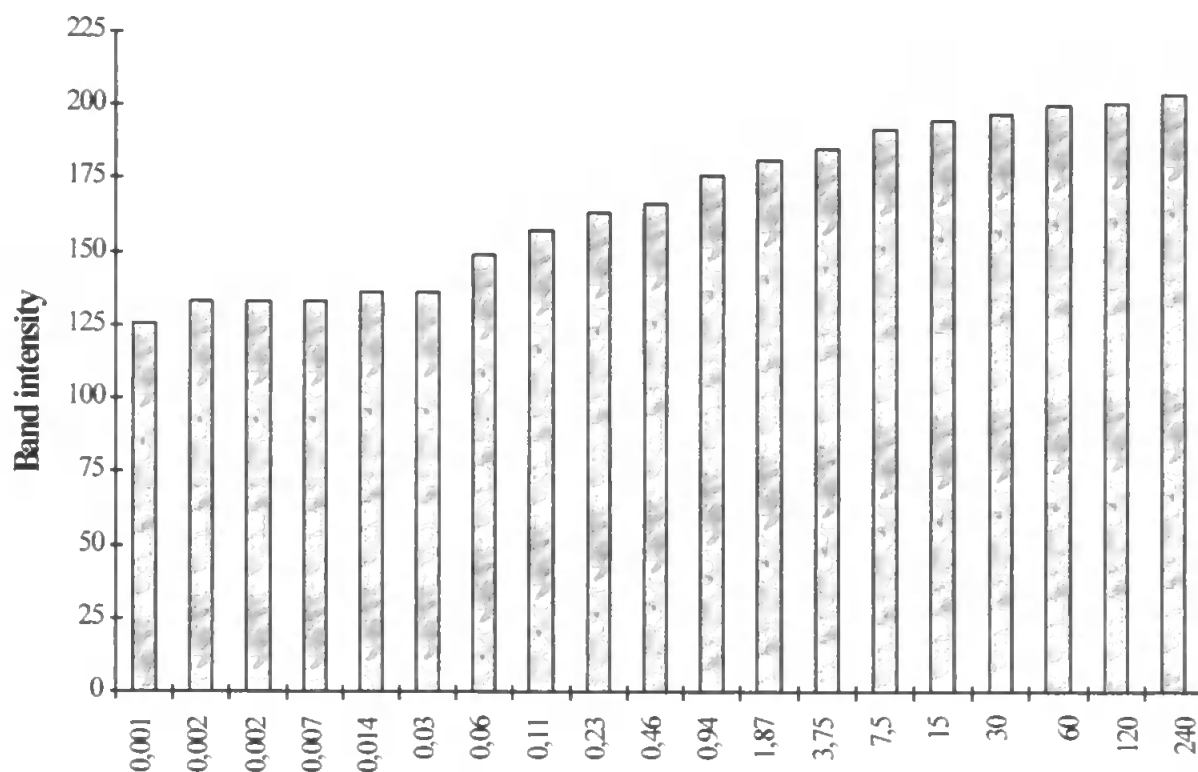
RAPD profiles of *Palmaria palmata* exposed to UV radiation



Palmaria palmata, primer OPB5. For more details refer to legend of Figure 6.2.

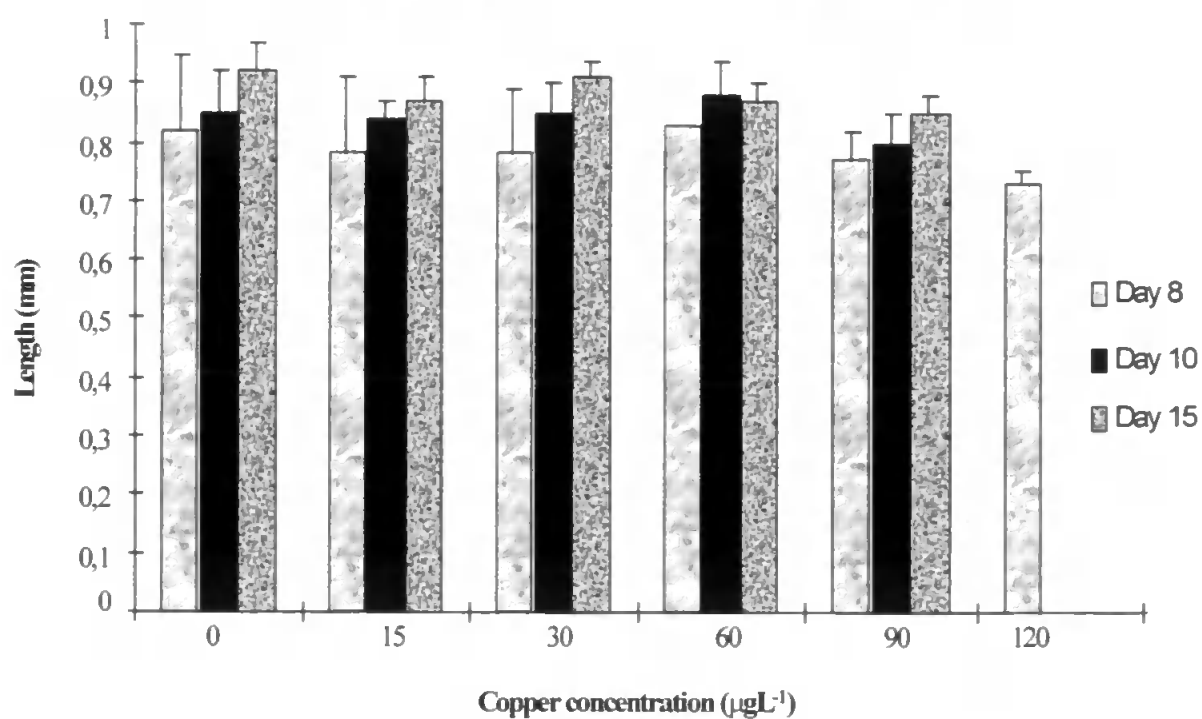
CHAPTER 6

Unusual effect of DNA concentration on band intensity (band B1-2 in Figure 6.4)



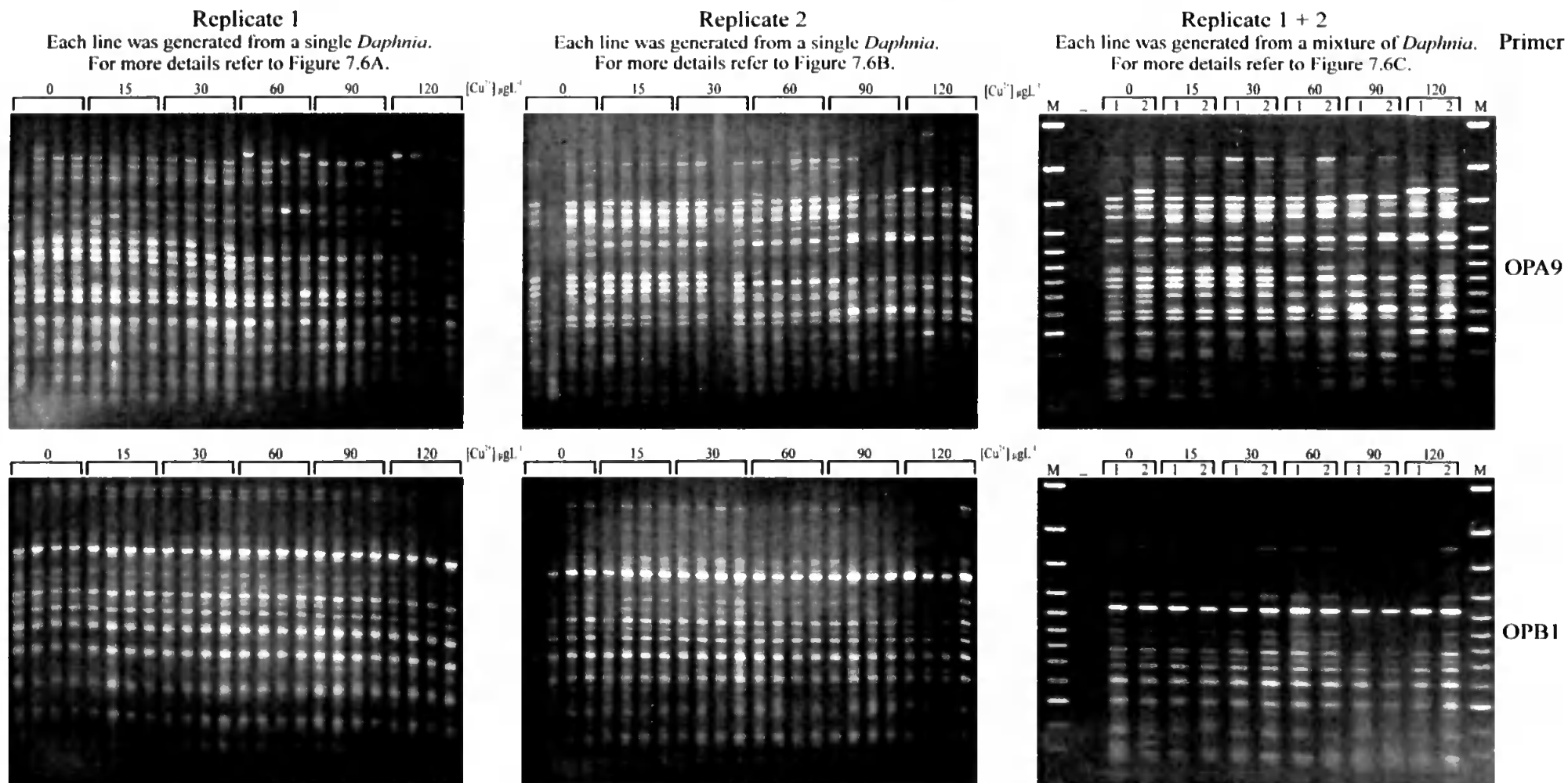
CHAPTER 7

Length of neonates (less than 24 h) produced by populations of *Daphnia magna* exposed to copper at day 8, 10, and 15



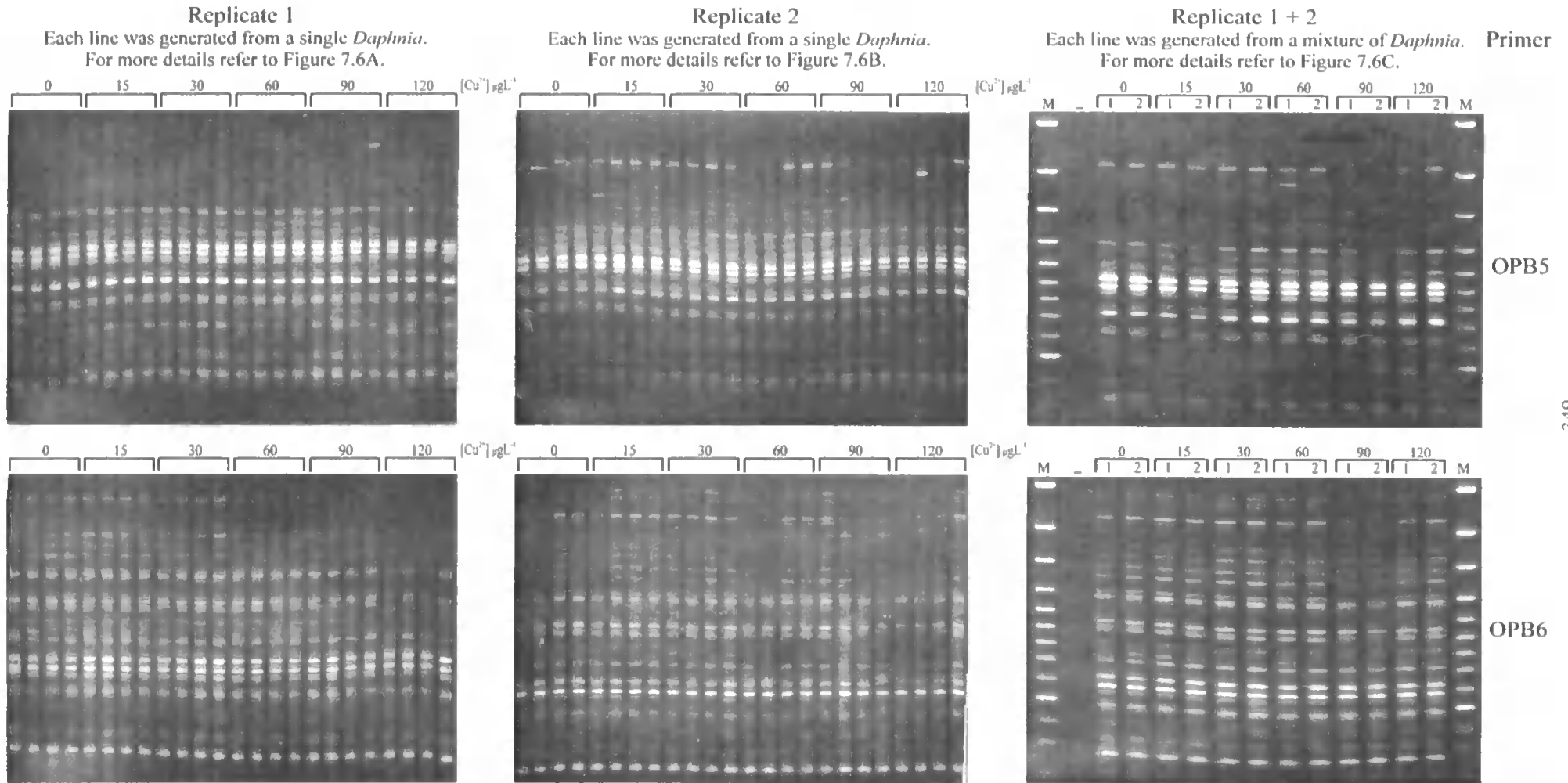
CHAPTER 7

RAPD profiles of individual and mixture of *Daphnia magna* exposed to copper



CHAPTER 7

RAPD profiles of individual and mixture of *Daphnia magna* exposed to copper



CHAPTER 7

RAPD profiles of individual and mixture of *Daphnia magna* exposed to copper

Replicate 1

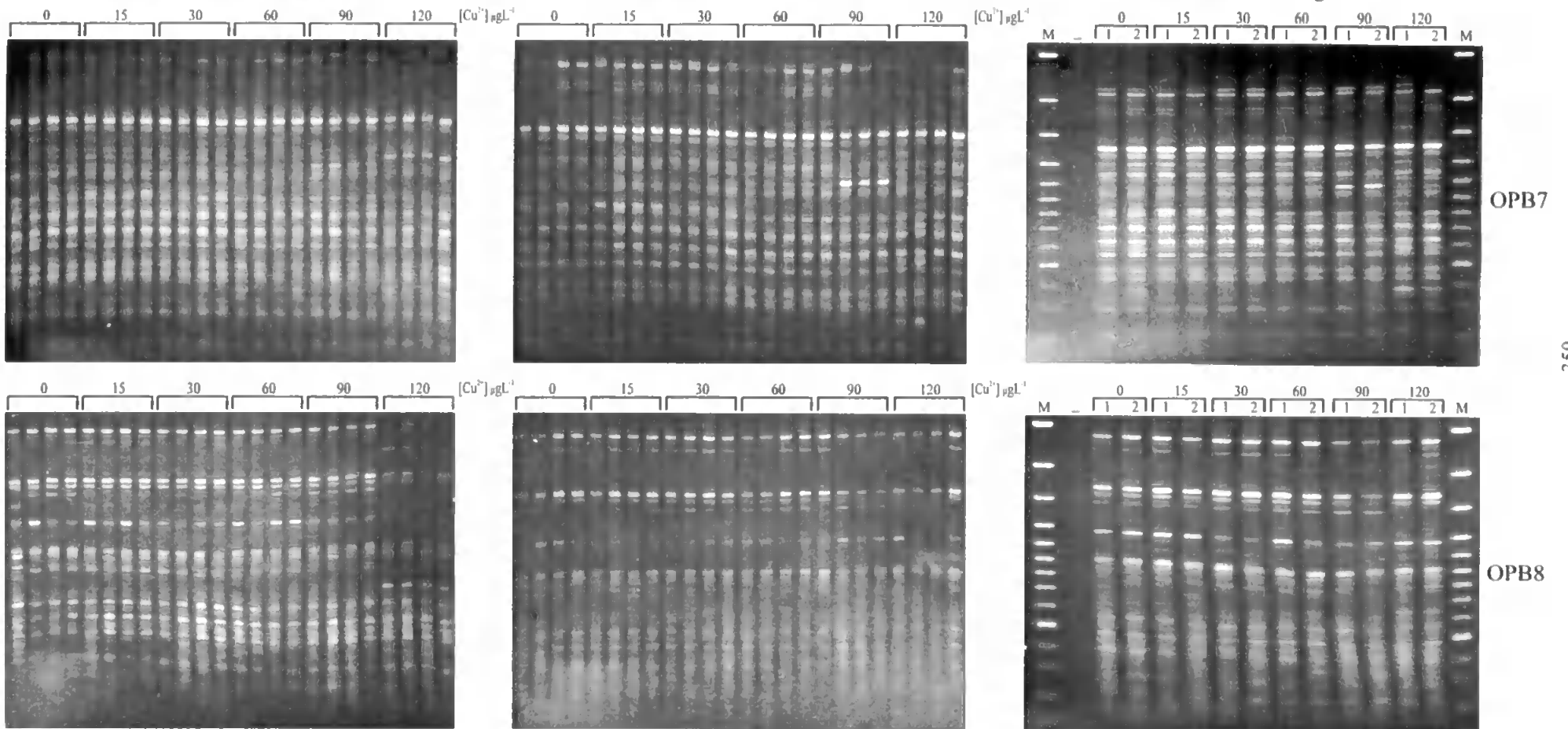
Each line was generated from a single *Daphnia*.
For more details refer to Figure 7.6A.

Replicate 2

Each line was generated from a single *Daphnia*.
For more details refer to Figure 7.6B.

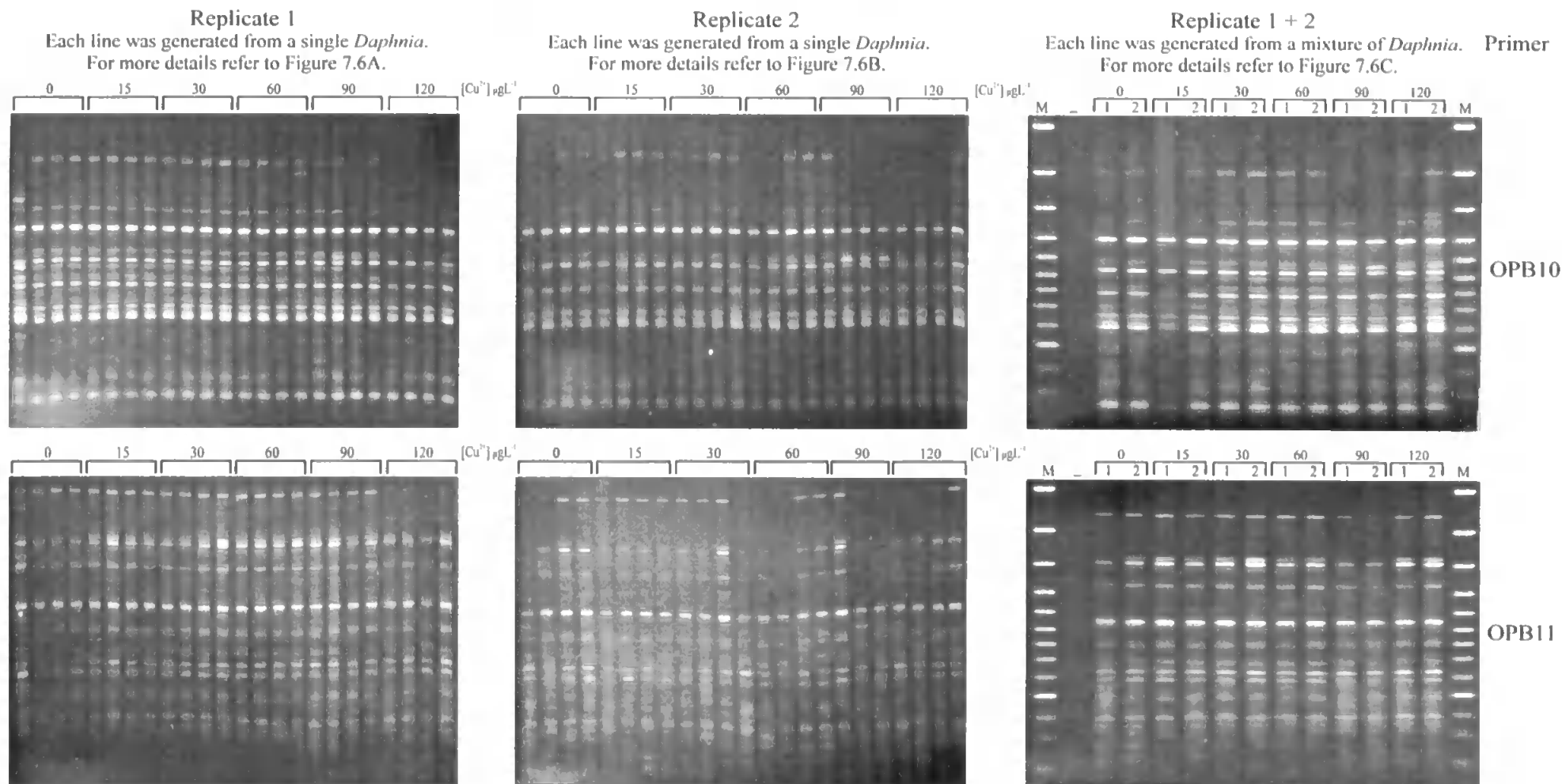
Replicate 1 + 2

Each line was generated from a mixture of *Daphnia*. Primer
For more details refer to Figure 7.6C.



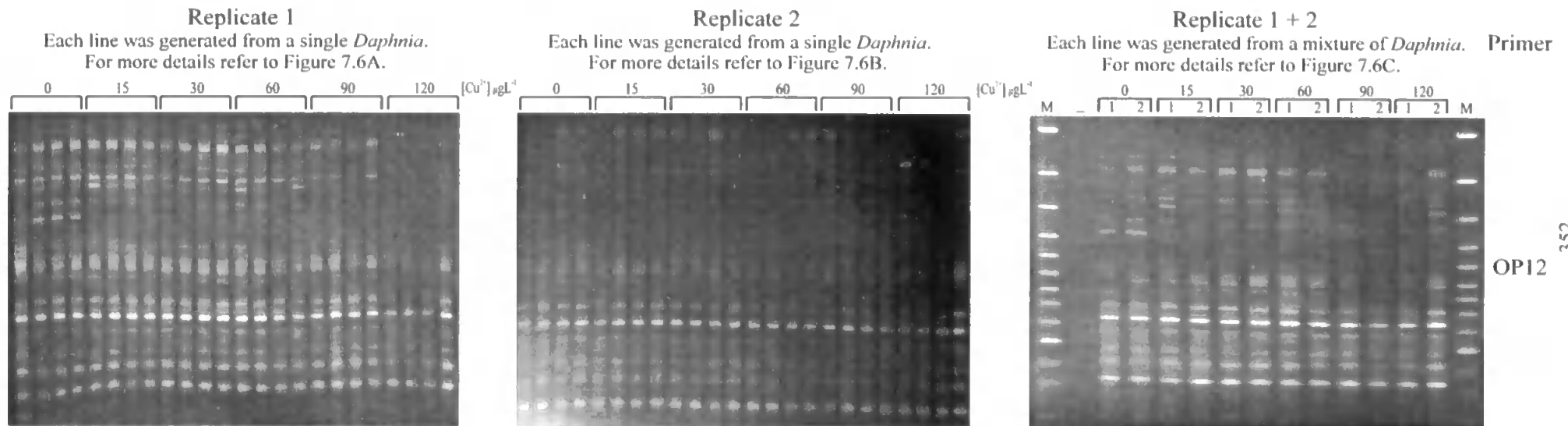
CHAPTER 7

RAPD profiles of individual and mixture of *Daphnia magna* exposed to copper



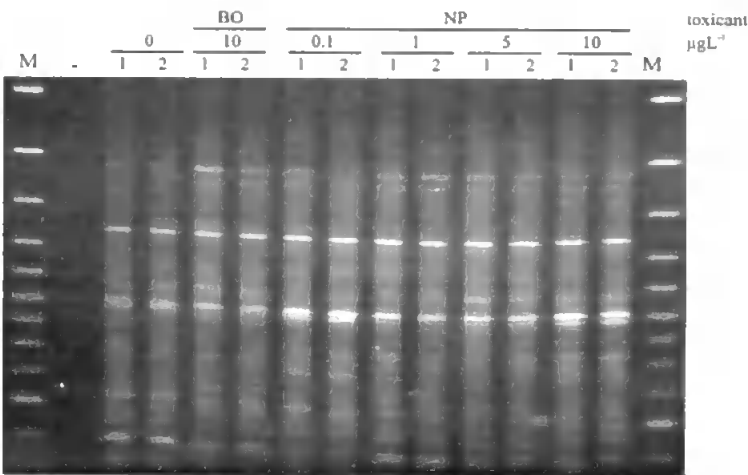
CHAPTER 7

RAPD profiles of individual and mixture of *Daphnia magna* exposed to copper



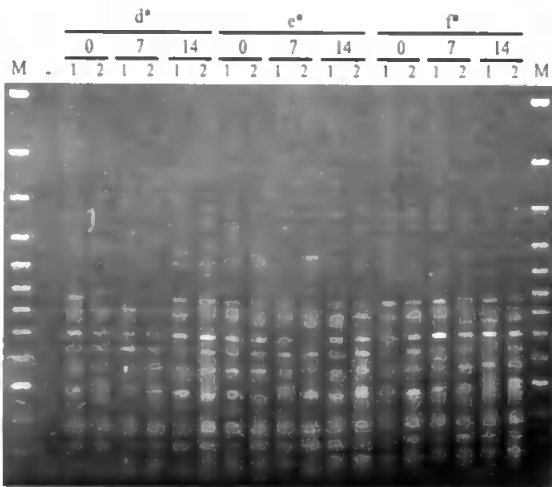
CHAPTER 8

RAPD profiles of barnacles larvae exposed to 17-β oestradiol (BO) and 4-*n*-nonylphenol (NP)

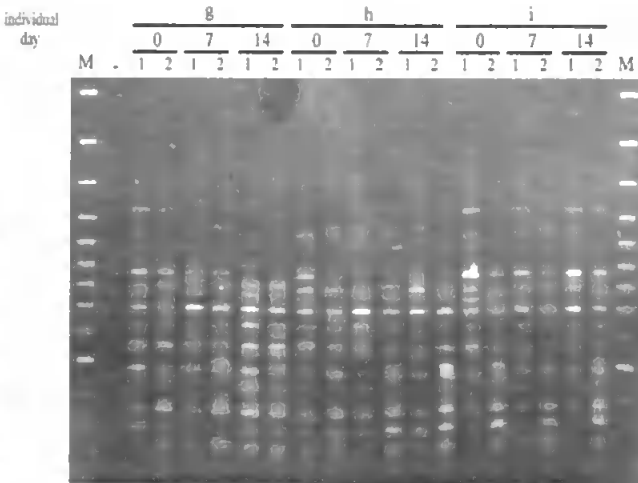


Profiles generated with primer OPB5. For more details refer to the legend of Figure 8.2C.

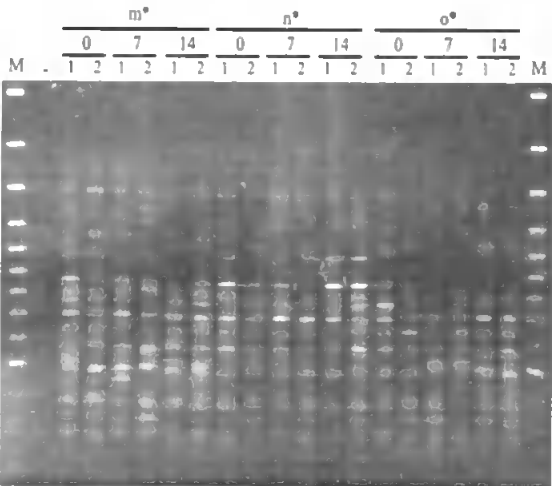
Reproducibility of RAPD profiles (primer OPA9) of control crabs and crabs exposed to 100 μg/L⁻¹ 4-*n*-nonylphenol or diethylstilbestrol using two DNA template concentrations



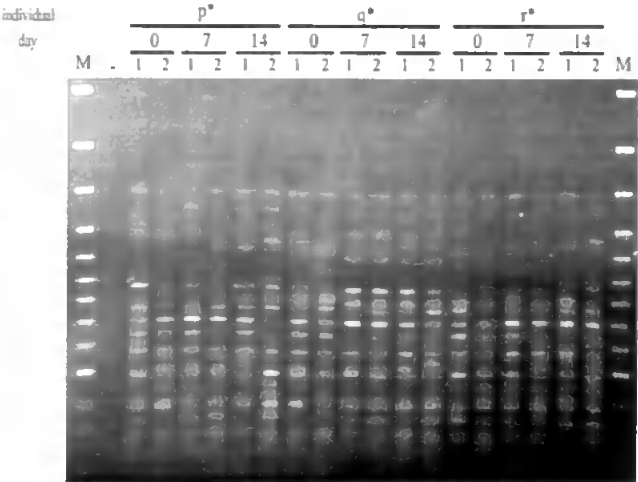
Control females. For more details refer to legend of Figure 8.3C.



Males exposed to 4-*n*-nonylphenol. For more details refer to legend of Figure 8.3C.



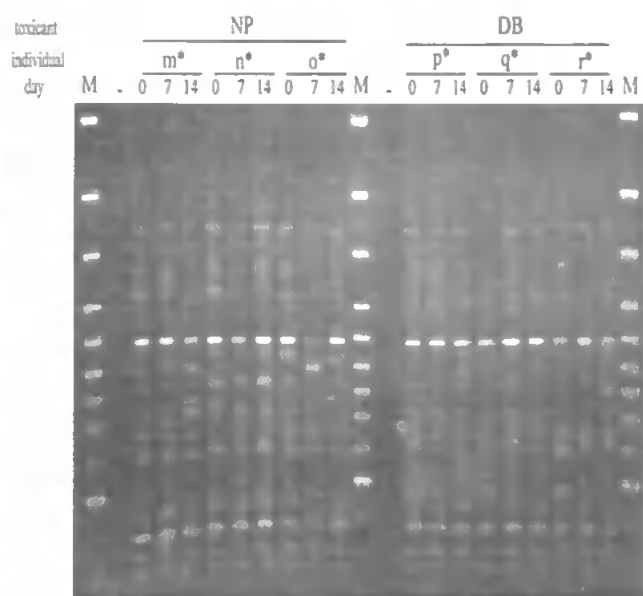
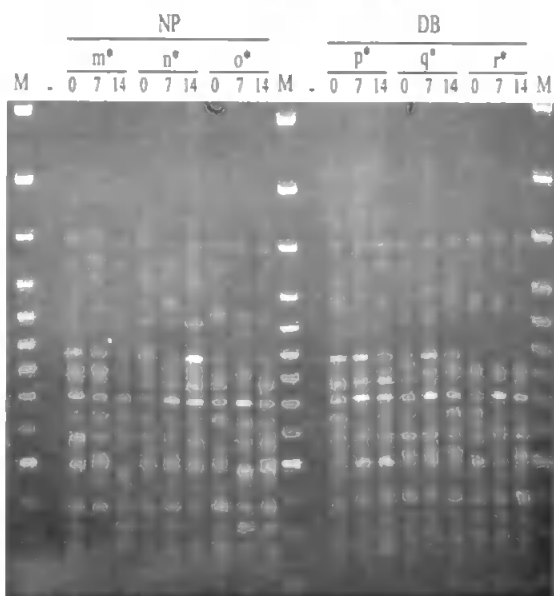
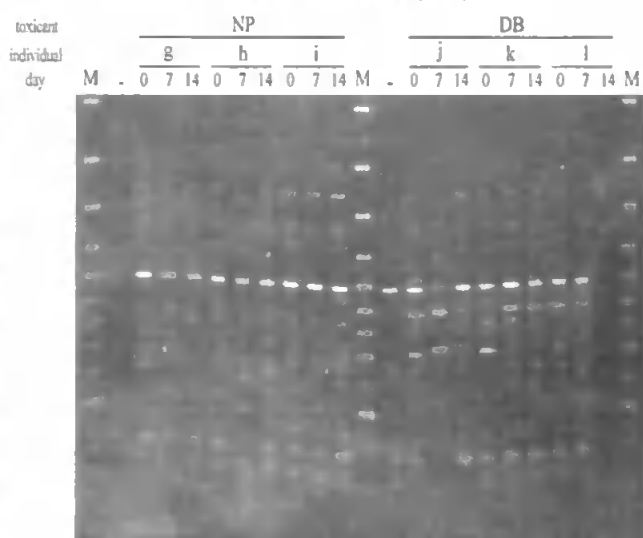
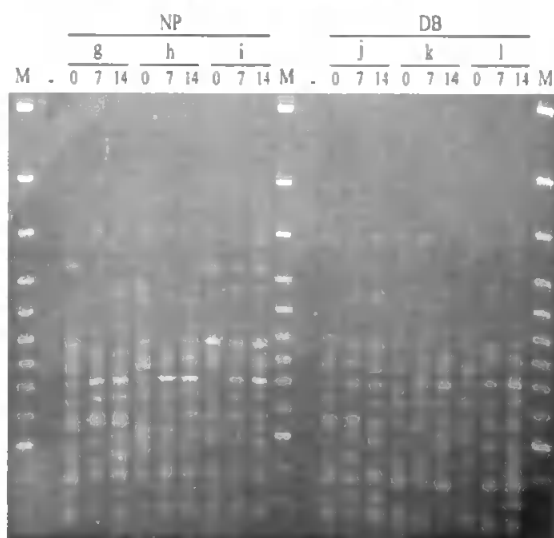
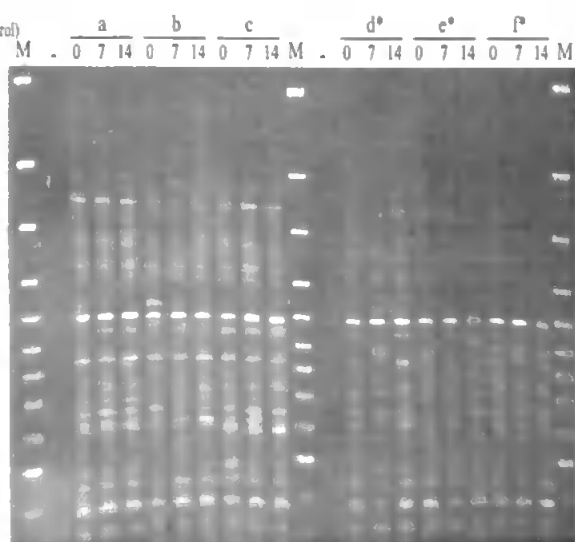
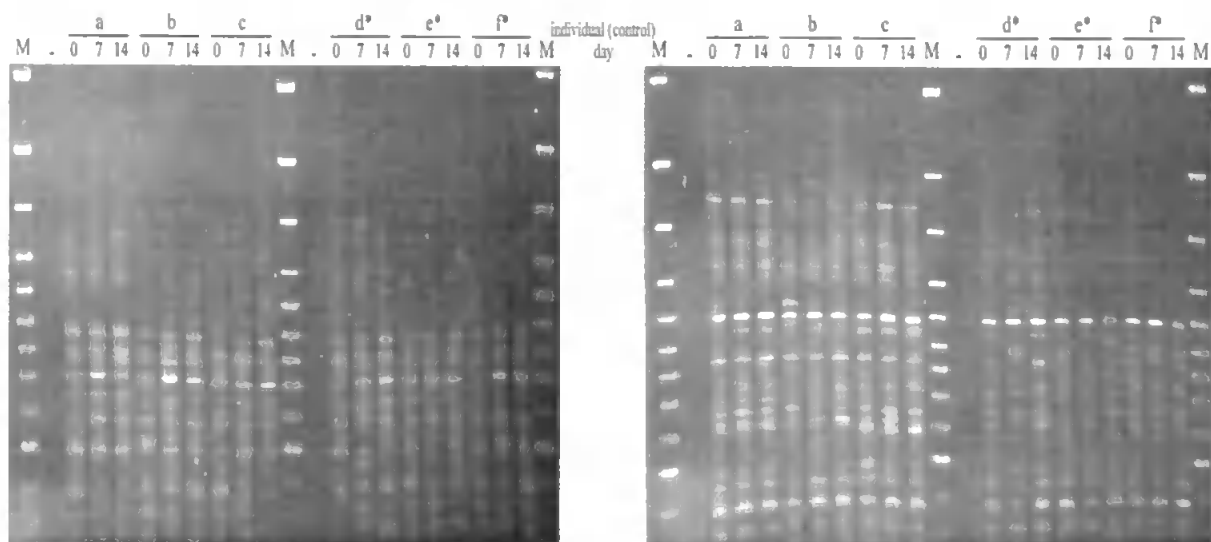
Females exposed to 4-*n*-nonylphenol. For more details refer to legend of Figure 8.3C.



Females exposed to diethylstilbestrol. For more details refer to legend of Figure 8.3C.

CHAPTER 8

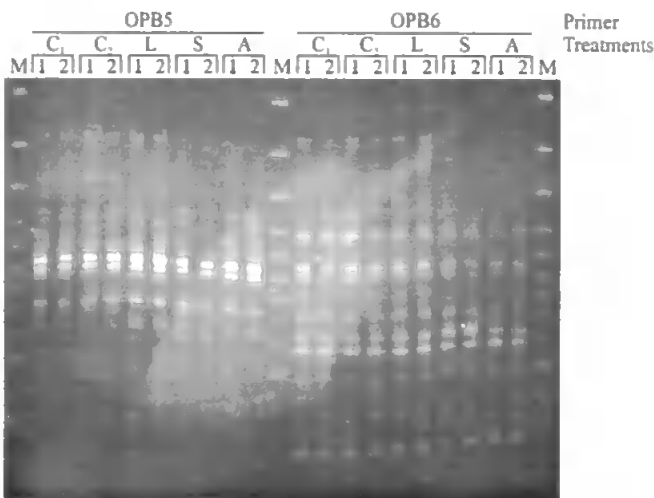
RAPD profiles of control crabs and crabs exposed to 100 μgL^{-1} of 4-*n*-nonylphenol (NP) and diethylstilbestrol (DB)



Primer OPA9. For more details refer to legend of Figure 8.3B.

CHAPTER 8

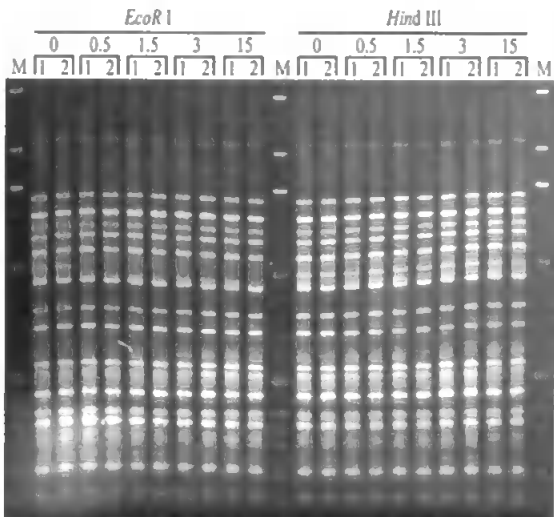
RAPD profiles of *Daphnia magna* exposed to different treatments



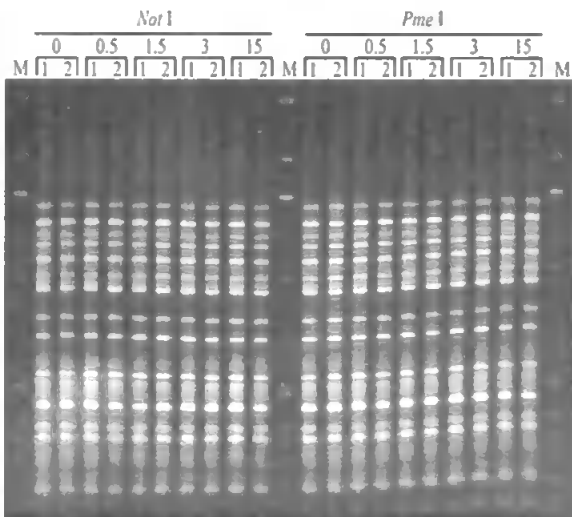
For more details refer to Figure 8.4.

CHAPTER 9

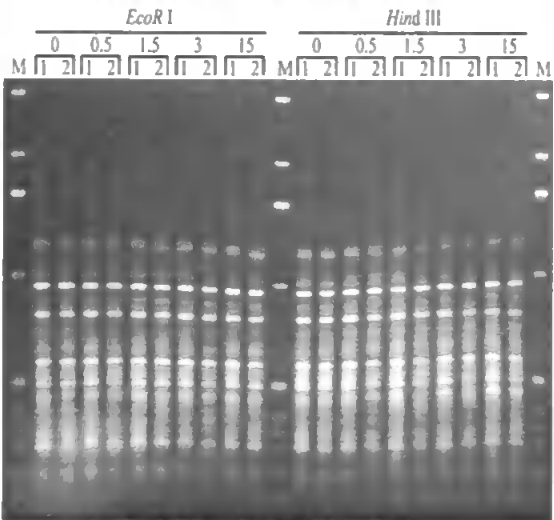
Effect of restricted *Escherichia coli* and calf thymus DNA on RAPD profiles



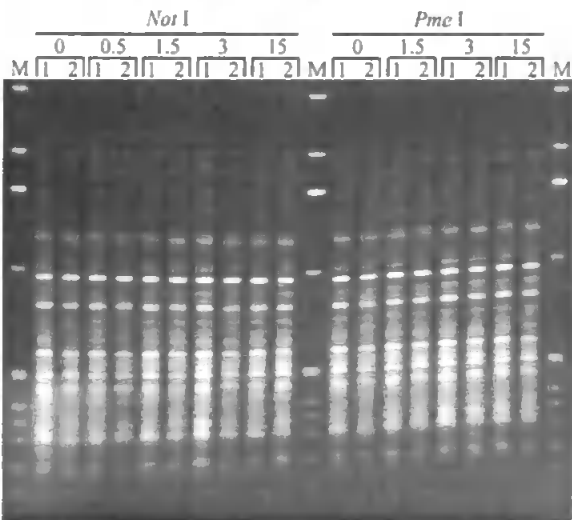
Escherichia coli, primer OPB7. For more details refer to legend of Figure 9.1B.



Escherichia coli, primer OPB7. For more details refer to legend of Figure 9.1C.



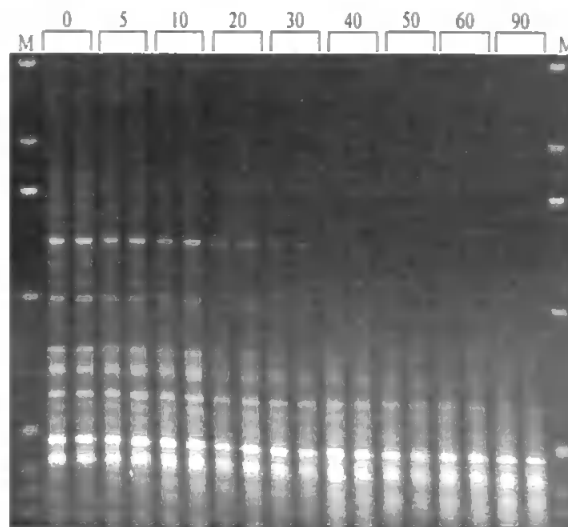
Calf thymus, primer OPB1. For more details refer to legend of Figure 9.2B.



Calf thymus, primer OPB1. For more details refer to legend of Figure 9.2C.

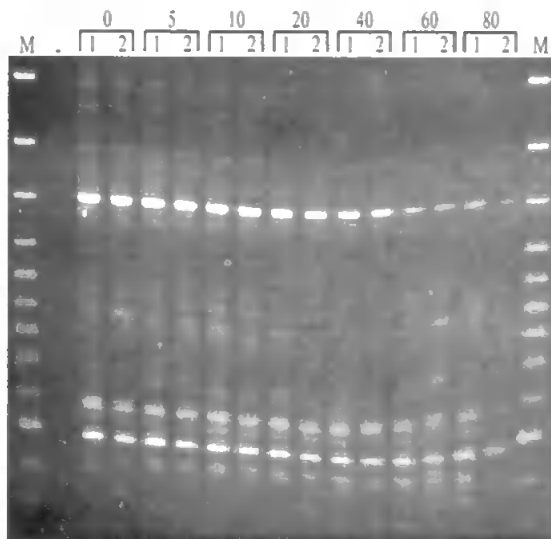
CHAPTER 9

Effect of sonicated genomic DNA on RAPD profiles

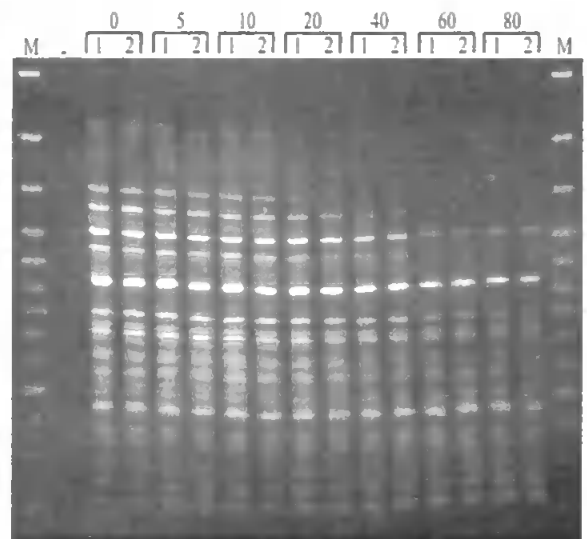


Palmaria palmata, primer OPB8. For more details refer to Figure 9.3B.

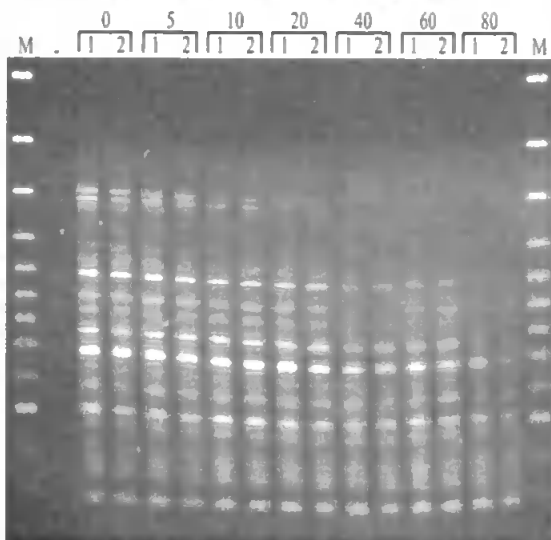
Effect of UV-C irradiated genomic DNA on RAPD profiles



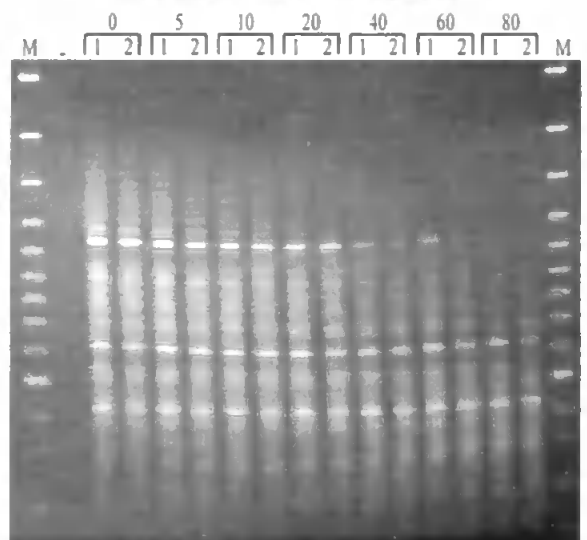
Escherichia coli, primer OPB5. For more details refer to legend of Figure 9.5.



Escherichia coli, primer OPB7. For more details refer to legend of Figure 9.5.



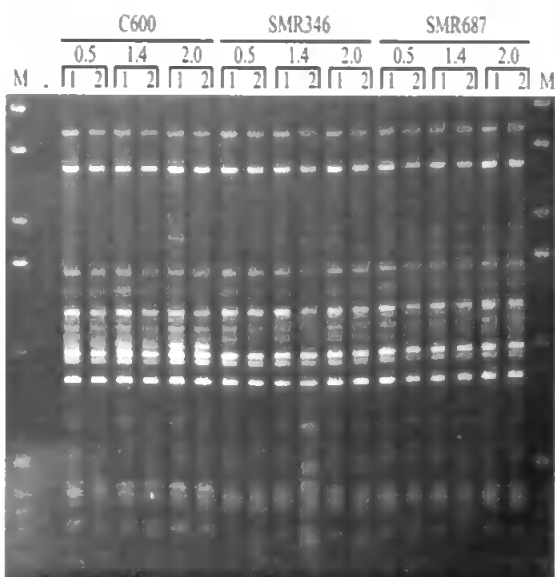
Calf thymus, primer OPB5. For more details refer to legend of Figure 9.5.



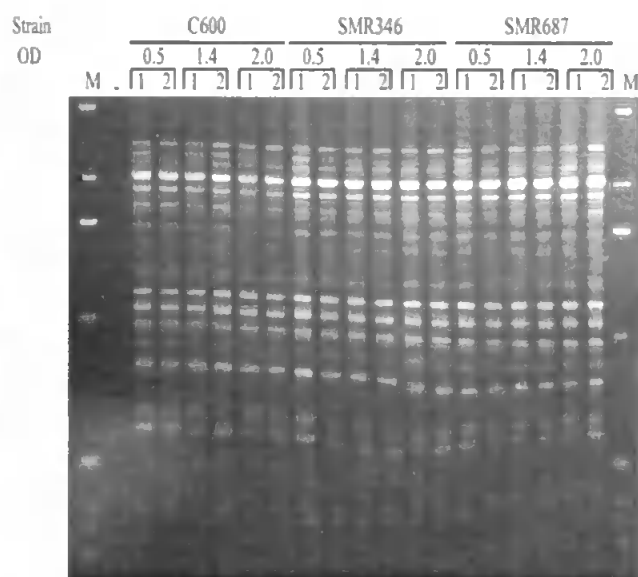
Calf thymus, primer OPB7. For more details refer to legend of Figure 9.5.

CHAPTER 9

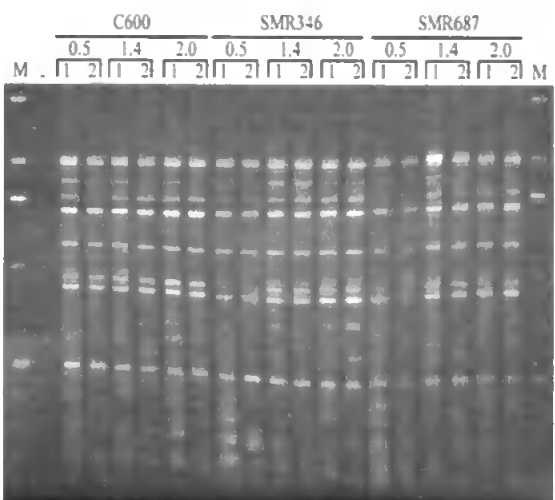
RAPD profiles of *Escherichia coli* control (C600) and mismatch repair defective strains (SMR346 and SMR687) at an approximate OD_{260nm} of 0.5, 1.4, and 2



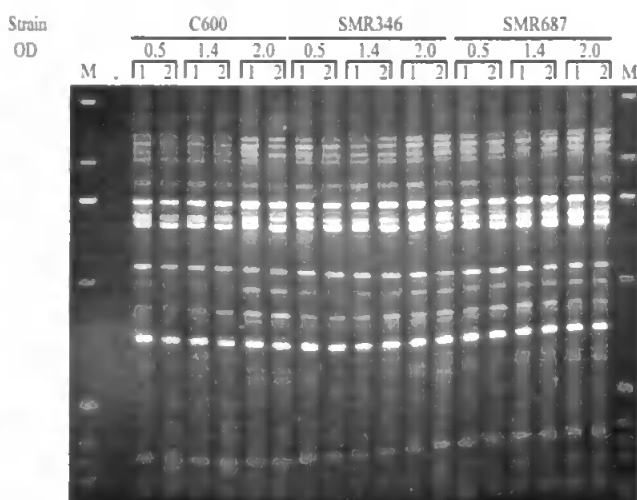
Primer OPB1. For more details refer to legend of Figure 9.7.



Primer OPB6. For more details refer to legend of Figure 9.7.



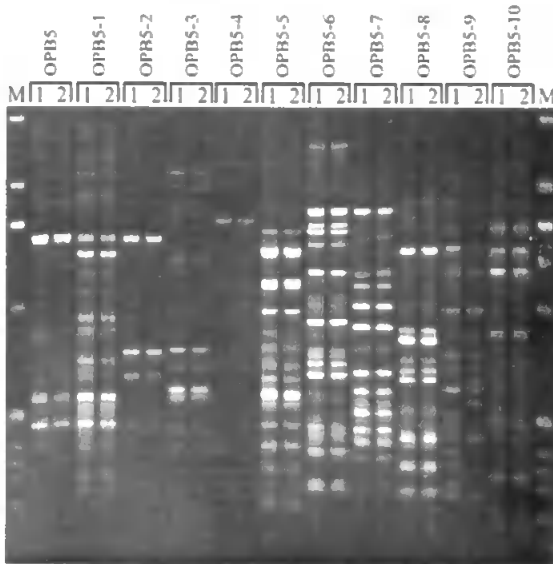
Primer OPB8. For more details refer to legend of Figure 9.7.



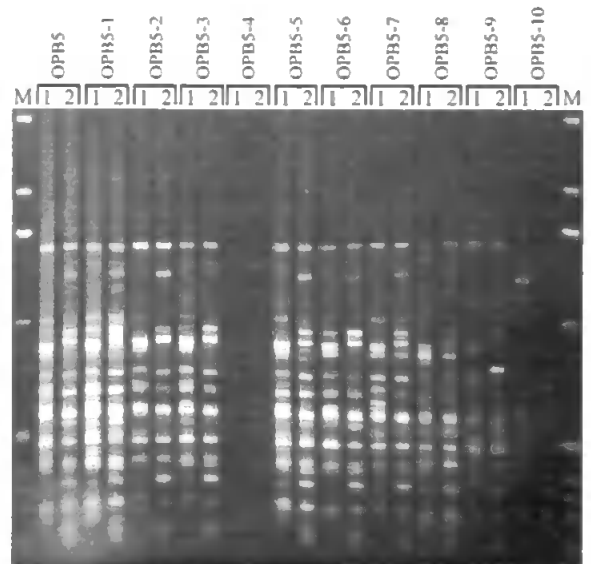
Primer OPB10. For more details refer to legend of Figure 9.7.

CHAPTER 9

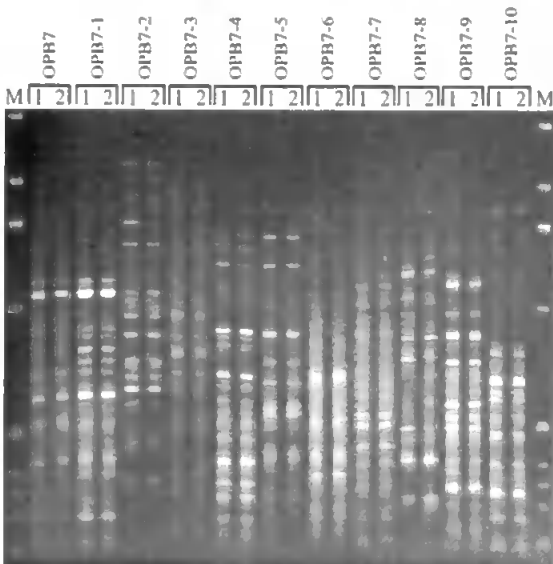
Effect of single nucleotide substitutions in 10-mer primers on RAPD profiles



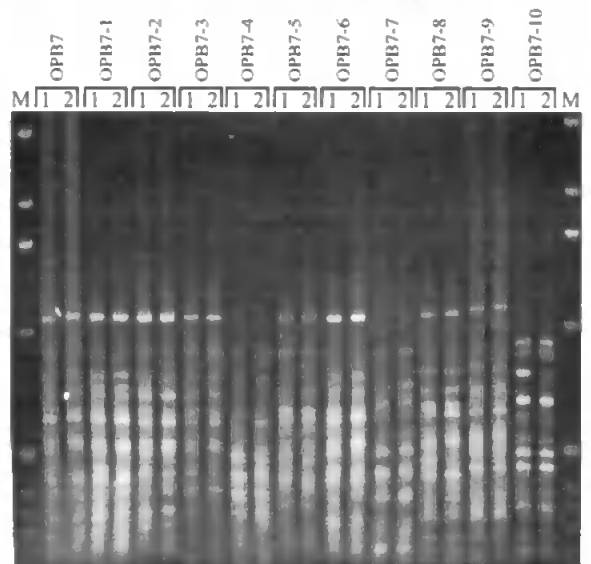
Escherichia coli. For more details refer to legend of Figure 9.8C.



Escherichia coli. For more details refer to legend of Figure 9.8D.



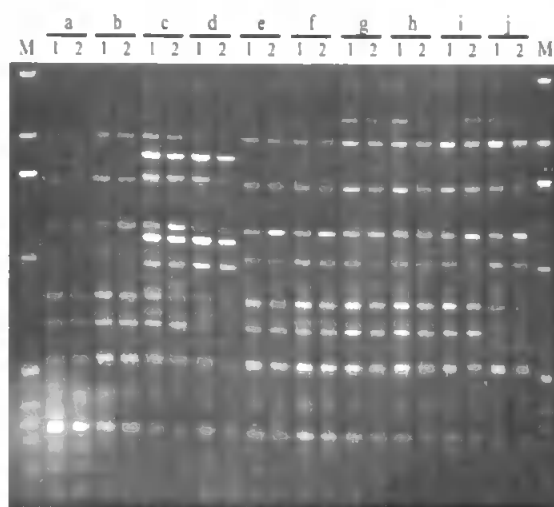
Calf thymus. For more details refer to legend of Figure 9.8A.



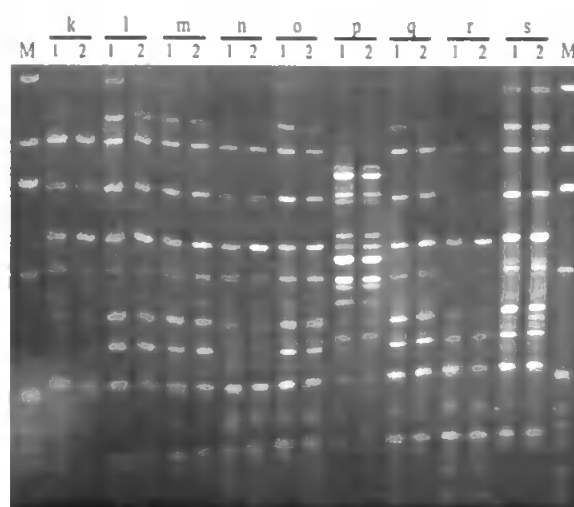
Calf thymus. For more details refer to legend of Figure 9.8B.

CHAPTER 9

RAPD profiles of 19 isolates of *Renibacterium salmoninarum* from a variety of geographic areas and host species



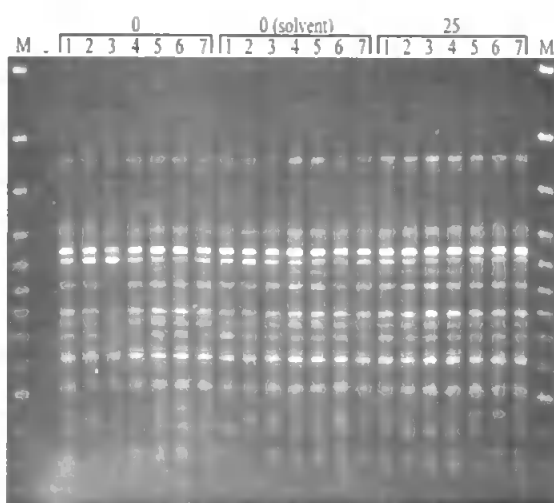
Primer OPB1. For more details refer to legend of Figure 9.9C.



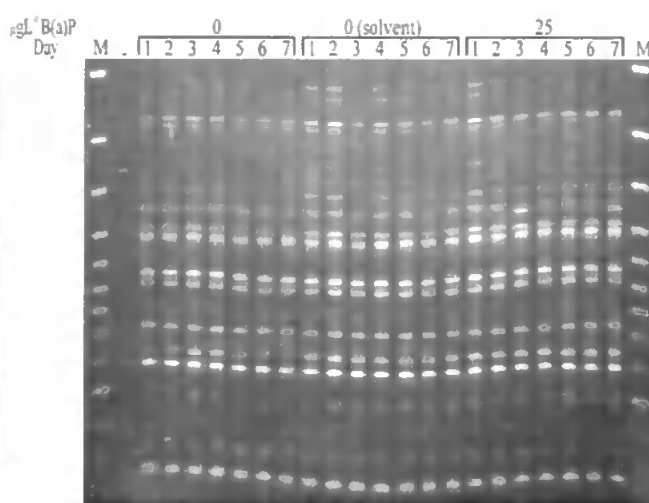
Primer OPB1. For more details refer to legend of Figure 9.9D.

CHAPTER 10

Kinetics of RAPD profiles of *Daphnia magna* exposed to 25 μgL^{-1} B(a)P



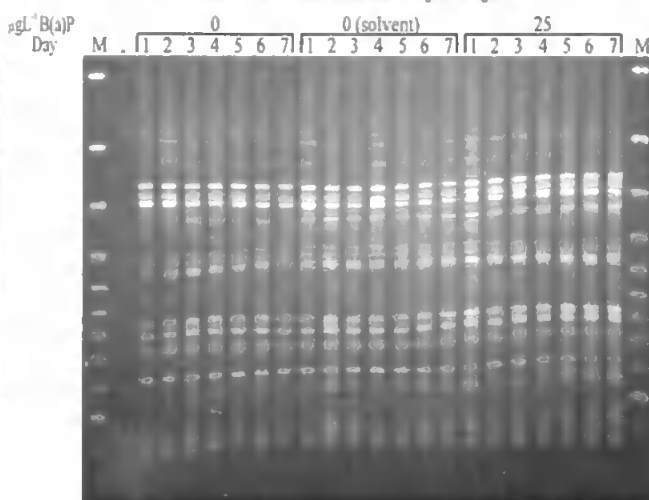
Primer OPB1. For more details refer to legend of Figure 10.3.



Primer OPB6. For more details refer to legend of Figure 10.3.



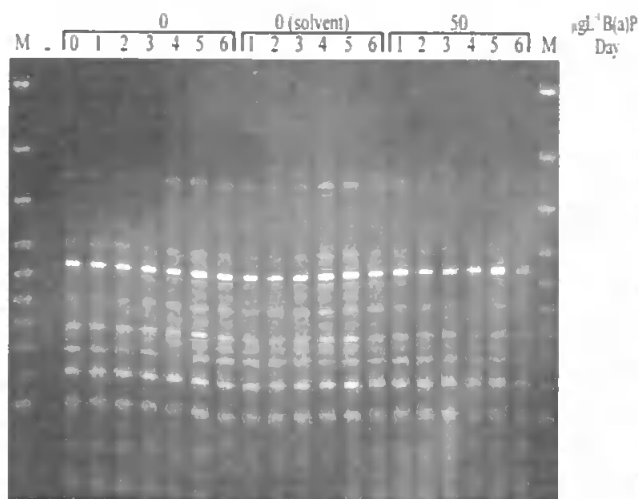
Primer OPB8. For more details refer to legend of Figure 10.3.



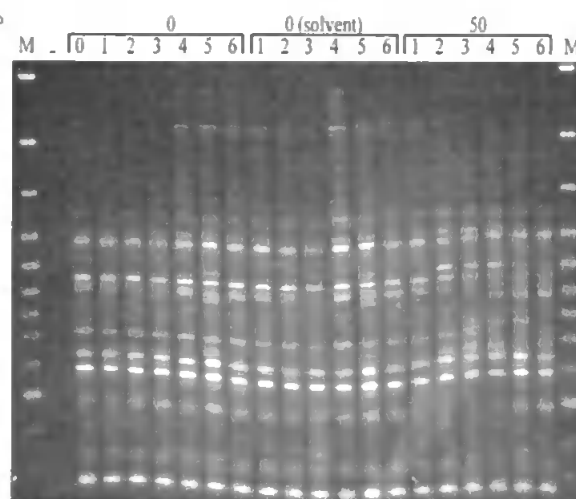
Primer OPB17. For more details refer to legend of Figure 10.3.

CHAPTER 10

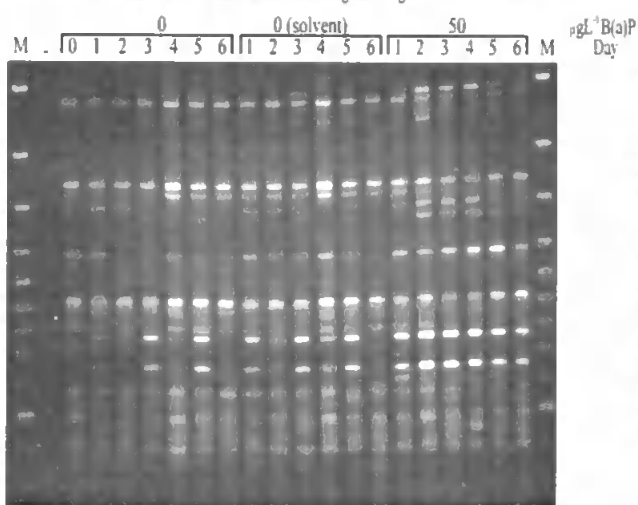
Kinetics of RAPD profiles of *Daphnia magna* exposed to 50 µg L⁻¹ B(a)P



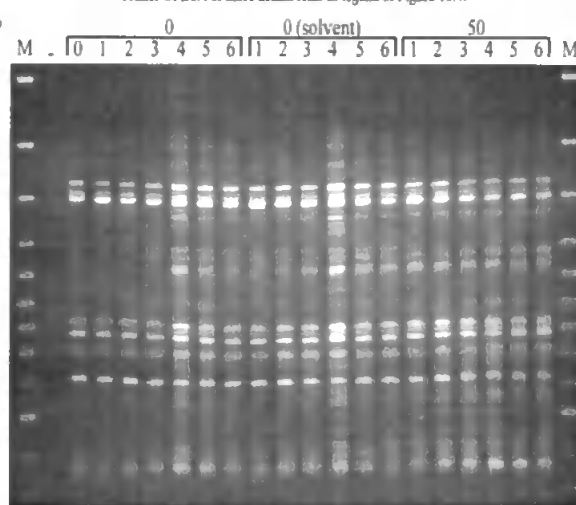
Primer OPB1. For more details refer to legend of Figure 10.4.



Printer OPB6. For more details refer to legend of Figure 10.4.



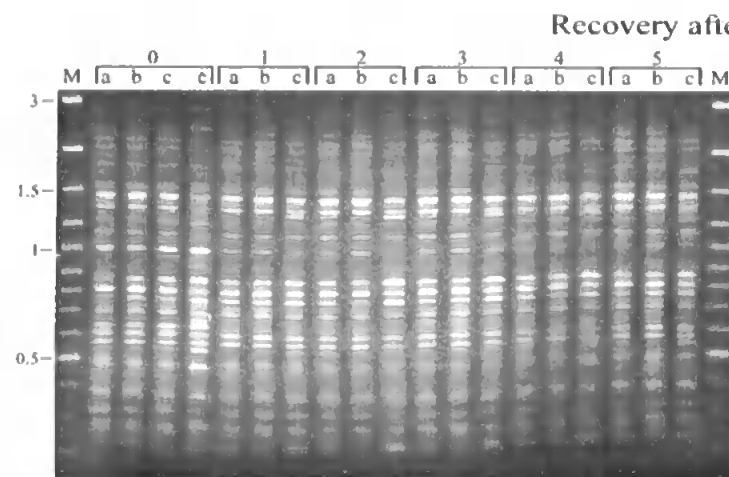
Primer OPB8. For more details refer to legend of Figure 10.4.



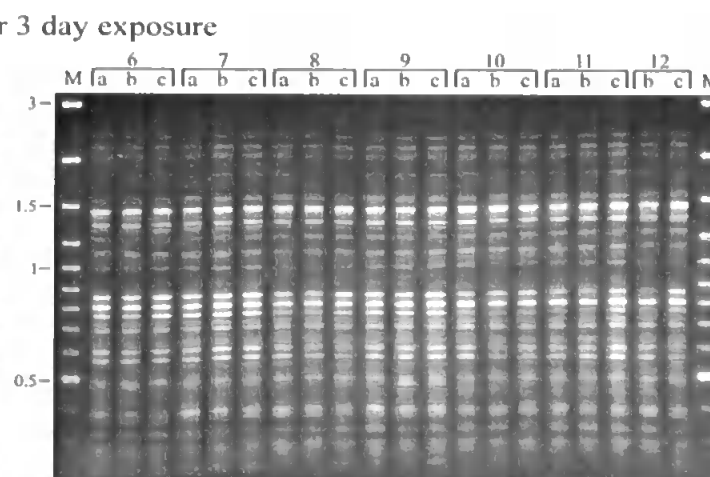
Primer OPB17. For more details refer to legend of Figure 10.4.

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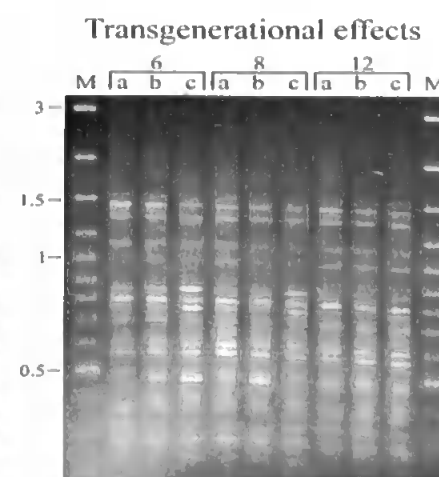
RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P for 3 and 6' days and recovered for 12 and 9' days, respectively, and their offspring, with primer OPA9



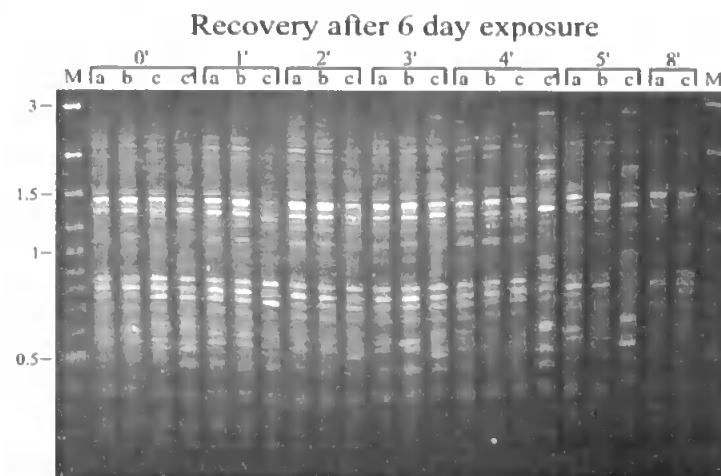
For more details refer to Figure 10.7.



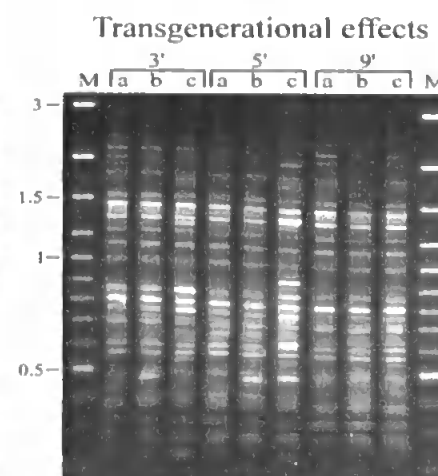
For more details refer to Figure 10.7.



For more details refer to Figure 10.9.



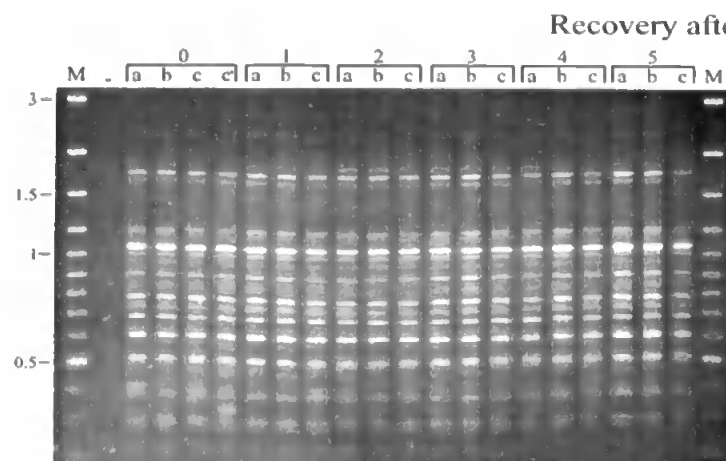
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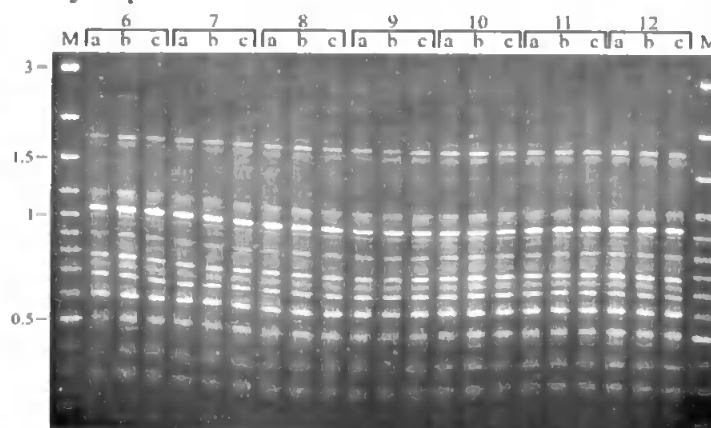
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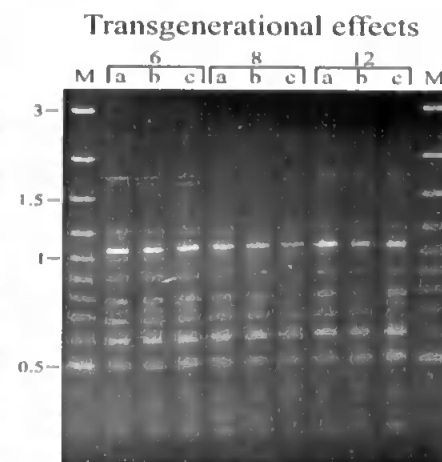
RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P for 3 and 6' days and recovered for 12 and 9' days, respectively, and their offspring, with primer OPB1



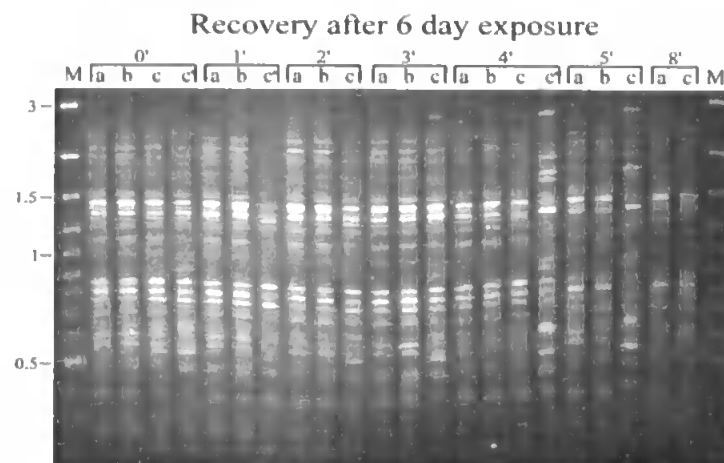
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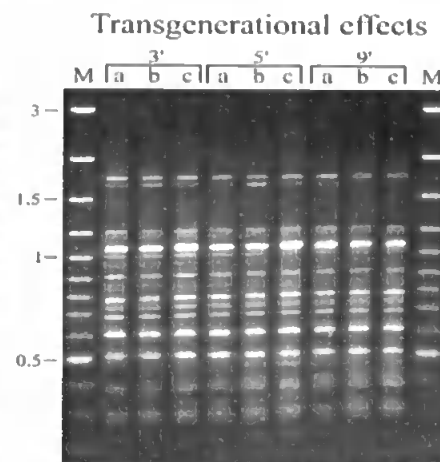
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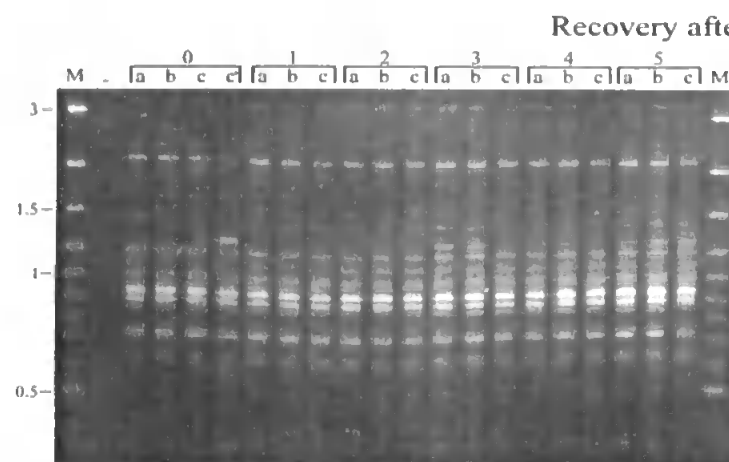
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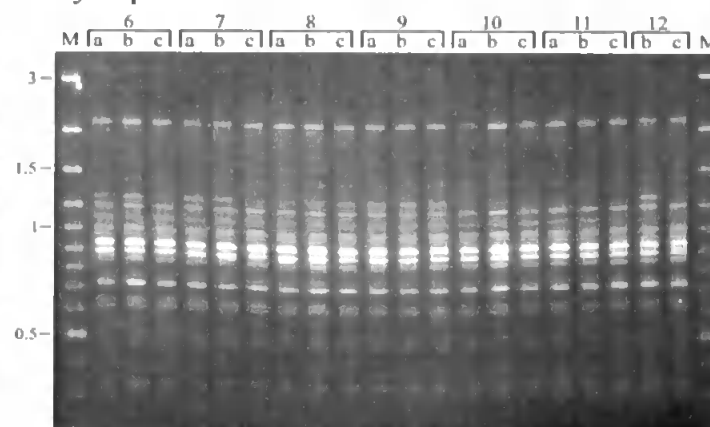
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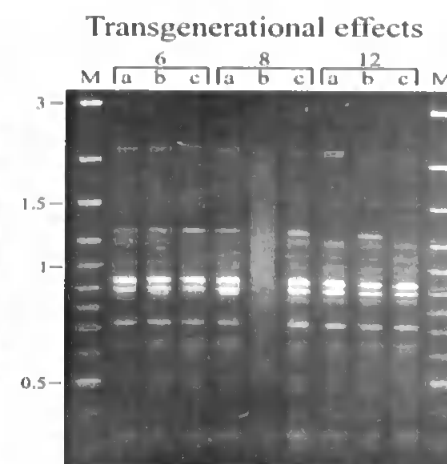
RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P for 3 and 6' days and recovered for 12 and 9' days, respectively, and their offspring, with primer OPB5



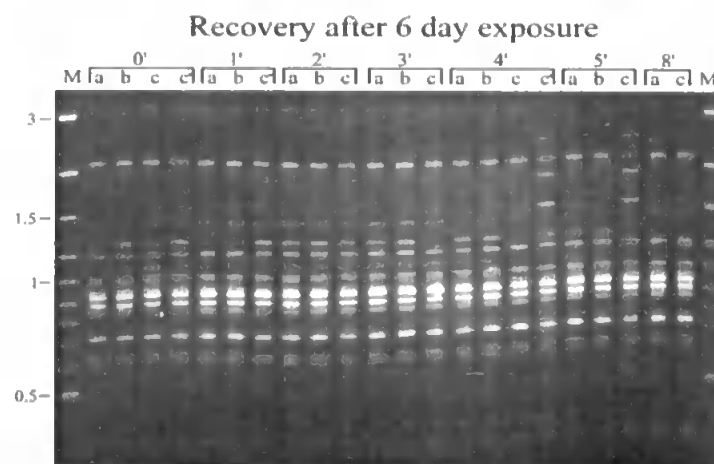
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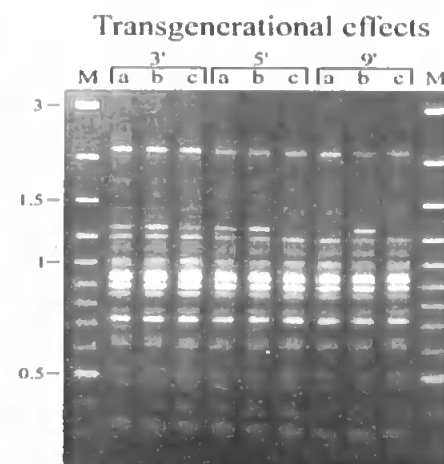
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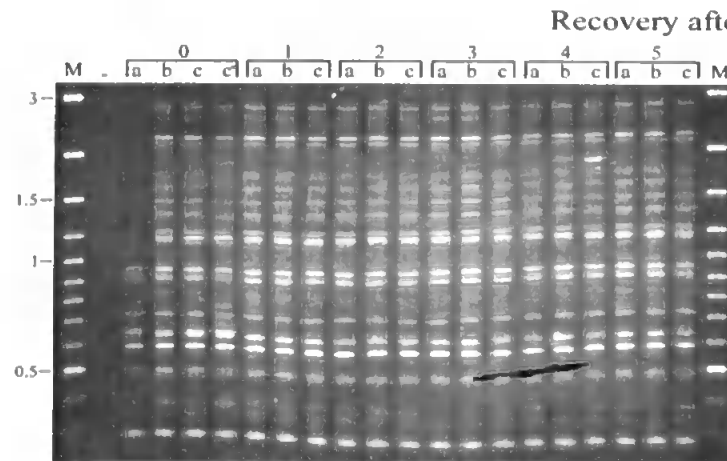
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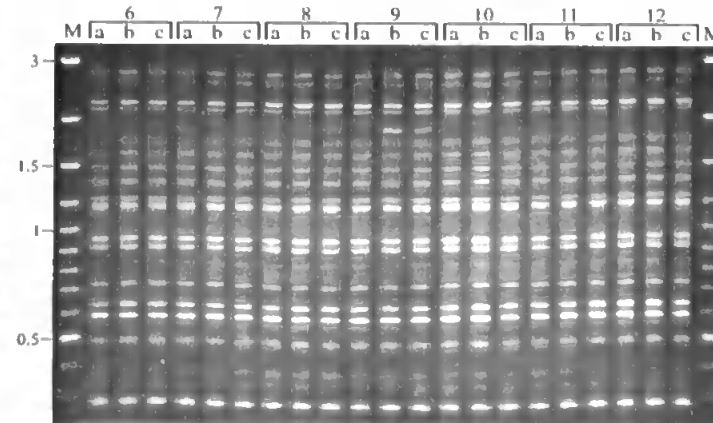
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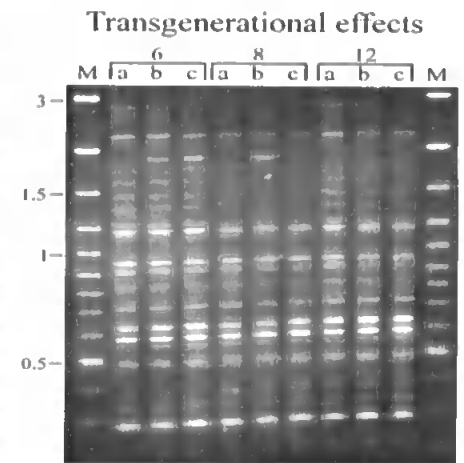
RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P for 3 and 6' days and recovered for 12 and 9' days, respectively, and their offspring, with primer OPB6



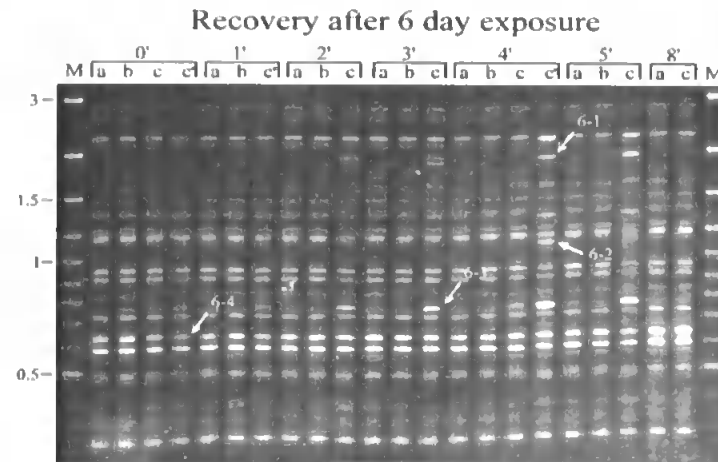
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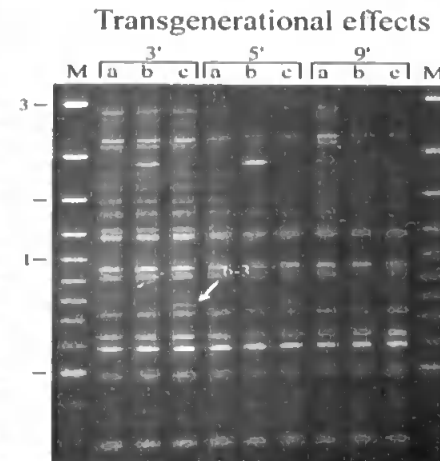
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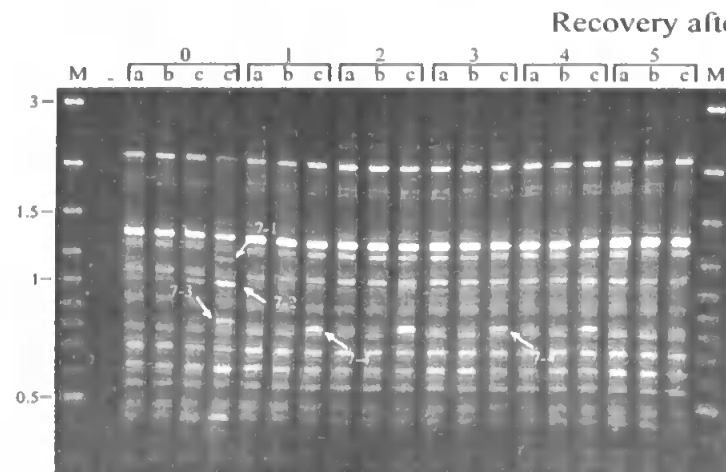
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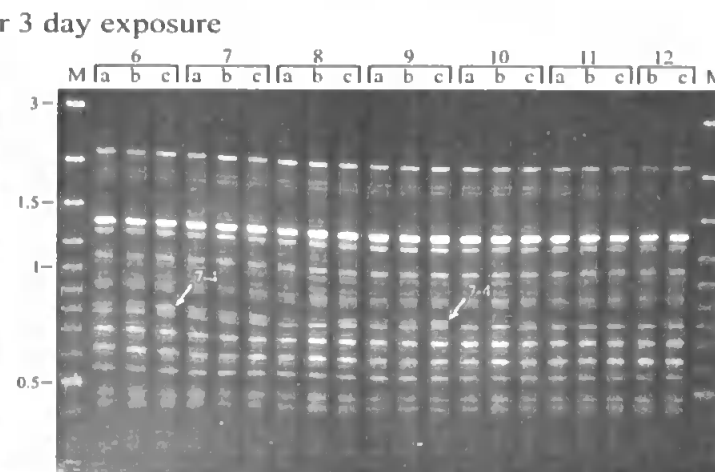
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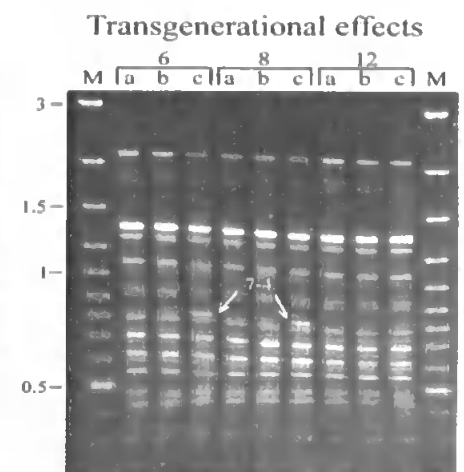
RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P for 3 and 6' days and recovered for 12 and 9' days, respectively, and their offspring, with primer OPB7



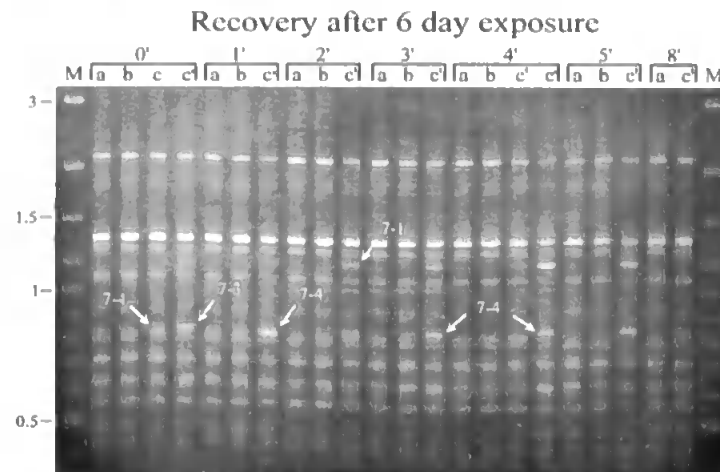
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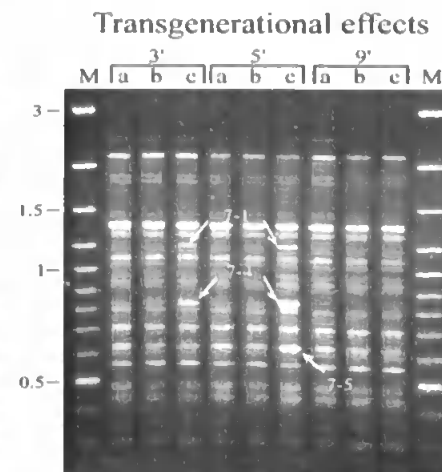
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For more details refer to Figure 10.9.



For more details refer to Figure 10.10.

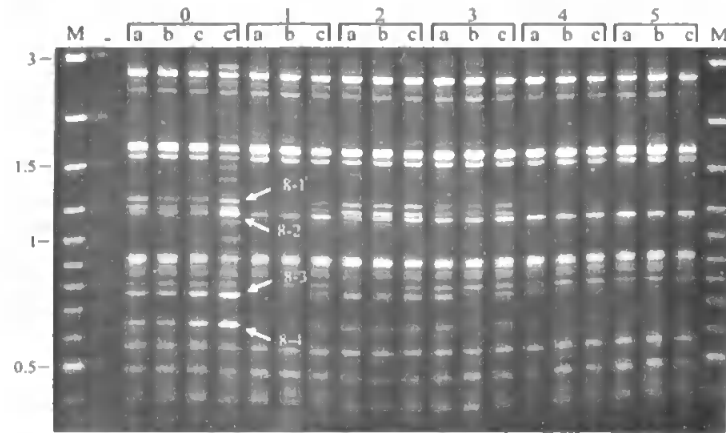


For more details refer to Figure 10.11.

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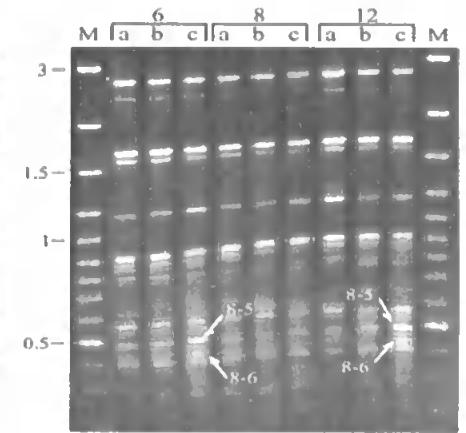
RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{g L}^{-1}$ B(a)P for 3 and 6' days and recovered for 12 and 9' days, respectively, and their offspring, with primer OPB8

Recovery after 3 day exposure



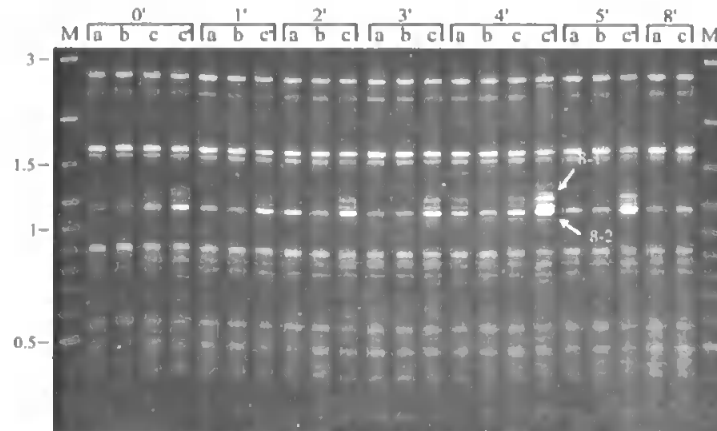
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Transgenerational effects



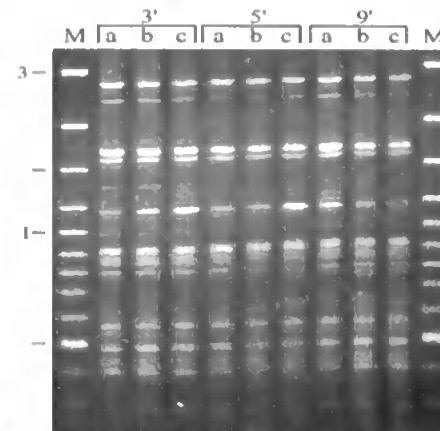
For more details refer to Figure 10.9.

Recovery after 6 day exposure



For more details refer to Figure 10.10.

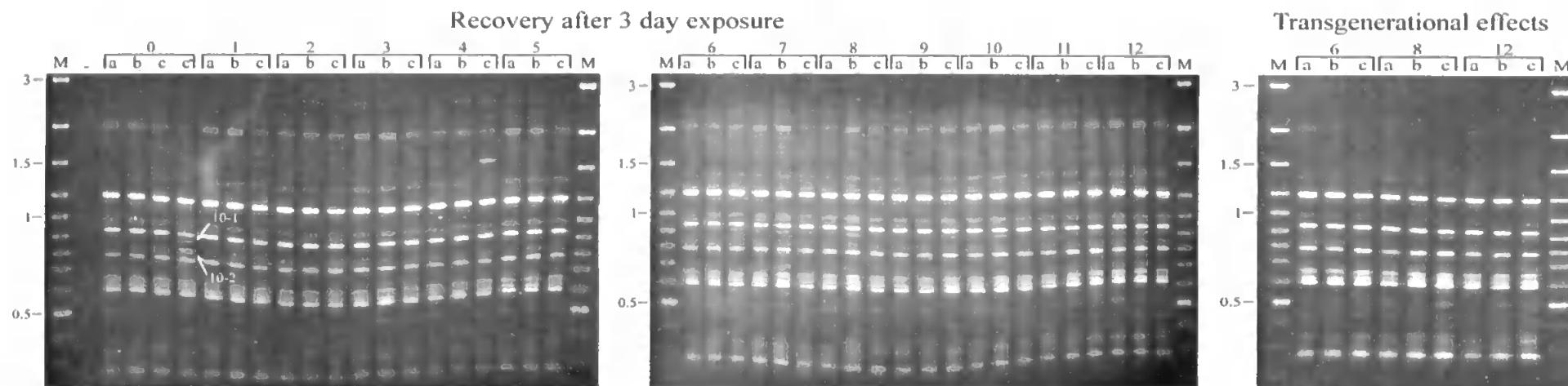
Transgenerational effects



For more details refer to Figure 10.11.

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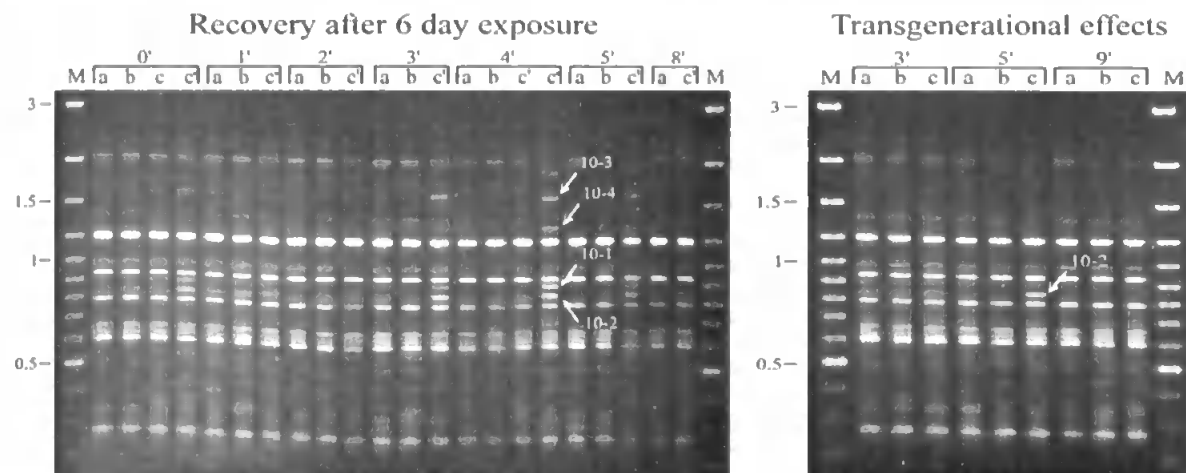
RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P for 3 and 6' days and recovered for 12 and 9' days, respectively, and their offspring, with primer OPB10



For more details refer to Figure 10.7.

For more details refer to Figure 10.7.

For more details refer to Figure 10.9.

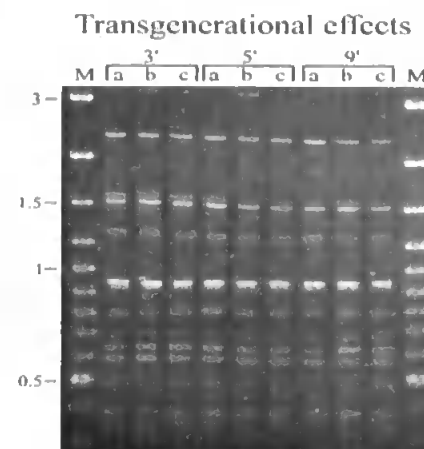
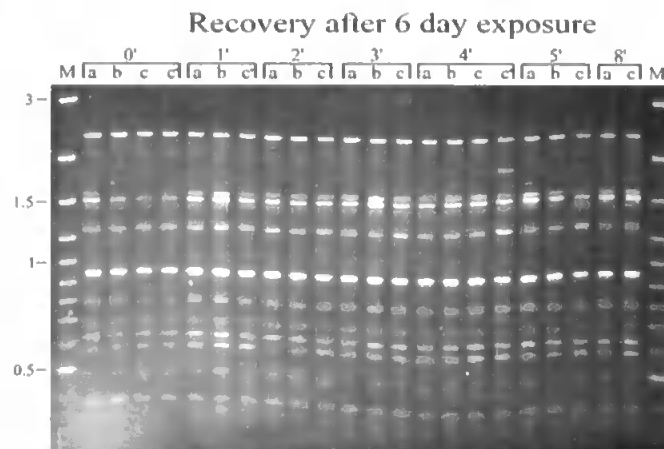
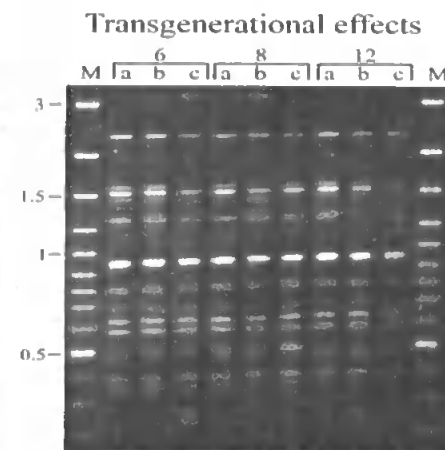
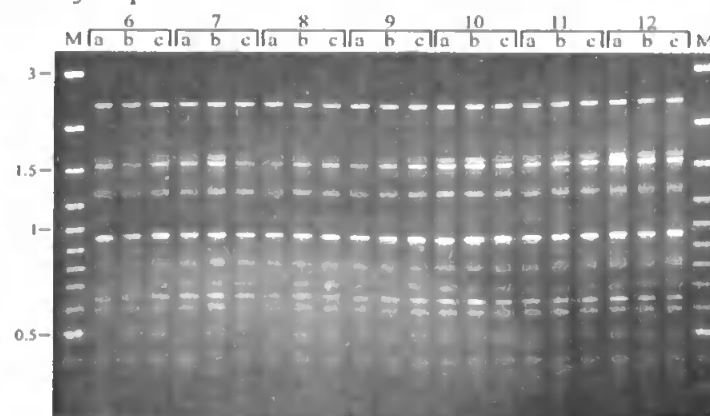
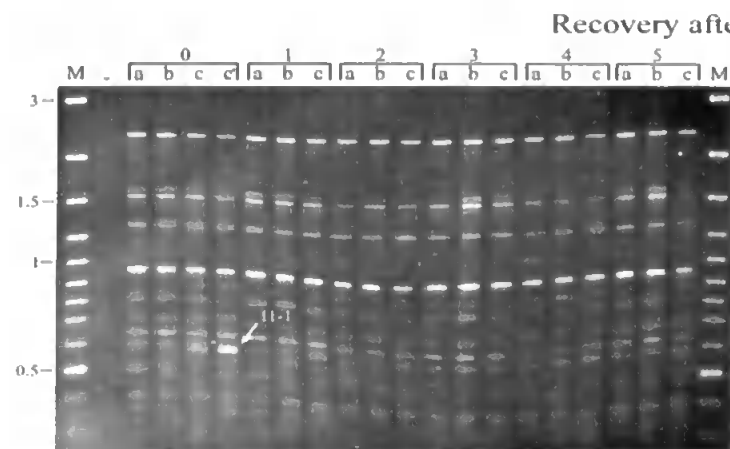


For more details refer to Figure 10.10.

For more details refer to Figure 10.11.

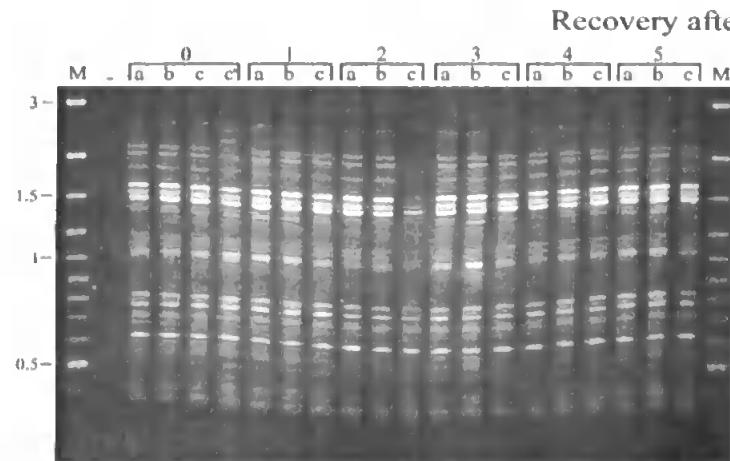
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RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P for 3 and 6' days and recovered for 12 and 9' days, respectively, and their offspring, with primer OPB11

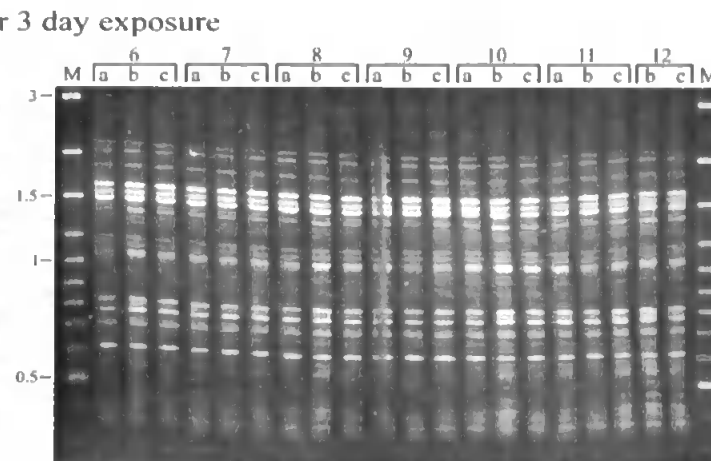


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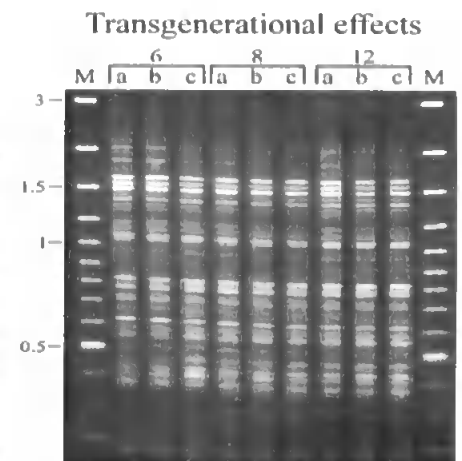
RAPD profiles of *Daphnia magna* exposed to $50 \mu\text{gL}^{-1}$ B(a)P for 3 and 6' days and recovered for 12 and 9' days, respectively, and their offspring, with primer OPB17



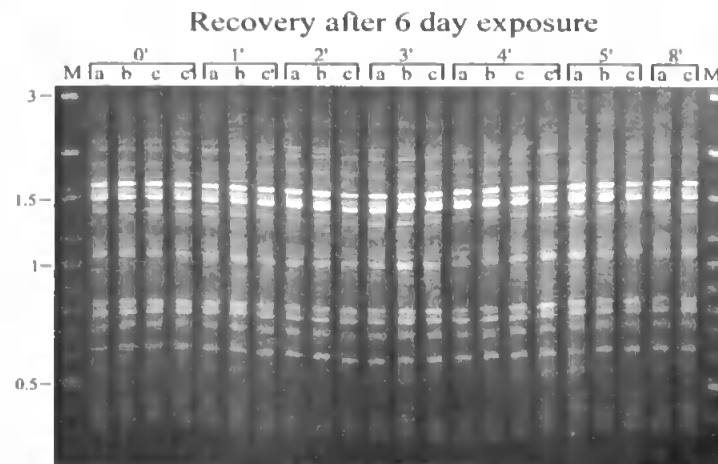
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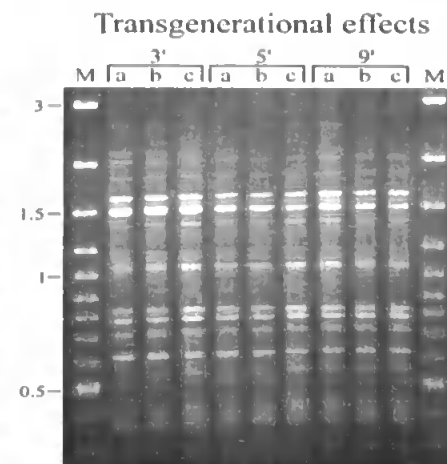
For more details refer to Figure 10.7.



For more details refer to Figure 10.9.



For more details refer to Figure 10.10.



For more details refer to Figure 10.11.

APPENDIX IV

Scientific contribution

Abstracts from conferences

- * 27-30 April 1997: "9th International symposium on pollutant responses in marine organisms (PRIMO 9)" - Bergen - Norway. Oral presentation entitled '*DNA fingerprinting in Daphnia magna*' Abstract: p. 40.

- * 7-12 Sept. 1997: "7th International Conference on Environmental Mutagens (7th ICEM)" - Toulouse - France. Poster presentation entitled '*Development of a genotoxicity assay in aquatic organisms using arbitrarily-primed Polymerase Chain Reaction*'. Abstract: ref. P XIII.10, p. S94.

- * 6-8 April 1998: "The United Kingdom Environmental Mutagen Society (UKEMS)" - York - England. Oral presentation entitled '*DNA fingerprints obtained by AP-PCR to detect DNA damage*'. Abstract p. 14.

- * 14-18 April 1998: "8th annual meeting of the Society of Environmental Toxicology and Chemistry (SETAC)" - Bordeaux - France. Oral presentation entitled '*The use of the arbitrarily-primed polymerase chain reaction to detect DNA damage*'. Abstract: ref. 3I/005, p. 71.

- * 7-11 September 1998: "28th European annual meeting on European Environmental Mutagen Society (EEMS)" - Salzburg - Austria. Poster presentation entitled '*Linking B(a)P-induced response detected by AP-PCR with fitness parameters in Daphnia magna*'. Abstract: ref. P150, p. 296.

DNA FINGERPRINTING IN *DAPHNIA MAGNA*

Atienzar F. *^{1,2}, P. Child¹, C. H. Walker¹, A. Evenden², A. Jha², M. H. Depledge² and D. Savva¹

¹ University of Reading, School of Animal and Microbial Sciences, Whiteknights, PO Box 228 Reading RG6 6AJ, England,

² University of Plymouth, Plymouth Environmental Research centre (Biological Sciences), Drake Circus, Plymouth; Devon, PL4 8AA, England.

DNA fingerprinting by arbitrarily-primed polymerase chain reaction (AP-PCR) has been used recently to differentiate between strains, between species and between individuals. In this study, we have applied this methodology to determine whether DNA fingerprints may be obtained from *Daphnia magna* and to assess whether such fingerprints may provide a new biomarker assay for genotoxic effects of chemicals.

DNA was extracted from individual *Daphnia* using a number of different methods and the best of these was found to be one that involved homogenisation, treatment with ribonuclease, extraction with phenol and with chloroform/isoamylol followed by ethanol precipitation; using spectrophotometric measurements and agarose gel electrophoresis the quality and purity of such DNA preparations was found to be good. AP-PCR was performed using a range of different 10-mer primers and when a fingerprint was obtained these were highly reproducible.

In some cases, reactions containing no DNA gave a "fingerprint" even at annealing temperatures as high as 55°C; this "fingerprint" was found not to be reproducible and to be due to annealing and extension of the primers themselves. However, addition of *Daphnia magna* DNA to the reaction generated clear and reproducible fingerprints. Studies are under way to examine DNA from *Daphnia magna* exposed to different genotoxic agents such as polyaromatic hydrocarbons (e.g. benzo-a-pyrene).

**DEVELOPMENT OF A GENOTOXICITY ASSAY IN AQUATIC ORGANISMS
USING ARBITRARILY-PRIMED POLYMERASE CHAIN REACTION**

Franck Atienzar, Britt Cordi, Andy Evenden, Awadhesh Jha and Michael Depledge. University of Plymouth, Plymouth Environmental Research Centre (Biological Sciences), Plymouth, Devon, PL4 8AA, UK.

DNA fingerprinting by arbitrarily-primed polymerase chain reaction (AP-PCR) has been used to differentiate between species, strains and individuals. In this study, we have applied this methodology to assess whether DNA fingerprints could provide a new biomarker assay for genotoxic effects of environmental agents.

There is a growing concern over increasing levels of ultraviolet (UV) radiation and its possible short and long term consequences to aquatic organisms. In a preliminary experiment, *Enteromorpha intestinalis*, a marine macroalgae, was exposed to UV B under *in vivo* conditions. After DNA extraction, AP-PCR reactions were performed; the *Taq* DNA polymerase amplified the DNA regions from 150 up to 500 base pairs with low efficiency compared to the controls where it amplified bands from 150 up to 1800 base pairs with high efficiency. In an another experiment, under *in vitro* conditions, DNA from *Daphnia magna* (water fleas) was exposed for different periods to UV C. A dose-dependent decrease for the appearance of bands was observed.

The studies suggested that following UV exposure, DNA amplification could not take place due to presence of thymine dimmers, which posed a physical barrier for the movement of the enzyme. It therefore appears that AP-PCR method could be used as a biomarker to detect the presence of genotoxins in the environment. Studies are underway to examine the usefulness of this method in *Daphnia magna* exposed to different genotoxic agents under *in vivo* conditions.

DNA FINGERPRINTS OBTAINED BY AP-PCR TO DETECT DNA DAMAGE

F. Atienzar, M. Conradi, B. Cordi, A. Evenden, A. Jha, and M. Depledge, PERC, University of Plymouth, Drake Circus, Plymouth, Devon, PL4 8AA, England.

Although DNA fingerprints obtained by the arbitrarily primed polymerase chain reaction (AP-PCR) technique was used originally to identify or distinguish between individuals, strains and species, we apply this method to detect DNA damage. The AP-PCR method does detect mutations and we do believe that this technique could also reveal the presence of DNA adducts, DNA breakage and possibly other DNA alterations.

The marine macroalga, *Palmaria palmata*, was exposed in vivo, to 5 different doses of ultraviolet (UV) radiations (1.3 W/m² UV A, 0.9, 1.6, 5.6, and 8.0 W/m² UV B) for 3 hours. DNA fingerprints were affected with respect to controls. The principal changes were a variation in intensity of bands as well as the presence of extra bands. The major event occurring for the 3 first UV doses (1.3 W/m² UV A, 0.9, and 1.6 W/m² UV B) was an increase of intensity of the bands compared to control with a frequency of 78, 53 and 41 % respectively. For the 2 highest UV B doses (5.6 and 8 W/m²), the major modification was a decrease in band intensity compared to control with a frequency of 62.5 and 81 % respectively. The occurrence of extra bands seen in the 3 lowest doses of UV, was absent in the higher doses. However, decrease in band intensity was only observed in the 3 highest UV doses. In an another in vivo study, *Daphnia magna* was exposed to benzo(a)pyrene (0.0125-0.2 ppm) for 14 days. DNA fingerprints obtained from exposed organisms also changed when compared to controls. It is believed that, in both studies, the modifications in the patterns are due to the generation of mutations (appearance of band), and to the interaction of *Taq* DNA polymerase with benzo(a)pyrene and UV-induced DNA damage (variation in intensities). In conclusion, the AP-PCR method appears to be a powerful and novel tool for the detection of a wide range of DNA damage at molecular level.

**THE USE OF THE ARBITRARILY-PRIMED POLYMERASE CHAIN
REACTION TO DETECT DNA DAMAGE**

Atienzar, F., A. Evenden, A. Jha, and M. Depledge, Plymouth Environmental Research Centre, University of Plymouth, Drake Circus, Plymouth, Devon, PL4 8AA, England.

DNA fingerprinting by arbitrarily-primed polymerase chain reaction (AP-PCR) was used originally to identify or distinguish individuals, strains and species. It also has the potential to detect DNA damage. In our study, the water flea, *Daphnia magna*, was exposed to benzo(a)pyrene (0.0125 - 0.2 ppm) for 14 days. After DNA extraction, AP-PCR reactions were performed. The results revealed that DNA fingerprints obtained from the treated animals were significantly affected; some bands disappeared or varied in intensity, and some new bands appeared. In another *in vivo* study, the marine macroalga, *Palmaria palmata*, was exposed to ultraviolet (UV) radiation (0.9 - 8 W/m²) for 3 hours. DNA fingerprintings were affected in relation to the level of exposure. The principal event occurring in the treated group was a variation in intensity of some high molecular weight bands, and eventually the disappearance of these bands. In some cases, new bands appeared. The modified patterns obtained in the DNA fingerprints of water fleas and UV-exposed algae are probably due to interactions between the *Taq* DNA polymerase (during amplification) and DNA damage, and to the gain or loss of annealing events of the primers due to mutations or modified DNA bases. In conclusion, the AP-PCR method appears to be a powerful and novel tool for the detection of a wide range of DNA damage at molecular level.

**LINKING B(A)P-INDUCED RESPONSE DETECTED BY AP-PCR
WITH FITNESS PARAMETERS IN *DAPHNIA MAGNA***

Franck Atienzar, Mercedes Conradi, Andrew Evenden, Awadhesh Jha and Michael
Depledge, Plymouth Environmental Research Centre (PERC), University of Plymouth,
Drake Circus, Plymouth, PL4 8AA, UK

There is a paucity of information on the potential effects of genotoxin-induced genetic damage in aquatic organisms at the population level. Our objective was to determine whether there is a possible link between genetic damage and fitness parameters. DNA damage as well as survival, growth and reproductive success were therefore evaluated in the freshwater flea, *Daphnia magna*, following exposure to varied concentrations of benzo(a)pyrene [B(a)P; 0.0125 to 0.2 mg l⁻¹]. Using the arbitrarily-primed polymerase chain reaction (AP-PCR), qualitative and quantitative modifications in DNA fingerprints, as a measure of genotoxin-induced DNA damage were observed in treated individuals compared to controls. B(a)P concentrations higher than 0.05 mg l⁻¹ reduced the age-specific survivorship and longevity of the organisms. Growth was affected at 0.1 and 0.2 mg l⁻¹. Exposures below 0.025 mg l⁻¹ did not delay the onset of first reproduction in the daphnids but the time between broods was prolonged, probably to reduce the negative impact on survival and brood size. Although age-specific fecundity was as sensitive as DNA changes detected using DNA fingerprints, the genetic endpoint appears to be more sensitive than survival and reproductive parameters. In summary, B(a)P induced genetic damage appears to have an impact on Darwinian fitness of the exposed individuals.

Publications in peer-reviewed international journals

Atienzar F, Child P, Evenden A, Jha A, Savva D, Walker C, Depledge M. 1998. Application of the arbitrarily primed polymerase chain reaction for the detection of DNA damage. *Marine Environmental Research* 46:331-335. Reprinted with permission from 'Elsevier Science'.

Atienzar F, Conradi M, Evenden AJ, Jha AN, Depledge MH. 1999. Qualitative assessment of genotoxicity using random amplified polymorphic DNA: comparison of genomic template stability with key fitness parameters in *Daphnia magna* exposed to benzo[a]pyrene. *Environmental Toxicology and Chemistry* 18:2275-2282. Reprinted with permission from 'Environmental Toxicology and Chemistry'.

Grayson TH, Cooper LF, **Atienzar FA**, Knowles MR, Gilpin ML. 1999. Molecular differentiation of *Renibacterium salmoninarum* isolates from worldwide locations. *Applied and Environmental Microbiology* 65:961-968. Reprinted with permission from 'The American Society for Microbiology'.

Atienzar F, Evenden A, Jha A, Savva D, Depledge M. 2000. Optimized RAPD analysis generates high-quality genomic DNA profiles at high annealing temperature. *BioTechniques* 28:52-54. Reprinted with permission from 'BioTechniques'.

Grayson TH, **Atienzar FA**, Alexander SM, Cooper LF, Gilpin ML. 2000. Molecular diversity of *Renibacterium salmoninarum* isolates determined by randomly amplified polymorphic DNA analysis. *Applied and Environmental Microbiology* 66:435-438. Reprinted with permission from 'The American Society for Microbiology'.

Atienzar FA, Cordi B, Donkin ME, Evenden AJ, Jha AN, Depledge MH. Comparison of ultraviolet-induced genotoxicity detected by random amplified polymorphic DNA with chlorophyll fluorescence and growth in a marine macroalgae, *Palmaria palmata*. *Aquatic Toxicology* (in press). Reprinted with permission from 'Elsevier Science'.



Application of the Arbitrarily Primed Polymerase Chain Reaction for the Detection of DNA Damage

F. Atienzar,^a P. Child,^b A. Evenden,^a A. Jha,^a D. Savva,^c C. Walker^c and M. Depledge^a

^aUniversity of Plymouth, Plymouth Environmental Research Centre (Biological Sciences), Drake Circus, Plymouth, Devon, UK, PL4 8AA

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ABSTRACT

*The technique of arbitrarily primed polymerase chain reaction (AP-PCR) shows potential as a selective and sensitive assay for the detection of xenobiotic-induced DNA damage. Problems, however, may occur in AP-PCR, diminishing its discriminative abilities. These problems include the presence of spurious amplification products in non-template-containing negative control reactions, and a lack of reproducibility amongst amplification patterns. Experiments designed to remove contaminated nucleic acids by ultraviolet (UV) treatment indicated that spurious bands are the result of aberrant primer-induced polymerisation, an event shown to be influenced by the concentration of deoxynucleotide triphosphates (dNTP) present in the reaction mixtures. Optimisation of dNTP concentration from 0.22 to 0.33 mM resulted in clear negative controls and highly reproducible amplification patterns with all DNA templates. As an example of the application of the method, in the present study, the macroalga *Palmaria palmata* (Rhodophyta) was exposed to UV A and B radiations. The study shows that the AP-PCR method can detect DNA damage and may be useful in detecting such damage following exposure of cells to xenobiotics. © 1998 Elsevier Science Ltd. All rights reserved*

Molecular biomarkers are potentially powerful tools for ecotoxicological studies (Peakall, 1992). Of particular interest are those biochemical changes that reflect the interaction of toxic components with genetic material, especially DNA (Anderson *et al.*, 1994). Advances in molecular biology have led to the development of a number of selective and sensitive assays for DNA analysis: DNA fingerprinting (Jeffreys, 1987); RFLP analysis (Botstein *et al.*, 1980) and a whole suite of techniques relying on the amplification of DNA sequences using the polymerase chain reaction (PCR) (Saiki *et al.*, 1988; Innis *et al.*, 1995).

All these methods have the potential to detect DNA damage by virtue of their ability to recognise changes in the structure of the DNA molecule.

One of the most promising techniques is that of arbitrarily primed PCR (AP-PCR), a semiquantitative method useful for genetic mapping, taxonomy, phylogenetics and the detection of mutations. The method relies on the comparison of amplification products from control (unexposed) and sample (exposed) DNA template produced by oligonucleotide primers of random sequence (Welsh *et al.*, 1995). The utility of AP-PCR for the detection of the effects of genotoxic exposure has been previously described (Savva *et al.*, 1994; Savva, 1996). In our laboratories, the AP-PCR technique is presently being investigated using DNA templates extracted from a number of plant and animal species, both from freshwater and marine habitats. In the present study, we aimed to develop the AP-PCR method as a generic first step technique for studying pollutant-induced DNA damage. An integral part of the development of AP-PCR genotoxicity assay, as in all PCR-based assays, is the optimisation of reaction conditions so as to facilitate the production of reproducible amplification profiles. It is also vital to prevent the influence of spurious amplification products on the accuracy of the method, by ascertaining the purity of reagents with respect to DNA contamination. This is normally achieved by including a negative control reaction containing all reagents except the DNA template.

Our experience suggests that with careful optimisation, especially regarding the purity and concentration of template, magnesium, deoxynucleotide triphosphates (dNTPs), primers, and DNA polymerase, reproducible amplification profiles can be obtained with the majority of samples. However, despite taking extensive precautions to ensure reagent purity, amplification products often appear in AP-PCR negative controls, specially when the primer concentration used is high.

The preliminary AP-PCR optimisation experiments carried out on DNA templates from the cladoceran *Daphnia magna*, suggested that the spurious amplification products were the result of either DNA or RNA contamination, or a polymerisation event involving the oligonucleotide primers, with the possible influence of one or more of the reagents used in the PCR reaction. It was initially thought that the pattern in the negative control was due to contamination of one of the reagents or the tubes, tips, pipettes, etc. All these were changed in turn but the problem still existed, suggesting that contamination was not the causative factor. The influence of contaminated DNA was investigated by exposing PCR reagents to high doses of UV radiation (254 nm, special UV box with three lamps; the distance from the lamps to the PCR tubes was 5 cm and the energy was 550 $\mu\text{W}/\text{cm}$) for 20 min, prior to the initiation of the reaction. DNA exposed to this high level of UV radiation cannot be amplified, due to the presence of DNA photoproducts such as thymine dimers, which seriously hamper the processivity of DNA polymerases. In this way, contaminating DNA is prevented from acting as a template for amplification. Differing concentrations of primer OPA9 (5' GGGTAACGCC 3') (Operon Technologies Inc.) ranging from 0.2 to 2 μM were used to amplify 20 ng of DNA isolated from *Daphnia*. Negative control reactions, using both 0.2 and 2 μM OPA9 primer were included in which template DNA was replaced with water (cell culture quality, Sigma). A non-UV exposed negative control PCR reaction was also performed. Lanes 2–9 in Fig. 1 demonstrate that the spurious amplification products are present in both UV-treated and untreated reactions and that the pattern is different at low and high primer concentrations. In the presence of DNA template, the bands corresponding to the major amplification products of the AP-PCR were shown to be reproducible at the varying primer concentrations.

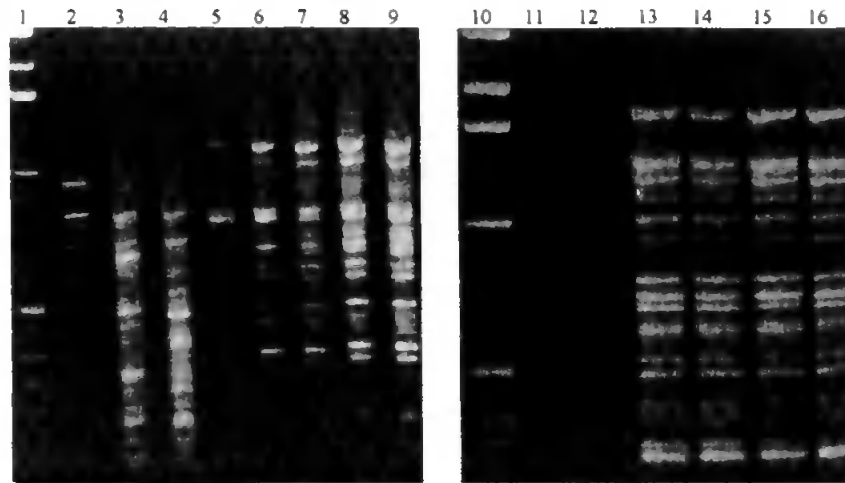


Fig. 1. Optimisation of AP-PCR reactions. Effect of UV radiation and primer concentration on reproducibility of the reactions using DNA extracted from *Daphnia* is shown in lanes 2–9. All the reactions (except lanes 1 and 4) were treated with UV radiation for 20 min (except DNA and enzyme) before performing PCR reactions. Lanes 2–4: no DNA control; 2: 0.2 μM primer; 3 and 4: 2 μM primer; 5–9: 20 ng DNA; 5: 0.2 μM primer; 6: 0.5 μM primer; 7: 1 μM primer; 8: 1.5 μM primer; 9: 2 μM primer. AP-PCR reactions were performed with 0.22 mM dNTP, 2.18 mM MgCl_2 and 2.8 U of *Taq* DNA polymerase (Promega). The PCR conditions were 5 min denaturation at 95°C, 10 cycles with an annealing temperature of 32°C, and 30 cycles with an annealing temperature of 35°C; each cycle also included 1 min at 74°C for extension and 1 min at 95°C for denaturation. The effect of dNTP concentrations on the reaction is shown in lanes 11–16. Lanes 11 and 12: no DNA control, 13–16: 20 ng DNA; 13 and 14: two DNA samples extracted from individual *Daphnia*; 15 and 16: two mixtures of DNA isolated from *Daphnia*. AP-PCR reactions were performed with 0.33 mM dNTP, 5.11 mM MgCl_2 , 2 μM primer, and 2.8 U of *Taq* DNA polymerase (MBI). Thermocycling was as before except that 40 cycles were performed with an annealing temperature of 50°C. Lanes 1 and 10: 1 kb marker (BRL), from top to bottom 3054, 2036, 1636, 1018, 506, 396, 344 and 298 bp.

Together, these results suggest that the spurious amplification within negative control reactions is not attributable to contamination by foreign DNA template, and as RNase-treated reagents produce the same result (results not shown), it is more likely that this is the result of non-specific reactions such as a primer-mediated polymerisation event. The apparent relationship between number and intensity of spurious bands and the concentration of the priming oligonucleotide supports this hypothesis.

In order to decrease the non-specific reactions, the annealing temperature was increased from 35 to 60°C; the results suggested that even at 50°C the negative control presented some bands whereas the positive control was not affected at all (data not shown). At 55 and 60°C, respectively, DNA fingerprintings were either partially or completely affected in both the controls. As a consequence, the annealing temperature used for further experiments was kept as 50°C, the highest suitable annealing temperature. Dimethyl sulfoxide (DMSO) was also used to decrease the background [various cosolvents such as DMSO have been shown to improve amplification in many applications (Landre *et al.*, 1995)]; it was not possible to obtain a clear negative control without affecting the quality of the positive control (data not shown).

By studying the results of numerous previous optimisation experiments for AP-PCR reactions and by reviewing a large body of diverse data from other PCR-based assays, it was found that a possible relationship exists between the number and intensity of

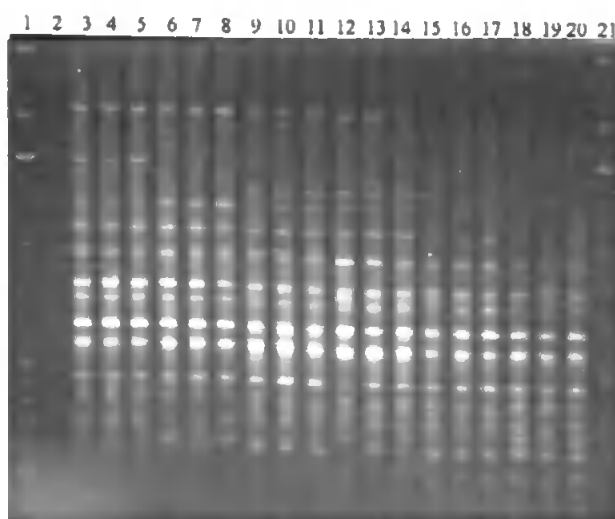


Fig. 2. DNA fingerprintings showing modified pattern due to UV radiation on the macroalga, *Palmaria palmata*. Lanes 1 and 21: 1 kb marker (BRL), from top to bottom 3054, 2036, 1636, 1018, 506, 396, 344 and 298 bp; 2: no DNA control; lanes 3–5: control alga [exposed to ambient level of UV (1)]; lanes 6–8: control alga [exposed to 1 + 1.3 W/m² UV A (2)]; lanes 9–11: alga exposed to 1 + 2 + UV B (0.9 W/m²); lanes 12–14: alga exposed to 1 + 2 + UV B (1.6 W/m²); lanes 15–17: alga exposed to 1 + 2 + UV B (5.6 W/m²); lanes 18–20: alga exposed to 1 + 2 + UV B (8.0 W/m²). AP-PCR conditions were the same as lanes 11 to 16 in Fig. 1.

amplification products in both control and sample reactions and the concentration of dNTPs. These data suggested that in reaction mixtures containing concentrations of dNTP at and above 0.33 mM, spurious amplification products are rarely found, but amplification patterns in reactions containing dNTPS above 0.33 mM are less reproducible. In general, the increase of the dNTP concentration increases the background but in our study 0.33 mM dNTP instead of 0.22 decreased the non-specific reactions in the negative control (and probably in the positive control). Taking into account all the results obtained, an experiment was performed using primer OPA9 to amplify template DNA from both individuals and populations of *Daphnia magna* under standard AP-PCR conditions with the dNTP concentration in all reactions adjusted to 0.33 mM. The results of this experiment, shown in lanes 11–16 in Fig. 1, indicate that at a concentration of 0.33 mM the negative control reaction is free from spurious bands and that the AP-PCR is extremely reproducible. These results were confirmed with 10 different primers (results not shown).

Whilst our experiments have shown the influence of primer and dNTP concentration on the AP-PCR reaction, and have alleviated the problems of 'dirty' negative controls and pattern reproducibility, we realise that many other factors might be involved. The random nature of the primer annealing segment of this particular variant of the PCR introduces many sources of variation. Therefore, we strongly advise that workers using AP-PCR based assays should be prepared to explore all components of the reaction. In this preliminary study, different concentrations of every PCR component were investigated in order to obtain a clear negative control and reproducible DNA fingerprints. The polymerisation of the primers or the formation of primer dimers has already been described in the literature. The 'Booster PCR' and the 'Hot-Start® PCR' (Ruano *et al.*, 1991) were used to solve the problem of the non-specific formation of the primers. Therefore, we suggest a modified protocol to avoid the polymerisation of the primers. A standard

protocol is presented for AP-PCR in conjunction with DNA isolated from a wide range of aquatic biota.

In order to further optimise and validate the method, the macroalga *Palmaria palmata* was exposed to different levels of UV radiation. Needless to mention, there is a growing concern over the increase of UV radiation due to depletion of the atmospheric ozone layer. In this context, DNA is considered to be the primary absorbing chromophore in the cell, which is likely to affect the short and long-term survival of the exposed populations (Anderson *et al.*, 1994). Two kinds of modification are due to UV radiation, i.e. the disappearance (main event) and the appearance of bands, which represents UV induced DNA damage (Fig. 2). In this study, the detection of DNA damage following UV radiation is an illustration of the application of the method, which suggests that AP-PCR technique could be used to detect other sources of DNA damage. In order to compare the sensitivity of the technique, it is planned to compare this method with other well-established methods, e.g. ^{32}P -post labelling. For the technique of AP-PCR to be adopted as a sensitive and selective method for the detection of xenobiotic-induced DNA damage, it is clear that the protocol needs standardisation. We hope that this initial study goes some way towards that goal.

ACKNOWLEDGEMENTS

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REFERENCES

- Anderson, S., Sadinski, W., Shugart, L., Brussard, P., Depledge, M., Ford, T., Hose, J., Stegeman, J., Suk, W., Wirgin, I. and Wogan, J. (1994) *Environmental Health Perspective* 102(Suppl. 12), 3-8.
- Botstein, D., White, R. L., Skolnick, M. and Davis, R. W. (1980) *American Journal of Human Genetics* 32, 314-331.
- Innis, M. A., Gelfand, D. H. and Sninsky, J. J. (eds) (1995) *PCR Strategies*. Academic Press, London, 373 pp.
- Jeffreys, A. J. (1987) *Biochemical Society Transactions* 15, 309-317.
- Landre, P. A., Gelfand, D. H. and Watson, R. M. (1995) In *PCR Strategies*, eds M. A. Innis, D. H. Gelfand, and J. J. Sninsky, pp. 3-16. Academic Press, London.
- Peakall, D. (1992) (ed) *Animal Biomarkers as Pollution Indicators*, Ecotoxicology Series 1, Chapman and Hall, London, 290 pp.
- Ruano, G., Brash, D. E. and Kidd, K. K. (1991) *Amplifications* 7, 1-3.
- Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988) *Science* 239, 487-491.
- Savva, D., Castellani, S., Mattei, N., Renzoni, A. and Walker, C. H. (1994) In *Environmental Contamination*, ed. S. P. Varnavas, pp. 105-110. CEP Consultants, Edinburgh.
- Savva, D. (1996) *TEN Review* 3, 110-114.
- Welsh, J., Ralph, D. and McClelland, M. (1995) In *PCR Strategies*, eds M. A. Innis, D. H. Gelfand and J. J. Sninsky, pp. 249-276. Academic Press, London.

QUALITATIVE ASSESSMENT OF GENOTOXICITY USING RANDOM AMPLIFIED POLYMORPHIC DNA: COMPARISON OF GENOMIC TEMPLATE STABILITY WITH KEY FITNESS PARAMETERS IN *DAPHNIA MAGNA* EXPOSED TO BENZO[a]PYRENE

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Abstract—A method of DNA profiling using the random amplified polymorphic DNA (RAPD) was used to assess toxicant-induced DNA effects in laboratory populations of *Daphnia magna* exposed to varying concentrations of the genotoxic hydrocarbon benzo[a]pyrene. These effects, represented by changes in the RAPD profiles, were compared with a number of key ecological fitness parameters (age-specific survival, age-specific fecundity, net reproductive rate, and intrinsic rate of population increase). Not only was the RAPD profiling method shown to be a rapid and reproducible assay of toxicant-induced DNA effects, but the qualitative measure of genomic template stability compared favorably with the traditional indices of fitness. The RAPD profiles, however, exhibited higher sensitivity in detecting toxic effects. The significance of these findings for future ecotoxicological studies is discussed.

Keywords—Random amplified polymorphic DNA profiles
Benzo[a]pyrene *Daphnia magna*

Genomic template stability

Fitness parameters

INTRODUCTION

Pollutants with genotoxic potential are of great concern to many ecotoxicologists. Once released, these agents have the capability not only to cause morbidity and/or mortality in the exposed organisms but potentially may induce higher order changes such as alterations to population dynamics and changes to biological diversity at both intra- and interspecies levels [1-3]. Such changes may initiate direct and catastrophic ecological consequences. The genotoxicity of pollutants is directly related to their effects on the structure and function of DNA molecules, which may be determined using a number of laboratory methods (e.g., [4,5]). However, while such pollutant-DNA interactions can be readily demonstrated using such methodology, there have been few direct experimental demonstrations of the wider relationships between DNA effects and their subsequent consequences at higher levels of biological organization [6,7]. To address this problem, it is necessary to develop reliable and reproducible genotoxicity assays that may then be used in conjunction with traditional assays detecting any impairment of classical Darwinian fitness parameters (e.g., growth, reproductive output, viability of offspring).

In the field of genotoxicity, advances in molecular biology have led to the development of a number of selective and sensitive assays for DNA analysis. The random amplified polymorphic DNA (RAPD) and the arbitrarily primed polymerase chain reaction (AP-PCR) techniques are two semiquantitative methods that have been used in genetic mapping, taxonomy, and phylogeny [8-10] and for the detection of various kinds of DNA damage and mutations [11-16]. Although RAPD and AP-PCR are very similar techniques (the methods have been often described as the same procedure, e.g., [15,17,18]), Meunier and Grimont [19] have defined procedural differences between the techniques. The detection of AP-PCR amplified product is facilitated by incorporation of $\alpha^{32}\text{P}$ -dATP during the last 10 cycles of the polymerase chain reaction (PCR) before electrophoresis is carried out in acrylamide gel prior to visualization by autoradiography. In contrast, RAPD fragments are detected after agarose gel electrophoresis and ethidium bromide staining. Therefore, to avoid any confusion, the definition described by Meunier and Grimont [19] has been adopted in this paper. Both techniques are based on the selective amplification of genomic sequences that, by chance, are flanked by adequate matches to an arbitrarily chosen oligonucleotide primer sequence. If two template genomic DNA sequences are different, the PCR products will display different banding patterns or profiles when these products are subjected to electrophoresis [9,20]. Detection of genotoxic effect using these techniques involves the comparison of profiles generated from control (unexposed) and treated (exposed) DNA.

The aims of this study were to further evaluate the potential of the RAPD technique to detect DNA damage as an ecotoxicological tool and to compare changes in RAPD profiles induced by the genotoxic polycyclic aromatic hydrocarbon, benzo[a]pyrene (BaP) with fitness parameters measured in a laboratory population of the cladoceran *Daphnia magna*.

MATERIALS AND METHODS

Culture of *Daphnia magna*

Daphnia magna (D. magna, clone 5) were provided by Zeneca's environmental laboratory at Brixham, United Kingdom, and were cultured in our laboratory. The animals were maintained in Elendt's medium [21] at a temperature of 20°C ($\pm 1^\circ\text{C}$) with a photoperiod of 16 h light (1,000 lux):8 h dark. Populations of 40 individuals were maintained in 1 L of me-

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dium contained in 2-L tall-form glass beakers (Sigma, Poole, UK). The medium was changed three times per week. Animals were fed the algae *Chlorella vulgaris* ($1.2\text{--}2.4 \times 10^7$ cells/daphnid/d) and a booster solution of Frippack microencapsulated food (Salt Lake Brine Shrimp, Grantsville, UT, USA). This extra source of carbon was incorporated into the culture medium at a ratio of 0.023 mg Frippack for every 1.2×10^7 algal cells.

Preparation of test solutions

Test solutions for chronic exposures were prepared from stock solutions of BaP at a concentration of 2 mg/ml in dimethylformamide (DMF). Once prepared, test solutions were stored at 4°C prior to use. Both BaP solutions and the DMF solvent controls were added to the *Daphnia* culture media in a volume of 100 µl of DMF in 1 L of medium (ensuring a level below the 0.05% maximum percentage of the solvent recommended by the U.S. Environmental Protection Agency) [22].

Toxicity tests

Acute toxicity. The acute toxicity of BaP was assessed by determining the LC50 of the chemical for *D. magna* over a period of 48 h. Freshly born neonates (less than 48 h) were exposed in replicate groups of 20 to concentrations of BaP equivalent to 0.1, 0.15, 0.2, 0.25, and 0.5 mg/L. Animals were fed (see culture of the organism) during the test, and surviving animals were counted to determine the 48-h LC50.

Chronic toxicity. The chronic toxicity test of BaP to *D. magna* was performed under the same experimental conditions as for the acute toxicity tests, with a blank control, DMF solvent control, and BaP concentrations of 0.0125, 0.025, 0.05, 0.1, and 0.2 mg/L, respectively. It is noteworthy that BaP concentrations used in the chronic test were lower than the 48-h LC50 determined in the acute toxicity (see the Results section). Surviving animals were counted at 0, 3, 5, 7, 10, and 14 d. Moribund, nonswimming animals were removed from culture at regular intervals on and between counting days. These animals were placed individually in 1.5-ml microcentrifuge tubes, snap frozen in liquid nitrogen, and stored at -80°C prior to DNA extraction and RAPD profiling.

Growth and reproductive measurements

The length of *D. magna* (apex to base) surviving at day 0, 3, 5, 7, 10, and 14 d was measured by video capture and image analysis using a Quantimet 570 image analyzer (Cambridge Instruments, Cambridge, UK). Neonates were counted and recovered at daily intervals. Once pooled, each batch of neonates was snap frozen in liquid nitrogen and stored at -80°C prior to DNA extraction and RAPD.

Calculation of fitness parameters

The intrinsic rate of natural increase of the *D. magna* population, r_m , was calculated using Lotka's equation, $\sum l_x m_x e^{-rx} = 1$ [23,24]. For a cohort of animals observed from birth to death at regular intervals, x is the age in days, l_x is the age-specific survival (number of living females on day x /number of females at start of life table), and m_x is the age-specific fecundity (number of new-born individuals produced on day x /number of living females on day x). Realized fecundity (U_x) was also calculated for the test populations ($U_x = l_x m_x$). Using this data, the net reproductive rate (R_0) can also be calculated, as R_0 represents U_x summarized over the entire

test period ($R_0 = \sum l_x m_x = \sum U_x$). Minimum generation time (T_{min}) was calculated by measuring the time that elapsed between birth and the deposition of the first batch of offspring. The interbrood time (Br) was measured as the time (in days) between clusters or broods.

Extraction of DNA from *Daphnia magna*

Total DNA from *D. magna* was extracted and purified using either a conventional phenol/chloroform method or a commercially available extraction kit (IGi Genie DNA extraction Kit, Immunogen International, Sunderland, UK). Two methods were used because traces of organic compounds may inhibit PCR reactions, and one of the methods could have failed to produce DNA of sufficient quality to obtain robust RAPD profiles. Furthermore, both protocols of DNA extraction proved to generate similar RAPD patterns (data not shown). The conventional DNA extraction procedure involved the homogenization of single *D. magna* in 400 µl of sperm lysis buffer (100 mM Tris-HCl, pH 8; 500 mM NaCl; 10 mM ethylenediaminetetraacetic acid [EDTA], pH 8; 1% SDS; 2% mercaptoethanol) followed by RNase treatment (40 µg, 37°C for 1.5 h). The DNA was then extracted in phenol (pH 8) and chloroform:isoamyl alcohol (1:1). The DNA was finally precipitated by two volumes of ice-cold ethanol in the presence of 3 M sodium acetate (1/10 of the DNA volume) and was incubated at -80°C overnight. Precipitated DNA was harvested by centrifugation, dried in air, and the final pellet dissolved in sterile analytic grade water.

In the second procedure, individual *D. magna* were homogenized in 100 µl of sperm lysis buffer and treated with RNase (10 µg, 37°C for 1.5 h). The DNA extraction was performed using the protocol supplied by the manufacturer (DNA binding silica resin, IGi, Sunderland).

Generation of *Daphnia magna* DNA profiles using RAPD

The DNA profiles of *D. magna* were generated in RAPD reactions performed in a reaction volume of 25 µl as described previously [18]. The decamer oligonucleotides, OPA9 (GGGTAACGCC) and OPB7 (GGTGACGCAG), were obtained from Operon Technologies (Southampton, United Kingdom). Approximately 20 ng of *D. magna* genomic DNA was subjected to RAPD amplification with a primer concentration of 2 µM, a deoxy-trinucleotide phosphate (dNTP) concentration of 0.33 mM, and a MgCl₂ concentration of 5.11 mM in the presence of 2.8 units of *Taq* DNA polymerase and 1× reaction buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 0.1% Triton-X 100, 0.01% gelatin, 2.5 µg bovine serum albumin). All PCR chemicals were obtained from Immunogen International (Sunderland) except when otherwise mentioned. Thermal cycling parameters consisted of 5-min denaturation (95°C) followed by 40 cycles of 1-min denaturation (95°C), 1-min annealing at 50°C, and 1-min extension [(74°C), with the final extension period adjusted to 10 min]. Reaction mixtures were stored at 4°C prior to use.

Agarose gel electrophoresis and analysis of DNA profiles

Electrophoresis of RAPD reaction products was performed in 1.2% w/v agarose, using a Tris-borate-EDTA buffer (TBE) system (1× TBE = 90 mM Tris-base, 90 mM boric acid, and 2 mM EDTA). Amplified DNA was mixed with one-fifth volume of gel loading buffer (analytical grade water containing 25% ficoll, 0.25% bromophenol blue, and 0.25% xylene cy-

anol), and 15 μ l of this solution was loaded onto the agarose gel. Chemicals used were from Sigma (Poole, UK) unless otherwise mentioned. For comparison, DNA molecular size marker (1 Kb marker; Gibco BRL (Paisley, UK)) was used for each agarose gel. In all gels, the bands visualized were, from top to bottom, 3,054, 2,036, 1,636, 1,018, 517, 506, 396, 344, and 298 bp. The DNA samples were subjected to electrophoresis at 80 volts for 7 h, after which the gels were stained in a 1X TBE solution containing ethidium bromide (0.015% v/v) for a period not less than 40 min. Gels were photographed under ultraviolet illumination using a Polaroid camera (CU-5) (Cambridge, MA, USA). Images of each gel were also captured using a Kodak DC40 digital camera (Eastman Kodak, New York, NY, USA) and the DNA profiles analyzed using Kodak Digital Science[®] 1 D Image Analysis Software (Eastman Kodak).

Estimation of genomic template stability and transformation of the data

Each separate DNA effect observed in RAPD profiles (disappearance of bands, appearance of new bands, and variation in band intensities in comparison to control profiles) was given the arbitrary score of +1 and the average calculated for each experimental group of animals with primers OPA9 and OPB7. The template genomic stability (percent) was calculated as $100 - (100a/n)$, where n is the number of bands detected in control DNA profiles and a is the average number of changes in DNA profiles. To compare the sensitivity of each parameter (genomic template stability, I_s , m_s , r_s , and R_d), changes in these values were calculated as a percentage of their control value (set to 100%).

Statistical analyses

The LC50 value, confidence limits, and Chi-square goodness of fit were determined according to the method of Finney [25]. Differences among growth rates were calculated using multiple regression analysis as in Conradi and Depledge [26]. Briefly, the data were log transformed to homogenize the variances. Because the number of surviving animals decreased as the toxic effect increased, the regressions were weighted with $n^{1/2}$ (where n is the number of the surviving animals). Correlation and analysis of variance (ANOVA) were performed using the computer software package Statgraphics (Statgraphics plus for Windows, version 2.1, Statistical Graphics, Princeton, NJ, USA). Changes in genomic template stability and key fitness parameters (age-specific survival [I_s], age-specific fecundity [m_s], net reproductive rate [R_d], and intrinsic rate of population increase [r_s]) were also statistically tested using ANOVA (Statgraphics). The least significant differences (LSD) test was used to reveal statistical differences.

RESULTS

Acute toxicity

Using survivorship data, the 48-h LC50 of BaP for *D. magna* (clone 5) was 0.25 ± 0.04 mg/L BaP ($\chi^2 = 63.6$, $p < 0.005$).

Chronic toxicity

Demographic trends for the populations of *D. magna* exposed to differing concentrations of BaP are shown in Table 1. From these results, it can be seen that, with increases in toxicant concentration in the range 0.025 to 0.2 mg/L BaP,

Table 1. Demographic trends of *Daphnia magna* populations under various benzo[a]pyrene (BaP) treatments; values representing mean \pm SD were calculated using both replicates (i.e., 40 *Daphnia* per concentration); all parameters were determined throughout the experiment until *Daphnia* were unable to swim properly; parameters such as minimum generation time (T_{min}), age-specific survival (I_s), age-specific fecundity (m_s), net reproductive rate (R_d), and intrinsic rate of natural increase (r_s) are described in the materials and methods section; parameters referring to reproduction could not always be calculated for the two highest BaP concentrations (i.e., when number of broods < 2)

BaP concentrations (mg/L)	Mean period to become ovigerous (days)	T_{min} (days)	Maxi-mum body size (mm)	Maxi-mum number of eggs/female	Number of broods	Inter-brood period (days)	Total number of neonates	Age-specific survival (I_s)	Age-specific fecundity (m_s)	Net productive rate (R_d)	Intrinsic rate of natural increase (r_s)
Blank control	5 \pm 0.00	6	4.03	12	3 \pm 0.00	3	826.5 \pm 71.4	5.97 \pm 0.04	42.45 \pm 1.97	42.35 \pm 2.13	0.59 \pm 0.22
Dimethylformamide control	5 \pm 0.00	6	3.89	12	3 \pm 0.00	3	921 \pm 101.8	5.95 \pm 0.07	47.55 \pm 2.97	47.17 \pm 3.51	0.66 \pm 0.21
0.0125	5 \pm 0.00	6	3.9	12	3 \pm 0.00	3.5	861.6 \pm 133.6	5.90 \pm 0.14	43.78 \pm 5.69	43.07 \pm 6.68	0.78 \pm 0.16
0.025	5 \pm 0.00	7	4.03	11	2.5 \pm 0.49	4	499 \pm 22.6	5.82 \pm 0.11	26.48 \pm 2.39	25.63 \pm 2.09	0.45 \pm 0.15
0.05	5.1 \pm 0.01	6	3.67	11	2 \pm 0.00	4	227 \pm 45.3	4.95 \pm 0.07	11.66 \pm 1.97	11.61 \pm 1.90	0.33 \pm 0.04
0.1	5.31 \pm 0.00	7	3.16	9	1 \pm 0.00	—	40.5 \pm 6.4	2.50 \pm 0.63	2.08 \pm 0.40	2.08 \pm 0.40	0.06 \pm 0.01
0.2	—	—	2.44	5	0	—	0	1.92 \pm 0.03	0	0	0

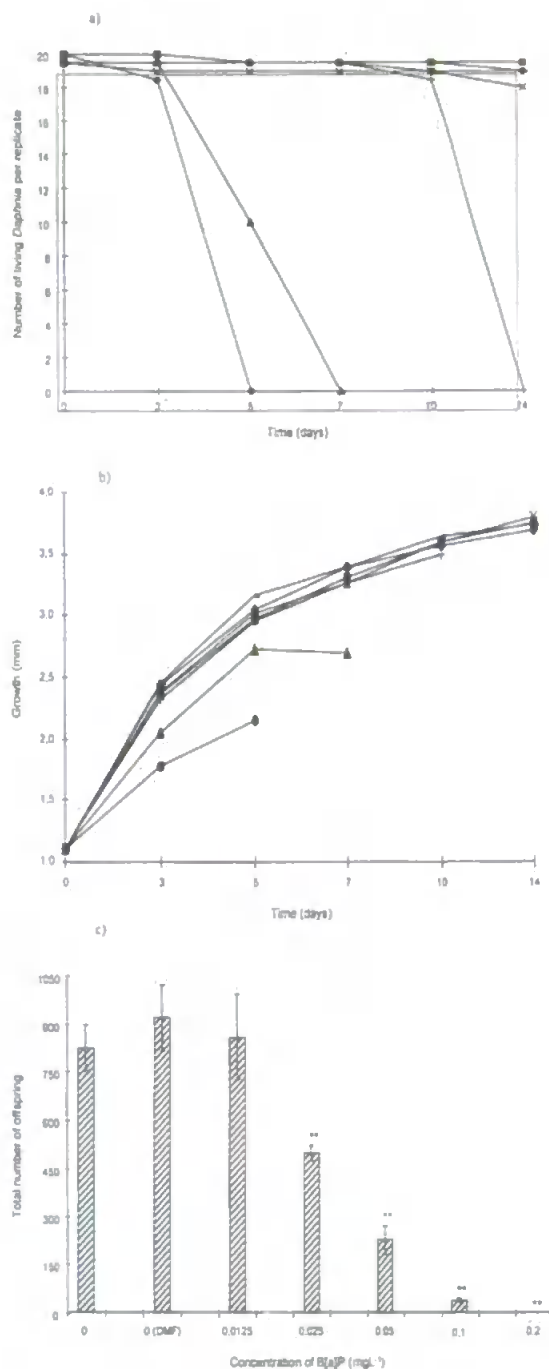


Fig. 1. Variation in (a) number of living *Daphnia magna* per replicate, (b) growth, and (c) total number of offspring in populations of *D. magna* exposed to varying concentrations of BaP, including 0 (control); \diamond , 0 (solvent control); \blacksquare , 0.0125; \times , 0.025; $+$, 0.05; \blacktriangle , 0.1; and \bullet , 0.2 mg/L BaP. ** indicates a significant difference from control ($p < 0.01$).

consistent reductions occurred in number of eggs per female, maximum body size, and number of broods. However, the mean period for *Daphnia* to become ovigerous was identical among groups. The number of living organisms was significantly reduced at the highest BaP concentrations; e.g., organisms could not survive more than 5, 7, and 14 d at 0.2, 0.1, and 0.05 mg/L BaP, respectively (Fig. 1a). In contrast, *Daph-*

nia from the other groups (i.e., control, control + DMF, 0.0125, and 0.025 mg/L BaP) survived throughout the exposure period. The data indicate as well that *D. magna* exposed to gradual increases in BaP concentrations displayed a lengthened time between the first and second brood. Although populations growing at 0.1 and 0.2 mg/L both had a smaller body size than controls ($p < 0.001$), there were no significant differences between the control groups and the other BaP-exposed groups (Fig. 1b). In addition to that, the size of sub-24-h juveniles was identical between all groups ($p < 0.05$, data not shown). Finally, the total number of offspring was significantly reduced at 0.025 mg/L and higher BaP concentrations (Fig. 1c and Table 1).

Changes in fitness parameters

Alterations to the key fitness parameters, i.e., age-specific survival (l_x), age-specific fecundity (m_x), net reproductive rate (R_0), and intrinsic rate of population increase (r_m) are presented in Table 1 and Figure 3, before and after transformation, respectively. The *D. magna* exposed to concentrations higher than 0.05 mg/L exhibited reduced life spans compared to the controls. Age-specific fecundity in blank and DMF controls was maximal on day 8, while animals exposed to both 0.0125 and 0.025 mg/L presented their maximal values on day 10 (data not shown). The intrinsic rate of natural increase and the net reproductive rate were also shown to be significantly reduced by increasing BaP concentrations above 0.0125 mg/L ($p < 0.001$). R_0 and m_x appear to be the most sensitive fitness parameters, as r_m was calculated to be only significantly different at 0.1 and 0.2 mg/L BaP. None of the fitness parameters appeared to be altered at the lowest hydrocarbon concentrations.

The RAPD DNA profiling

The DNA amplified was extracted from moribund, non-swimming (but alive) organisms after 14, 7, and 5 d at 0.05, 0.1, and 0.2 mg/L BaP, respectively. For the other groups (i.e., control, control + DMF, 0.0125, and 0.025 mg/L BaP), organisms were sacrificed after 14 d. In total, 10 oligonucleotide primers were used to analyze the results. However, depending on the sequence of the primers, changes in RAPD profiles obtained from the exposed population occurred or not. The DNA profiles generated by two of them (primers OPA9 and OPB7) are shown in Figure 2. The patterns show significant differences between unexposed and exposed individuals, with visible changes in the number and size of amplified DNA fragments and both increases and decreases of DNA band intensities. Extra bands appeared with both primers OPA9 (two new PCR amplification products labeled 9-1 and 9-2; Fig. 2A) and OPB7 (three new main bands labeled 7-1, 7-2, and 7-3; Fig. 2B). The reproducibility of the RAPD profiling method in detecting BaP-induced DNA changes was also determined using both replicates R1 and R2 (Fig. 2C and D). This experiment was performed to confirm if extra bands 9-1 and 9-2 appeared in the majority of the organisms exposed to 0.025 (Fig. 2C) and 0.05 (Fig. 2D) mg/L BaP. Results suggested that both bands were very reproducible between individuals and mixtures of *Daphnia*. The frequency of bands 9-1 and 9-2 was calculated to be at least of 95% at 0.025 and 0.05 mg/L BaP. When the same samples were subjected to analysis with primer OPB7, the bands 7-1, 7-2, and 7-3 appeared all together or not at all (data not shown). The frequency of appearing bands at 0.025 and 0.05 mg/L BaP was calculated to be 42 and 87%, respectively. In addition to that, further experiments confirmed

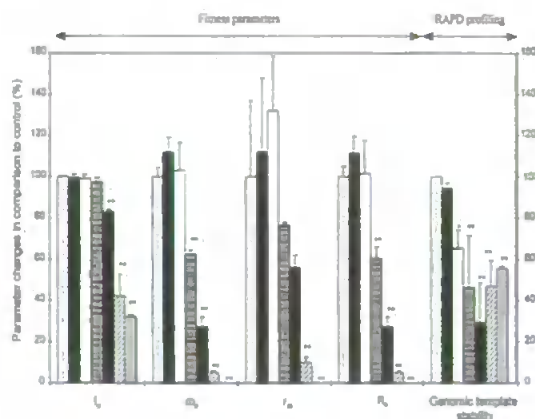


Fig. 3. Comparison between key fitness parameters and genomic template stability in populations of *Daphnia magna* exposed to BaP. l_1 = age-specific survival, m_1 = age-specific fecundity, R_0 = net reproductive rate, and r_0 = intrinsic rate of natural increase (see materials and methods and results). □, 0 (control); ■, 0 (solvent control); ▤, 0.0125; ▥, 0.025; ▦, 0.05; ▧, 0.1; and ▨, 0.2 mg/L BaP. * and ** indicate a significant difference from control ($p < 0.05$ and $p < 0.01$, respectively). Error bars represent standard deviation.

range of biota. Nevertheless, the value of such procedures would be further enhanced by linking molecular/cellular effects to higher order changes such as reductions in Darwinian fitness and decreases in species diversity [1–3]. This study evaluates the suitability of a DNA profiling/fingerprinting assay combined with the measurement of parameters at the population level with the objective of better understanding the impact of BaP on *D. magna*. The RAPD profiles detect alterations to genomic DNA through the use of randomly primed PCR reactions. These effects include changes in oligonucleotide priming sites and variations in the activity of the *Taq* DNA polymerase. Such effects lead to visible changes in the electrophoretic profiles of RAPD reaction products. Changes include the appearance of extra amplified bands, the apparent disappearance of amplified bands, and the changes in amplified band fluorescence. However, although obvious changes occurred in RAPD profiles obtained from the exposed population, there were no obvious trends among patterns obtained at the different BaP concentrations. New PCR amplification products may reveal a change in the DNA sequence due to mutations (resulting in [a] new annealing event[s] and/or large deletions (bringing two preexisting annealing sites closer) and/or homologous recombination (juxtaposing two sequences that match the sequence of the primer). Indeed, this method (in fact, the AP-PCR technique) has already been successfully used for the detection of mutations [12,13]. Following exposure to mutagens, DNA replication [27] and error-prone DNA repair [28] are generally implicated in generating mutations [29]. Although the experiment did not exceed 14 d, DNA replication probably occurred at an intensive rate since *D. magna* have a rapid growth rate. Thus, the frequency of mutations in the exposed populations may be elevated compared to control *Daphnia*. Mutations can only be responsible for the appearance of new bands if they occur at the same locus in at least a minimum number of cells to be amplified by PCR. In this context, this is likely, as Boles and Hogan [30] reported hot spot interactions between DNA and metabolized BaP products. Appearing bands may also be the result of structural changes induced by BaP adducts and/or by nongenotoxic

events such as transposition, DNA amplification, and so forth. Other changes in DNA patterns such as the variation in band intensities can be attributed principally to the presence of bulky adducts [31] that potentially block the PCR enzyme. Indeed, although BaP adducts block DNA replication and transcription [27], the local sequence context, the conformation of the adduct, and the nature of the polymerase play important roles in the bypass event [32]. Furthermore, the disappearance of bands is not likely to be due to mutations because the same event must happen in most of the cells if not all. Finally, the third observed change in RAPD patterns (the variation in band intensity) is likely to be due to the sum of all DNA alterations (e.g., BaP adducts, mutations, rearrangements, structural changes) induced by BaP.

The genomic template stability is directly related to the extent of DNA damage and also to the efficiency of DNA repair and replication. For example, a high level of DNA damage does not necessarily decrease the genomic template stability (in comparison to a low level of DNA alterations) because DNA repair and replication may be inhibited due to excessive, lethal actions of the BaP-induced adducts. If the survivorship of a population is affected, a toxic effect can completely inhibit a biological response (e.g., fitness parameters); in contrast, the genomic template stability cannot be completely affected because the induction of DNA damage may not increase linearly (plateau effect) (Fig. 3). Furthermore, since genomic template stability may be related to different kinds of DNA damage, such as DNA adducts, mutations, rearrangements, etc., it would be difficult to anticipate a dose-response relationship.

It is proposed that alterations to RAPD profiles due to genotoxic exposure can be regarded as alterations in genomic DNA template stability and that this qualitative measure of genotoxic effect can be directly compared with changes in key Darwinian fitness parameters. The results from this experiment clearly suggest that genomic DNA template stability can be more sensitive than growth parameters and of at least equal or even greater sensitivity than other measures of fitness such as age-specific survival, age-specific fecundity, net reproductive rate, and the intrinsic rate of population increase. It is also important to note from this series of experiments that RAPD profiling can detect DNA changes earlier than other changes in conventional toxicity assays measuring fitness parameters. After exposing larval *Xenopus laevis* to BaP, Sadinski et al. [7] suggested that DNA adducts and micronuclei were sensitive measures of sublethal DNA damage as well as possible short-term indicators of indirect effects on fitness parameters. The present study, using a novel and simple approach, also supports this notion in a clonal freshwater invertebrate species.

The growth experiment suggested that *Daphnia* exposed to 0.1 and 0.2 mg/L BaP had a size significantly reduced. By attaining reproductive maturity at a smaller body size, the *Daphnia* are able to buffer the impact of lower body growth rate on the age at the first reproduction [33]. Generally, when food is limited, *Daphnia* generate fewer but larger neonates compared with organisms fed with adequate amounts of food [34]. Thus, the identical size of the neonates at first reproduction plus the fact that growth rates were a good indicator of the intrinsic rate of population increase (r_m) [35] suggest that the decrease in size at maturity was more readily ascribed to a direct toxic effect of BaP. In other words, the effects on growth were not due to a limitation in food levels. The data also indicate that *D. magna* exposed to gradual increases in

BaP concentrations lengthened the time between the first and second brood. This response is generally known to lessen the impact of adverse effects on survival and the number of offspring at each breeding [36].

The measure of molecular and population parameters present several advantages. First, in ecotoxicology, it is fundamental to accumulate data at different levels of biological organization in order to fully understand the effect of a toxicant on organisms. Second, the measure of some parameters at the population level facilitates the interpretation of the data at the molecular level. For instance, a significant reduction in growth correlates with a significant inhibition in DNA replication, suggesting that the extent of DNA damage may be important in the majority of the cells.

The random nature of the DNA amplification events that form the basis of the RAPD profiling technique has often attracted criticism. The generation of profiles has regularly been considered to exhibit poor reproducibility [37]. However, after suitable optimization of amplification reaction conditions [18] and the judicious choice of oligonucleotide primers for each species-specific DNA template, the assay performs well, even for nonclonal organisms. For example, we successfully applied the RAPD technique to a wide range of species such as *Platynereis dumerilii* (worm), *Mytilus edulis* (mussel), *Palmaria palmata* (red macroalgae), *Enteromorpha intestinalis* (green macroalgae), *Escherichia coli* (bacteria), *Renibacterium salmoninarum* [38] (bacteria), together with calf thymus and human placental tissue (unpublished manuscript). While the RAPD technique clearly shows promise in the detection of pollutant-induced DNA effects, a great deal of further experimentation and validation is required. Future work will focus on the determination of sensitivity and selectivity of the RAPD technique by comparison with preexisting DNA analysis methodologies. In particular, the performance of RAPD will be compared with ³²P postlabelling for the detection of BaP adducts in *Daphnia* DNA. It is also planned to assess the performance of this assay in the detection of chromosomal aberrations, DNA strand breaks, and DNA sequences containing multiple and defined mutations. The latter will be performed using material obtained from both hypermutating cultured animal cells and from bacterial isolates. Using this approach, it should then be possible to determine the general applicability of this method to the detection of DNA effect in both in vitro and in vivo systems and the specific ability of RAPD to evaluate pollutant-induced genotoxicity in natural populations of invertebrate animals.

In conclusion, the RAPD and AP-PCR techniques show potential as reliable and reproducible assays for genotoxicity. These techniques are therefore being increasingly adopted as sensitive methods for the detection of induced genetic damage at the molecular level in both somatic and germ cells of aquatic organisms (e.g., [16,39]). The present study suggests that, when coupled with the measurement of pollutant-induced effects at higher levels of biological organization, this technique would prove to be a powerful ecotoxicological tool.

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REFERENCES

- Wurgler FE, Kramers PGN. 1992. Environmental effects of genotoxins (ecogenotoxicology). *Mutagenesis* 7:321-327.
- Anderson SL, et al. 1994. Genetic and molecular ecotoxicology—A research framework. *Environ Health Perspect* 102:3-8.
- Depledge MH. 1998. The ecological significance of genotoxicity in marine invertebrates. *Mutat Res* 399:109-122.
- Jha AN, Hutchinson TH, Mackay JM, Elliot BM, Dixons DR. 1997. Evaluation of the genotoxicity of municipal sewage effluent using the marine worm *Platynereis dumerilii* (Polychaeta, Nereidae). *Mutat Res* 391:179-188.
- Chipman JK, Marsh JW. 1991. Bio-techniques for the detection of genetic toxicity in the aquatic environment. *J Biotechnol* 17:199-208.
- Anderson SL, Wild GC. 1994. Linking genotoxic responses and reproductive success in ecotoxicology. *Environ Health Perspect* 102:9-12.
- Sadinski WJ, Levay G, Wilson MC, Hoffman JR, Bodell WJ, Anderson SL. 1995. Relationships among DNA adducts, micro-nuclei, and fitness parameters in *Xenopus laevis* exposed to benzo(a)pyrene. *Aquat Toxicol* 32:333-352.
- Welsh J, McClelland M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* 19:5275-5279.
- Welsh J, Petersen C, McClelland M. 1991. Polymorphisms generated by arbitrarily primed PCR in the mouse: Application to strain identification and genetic mapping. *Nucleic Acids Res* 19:303-306.
- Williams JGK, Rubelik AR, Livak KJ, Rafalski JA, Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 18:6531-6535.
- Kubota Y, Shimada A, Shima A. 1992. Detection of γ -ray-induced DNA damages in malformed dominant lethal embryos of the Japanese medaka (*Oryzias latipes*) using AP-PCR fingerprinting. *Mutat Res* 283:263-270.
- Peinado MA, Malkhosyan S, Velazquez A, Perucho M. 1992. Isolation and characterization of allelic losses and gains in colorectal tumors by arbitrarily primed polymerase chain reaction. *Proc Natl Acad Sci USA* 89:10065-10069.
- Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. 1993. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 363:558-561.
- Welsh J, Ralph D, McClelland M. 1995. DNA and RNA fingerprinting using arbitrary primed PCR. In Innis MA, Gelfand DH, Sninsky J, eds. *PCR Strategies*. Academic, San Diego, CA, USA, pp 249-275.
- Savva D. 1996. DNA fingerprinting as a biomarker assay in ecotoxicology. *Toxicol Ecotoxicol News* 3:110-114.
- Shimada A, Shima A. 1998. Combination of genomic DNA fingerprinting into the medaka specific-locus test system for studying environmental germ-line mutagenesis. *Mutat Res* 399:149-165.
- Akopyanz N, Bukanov NO, Westblom TU, Kresovich S, Berg DE. 1992. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res* 20:5137-5142.
- Atienzar F, Child P, Evenden A, Jha A, Savva D, Walker C, Depledge M. 1998. Application of the arbitrarily primed polymerase chain reaction for the detection of DNA damage. *Mar Environ Res* 46:331-335.
- Meunier JR, Grimont PAD. 1993. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. *Res Microbiol* 144:373-379.
- White JW, Neuwirth H, Miller CD, Schneider EL. 1990. DNA alterations in prostatic adenocarcinoma and benign prostatic hyperplasia: Detection by DNA fingerprint analyses. *Mutat Res* 237:37-43.
- Elendt BP, Bias WR. 1990. Trace deficiency in *Daphnia magna* cultured in standard medium for toxicity testing. Effects of the optimization of culture conditions on the life history parameters of *D. magna*. *Water Res* 24:1157-1167.
- U.S. Environmental Protection Agency. 1975. Method for acute toxicity tests with fish, macro-invertebrates and amphibians. EPA-660/3-75-009. Technical Report. Springfield, IL.
- Lotka AJ. 1925. *Elements of Physical Biology*. Williams & Wilkins, Baltimore, MD, USA.
- Krebs CJ. 1978. *Ecology*. Harper and Row, New York, NY, USA.
- Finney DJ. 1971. *Probit Analysis*. London Cambridge University Press, London, UK.
- Conradi M, Depledge MH. 1998. Population responses of the

- marine amphipod *Corophium volutator* (Pallas, 1766) to copper. *Aquat Toxicol* 44:31–45.
27. Christner FD, Lakshman MK, Sayer JM, Jerina DM, Dipple A. 1994. Primer extension by various polymerases using oligonucleotide templates containing stereoisomeric benzo[a]pyrene-deoxyadenosine adducts. *Biochemistry* 33:14297–14305.
28. Sancar A, Sancar GB. 1988. DNA repair enzymes. *Rev Biochem* 57:29–67.
29. Livneh Z, Cohen-Fix O, Skaliter R, Elizur T. 1993. Replication of damaged DNA and the molecular mechanism of ultraviolet light mutagenesis. *Crit Rev Biochem Mol Biol* 28:465–513.
30. Bales TC, Hogan ME. 1984. Site-specific carcinogen binding to DNA. *Proc Natl Acad Sci USA* 81:5623–5627.
31. Shugart L, McCarthy J, Jimenez B, Daniels J. 1987. Analysis of adduct formation in the bluegill sunfish (*Lepomis macrochirus*) between benzo[a]pyrene and hemoglobin of the erythrocyte. *Aquat Toxicol* 9:319–325.
32. Ide H, Petruccio LA, Hataet Z, Wallace SS. 1991. Processing of DNA base damage by DNA polymerases dihydrothymine and β -ureidobutyric acid as models for instructive and noninstructive lesions. *J Biol Chem* 266:1469–1477.
33. Lynch M. 1985. Elements of mechanistic theory for the life history consequences of food limitation. *Archiv für Hydrobiol Limnol* 21:351–362.
34. Enserik L, Haye M, Maas M. 1993. Reproductive strategy of *Daphnia magna*: Implications for chronic toxicity test. *Aquat Toxicol* 25:111–124.
35. Lampert W, Trubetskova I. 1996. Juvenile growth rate as a measure of fitness in *Daphnia*. *Funct Ecol* 10:631–635.
36. Calow P, Sibly RM, Forbes V. 1997. Risk assessment on the basis of simplified life-history scenarios. *Environ Toxicol Chem* 16:1983–1989.
37. Ellsworth DL, Rittenhouse KD, Honeycutt RL. 1993. Artefactual variation in randomly amplified polymorphic DNA banding patterns. *Biotechniques* 14:214–217.
38. Grayson TH, Cooper LF, Atienzar FA, Knowles MR, Gilpin ML. 1999. Molecular differentiation of *Renibacterium salmoninarum* isolates from worldwide locations. *Appl Environ Microbiol* 65:961–968.
39. Kubota Y, Shimada A, Shima A. 1995. DNA alterations detected in the progeny of paternally irradiated Japanese medaka fish (*Oryzias latipes*). *Proc Natl Acad Sci USA* 92:330–334.

Molecular Differentiation of *Renibacterium salmoninarum* Isolates from Worldwide Locations

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Renibacterium salmoninarum is a genospecies that is an obligate pathogen of salmonid fish and is capable of intracellular survival. Conventional typing systems have failed to differentiate isolates of *R. salmoninarum*. We used two methods to assess the extent of molecular variation which was present in isolates from different geographic locations. In one analysis we investigated possible polymorphisms in a specific region of the genome, the intergenic spacer (ITS) region between the 16S and 23S rRNA genes. In the other analysis we analyzed differences throughout the genome by using randomly amplified polymorphic DNA (RAPD). We amplified the spacer region of 74 isolates by using PCR and performed a DNA sequence analysis with 14 geographically distinct samples. The results showed that the 16S-23S ribosomal DNA spacer region of *R. salmoninarum* is highly conserved and suggested that only a single copy of the rRNA operon is present in this slowly growing pathogen. DNA sequencing of the spacer region showed that it was the same length in all 14 isolates examined, and the same nucleotide sequence, sequevar 1, was obtained for 11 of these isolates. Two other sequevars were found. No tRNA genes were found. We found that RAPD analysis allows reproducible differentiation between isolates of *R. salmoninarum* obtained from different hosts and different geographic regions. By using RAPD analysis it was possible to differentiate between isolates with identical ITS sequences.

Phylogenetically, *Renibacterium salmoninarum* is a member of the *Micrococcus-Arthrobacter* subdivision of the actinomycetes, a heterogeneous group of bacteria typified by high G+C contents (5, 22, 27, 38). *R. salmoninarum* is a slowly growing, fastidious organism with a narrow temperature range for optimal growth (10 to 20°C) and is an obligate pathogen of salmonid fish. This organism is distributed in much of the Northern Hemisphere and Chile and usually causes a chronic, systemic, granulomatous infection, bacterial kidney disease (BKD), which can be fatal under the appropriate conditions (14). The pathogen survives intracellularly and can be transmitted vertically within an ovum, as well as horizontally between cohabiting fish. There is no effective vaccine or chemotherapy. Furthermore, the presence of subclinical infections complicates attempts to control the disease through eradication programs.

The epidemiology of BKD, particularly the interactions which occur between wild and farmed salmonids, is unclear. This is mainly because attempts to differentiate between isolates of *R. salmoninarum* so far have been unsuccessful. This bacterium appears to possess remarkable biochemical uniformity, and no reliable serological means of distinguishing between isolates has been found (8, 19). A recent study of 40 isolates of *R. salmoninarum* from North America in which multilocus enzyme electrophoresis was used indicated that the level of genetic diversity was low (39). The lengthy periods required for growth of the bacterium (often 6 weeks or more) and the consequent degradation of antigenic or enzymically active components cause problems for studies which rely on the use of such components.

There are a variety of DNA-based methods available for differentiating between isolates, strains, and species of bacteria. The 16S-23S rRNA intergenic spacer (ITS) has proven to be useful for such differentiation in many cases (6, 17, 25, 28). The ITS appears to have a higher evolutionary rate than either 16S ribosomal DNA (rDNA) or 23S rDNA (28, 30) has, and there are variations in the ITS length and nucleotide sequence which make it possible to distinguish between closely related bacterial species and, sometimes, between strains and isolates (21). Incomplete 16S rRNA gene sequences of two isolates of *R. salmoninarum* have been determined (22, 29), but there have been no previous studies of either the 23S rRNA gene or the ITS of *R. salmoninarum*.

An alternative to using species-specific DNA sequences for isolate or strain differentiation involves a PCR-based method, randomly amplified polymorphic DNA (RAPD) analysis. Usually with this method, short random primers are used to rapidly detect genomic polymorphisms under low-stringency conditions (43, 45). RAPD analysis is widely used for differentiating between bacterial isolates (26, 42) and relies upon small quantities of genomic DNA, which makes it ideally suited to the study of slowly growing and fastidious organisms. We investigated the ITS and also performed a RAPD analysis of the *R. salmoninarum* genome in order to assess the potential of these methods for examining the molecular variability between isolates.

MATERIALS AND METHODS

Bacterial isolates. Seventy-four isolates of *R. salmoninarum* were used in this study. Table 1 shows the isolate designations, countries of origin, and sources of isolation and the GenBank accession numbers for the 16S-23S rRNA ITS sequences which were determined. *R. salmoninarum* was cultured in SKDM broth supplemented with 5% spent culture broth at 15°C (4, 15). The specificity control species (Table 2) were cultured on nutrient agar at 25°C.

DNA preparation and amplification of the ITS and specific *R. salmoninarum* genes. Genomic DNA was isolated by using a Puregene D-4000 DNA isolation kit according to the instructions of the manufacturer (Gentra Systems Inc.). DNA extracted by this method was electrophoresed on 1.2% agarose gels. Im-

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TABLE 1. *R. salmoninarum* isolates used in this study

Isolate	Geographic origin	Source ^a	Isolate	Geographic origin	Source ^a
970083-88	Southern England	Rainbow trout (f)	F-120-87(P-2)	Iceland	Atlantic salmon (f)
970083-102	Southern England	Rainbow trout (f)	F-130-87(P-4)	Iceland	Rainbow trout (f)
980106 #1.1.5 ^b	Southern England	Rainbow trout (f)	F-138-87(U-78)	Iceland	Atlantic salmon (f)
980036-150	Wales	Rainbow trout (f)	F-260-87(P-16)	Iceland	Atlantic salmon (f)
980036-87	Wales	Rainbow trout (f)	F-273-87(P-19)	Iceland	Atlantic salmon (f)
970419-1.2.3 ^b	Southern England	Atlantic salmon (w)	F-283-87(P-10)	Iceland	Atlantic salmon (f)
970153-19 ^c	Southern England	Grayling (w)	F-358-87(P-13)	Iceland	Atlantic salmon (f)
A6	Southern England	Rainbow trout (f)	S-182-90(P-7) ^r	Iceland	Atlantic salmon (f)
A60	Southern England	Rainbow trout (f)	Iwate	Japan	Coho salmon
W2	Northern England	Rainbow trout (f)	Siletz	Oregon	Coho salmon (f)
W6 ^d	Northern England	Rainbow trout (f)	Marion Forks ^b	Oregon	Chinook salmon (f)
WMV1	Southern England	Rainbow trout (f)	Little Goose	Washington	Chinook salmon (f)
MT409	Scotland	Unknown	CCM6205	Washington	Coho salmon (f)
MT417 ^e	Scotland	Atlantic salmon (f)	84-019-OC	Washington	Chinook salmon (w)
MT239	Scotland	Atlantic salmon (f)	SS-ChS-94-1	Oregon	Chinook salmon
MT426	Scotland	Unknown	Cow ChS94 P22	Washington	Chinook salmon (f)
NCIMB1111	Scotland	Atlantic salmon (w)	Idaho 91-126	Idaho	Sockeye salmon (f)
NCIMB1112	Scotland	Atlantic salmon (w)	RFL-643.94 #1	Washington	Sockeye salmon (f)
NCIMB1113	Scotland	Atlantic salmon (w)	CCM6206	Oregon	Chinook salmon (f)
NCIMB1114	Scotland	Atlantic salmon (w)	Round Butte	Oregon	Chinook salmon (f)
NCIMB1115	Scotland	Atlantic salmon (w)	NCIMB2196	Wyoming	Brook trout (f)
NCIMB1116	Scotland	Atlantic salmon (w)	ATCC 33209 ^f	Oregon	Chinook salmon (f)
MT420	Scotland	Atlantic salmon (f)	D-6	Oregon	Coho salmon
MT452	Scotland	Rainbow trout (f)	Cole River	Oregon	Unknown
MT1363	Scotland	Rainbow trout (f)	Looking Glass	Oregon	Unknown
MT410	Scotland	Unknown	AcF6-1 ^g	Northwest Territories, Canada	Arctic char (w)
FT-10	Scotland	Atlantic salmon	Rs 9 ^h	Sweden	Rainbow trout
BA99	Scotland	Atlantic salmon	Rs 19	Sweden	Atlantic salmon
DR143 ^b	Alberta, Canada	Brook trout (f)	Rs 61	Sweden	Arctic char
DR384	British Columbia, Canada	Coho salmon (f)	Rs 116	Sweden	Grayling
3784	British Columbia, Canada	Sockeye salmon (f)	Rs 122	Sweden	Rainbow trout (f)
980002	British Columbia, Canada	Chinook salmon (f)	Rs 125	Sweden	Rainbow trout
960023	British Columbia, Canada	Coho salmon (f)	Rs 126	Sweden	Rainbow trout
960046	British Columbia, Canada	Coho salmon (f)	3015-86 ^h	Norway	Atlantic salmon
GC96-1	British Columbia, Canada	Sockeye salmon (w)	4451-86	Norway	Atlantic salmon
DR-128	British Columbia, Canada	Rainbow trout (w)	DK98951714	Denmark	Rainbow trout (f)
RS-TSA	Nova Scotia, Canada	Atlantic salmon (f)	BY1996 ^h	Alaska	Chinook salmon (f)

^a Isolates were obtained from wild fish (w) or from farmed (captive reared) fish (f). The complete histories of some isolates are not known.

^b SV1 isolates whose 16S-23S rRNA ITS region was sequenced. The sequences have been deposited in the GenBank database under accession no. AF093462, AF093465, AF093466, AF093467, AF093468, AF093469, AF093470, AF093471, AF093472, and AF093473.

^c SV2 isolates whose 16S-23S rRNA ITS region was sequenced. The sequences have been deposited in the GenBank database under accession no. AF093463 and AF093474.

^d SV3 isolate whose 16S-23S rRNA ITS region was sequenced. The sequence has been deposited in the GenBank database under accession no. AF093464.

ages of each gel were captured with a Kodak model DC40 digital camera, and the DNA concentration was determined for each isolate by using Kodak Digital Science 1D Image Analysis software.

PCR amplification was performed with a DNA thermal cycler (Perkin-Elmer). The primers used to amplify the ITS sequence were selected from region 2 of the 16S rRNA gene sequence for *R. salmoninarum* and from two highly conserved regions corresponding to regions 5 and 7 of the 23S rRNA gene sequence (21) for *Micrococcus luteus* obtained from the GenBank database (29, 36). All of the primers (Table 3), including those used for amplification of the *R. salmoninarum* *msa*, *hly*, and *rsh* genes (9, 16, 20), were chosen by using Amplify software (13). Each 50- μ l reaction mixture contained 1 U of *Taq* polymerase (Boehringer Mannheim), reaction buffer (Boehringer Mannheim), 1.5 mM MgCl₂, 24 pmol of each primer, each deoxynucleoside triphosphate at a concentration of 0.2 mM, and 10 ng of bacterial DNA. The reaction mixtures were overlaid with mineral oil (Sigma), incubated at 96°C for 2 min, and then subjected to 25 cycles consisting of 96°C for 30 s, 65°C for 30 s, and 72°C for 90 s. Amplification products were analyzed on 1.5 and 2% agarose gels.

RAPD PCR. The RAPD analysis was performed with 19 isolates, and two separate methods were employed. First, a Ready-To-Go RAPD Analysis Beads kit (Pharmacia Biotech) containing six distinct random 10-mer primers, including primer P1 (GGTGGCGGAA), primer P2 (GTTTCGCTCC), primer P3 (GTA GACCCGT), primer P4 (AAGAGCCCGT), primer P5 (AACGCGCAAC), and primer P6 (CCCGTCAGCA), was used according to the manufacturer's instructions. Each 25- μ l reaction mixture contained 25 pmol of primer and 2.5 or 10 ng of template DNA. The reactions were performed in a Perkin-Elmer thermal cycler by using one cycle consisting of 95°C for 5 min and then 45 cycles consisting of 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min.

Second, the method described by Aizenar et al. (3) was used. Briefly, the following two primers were selected from the 10 primers in a kit obtained from Operon Technologies Inc.: primer OPA9 (GGGTAAACGCC) and primer OPB1 (GTTTCGCTCC). Each 25- μ l reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5.11 mM MgCl₂, 0.1% Triton X-100, 0.1% gelatin, each deoxynucleoside triphosphate at a concentration of 0.33 mM, 2 μ M primer, 2.5 μ g

TABLE 2. Reference organisms used

Species or group	Strain or source ^a
<i>Arthrobacter aureus</i>	NCIMB 8912
<i>Arthrobacter globiformis</i>	NCIMB 8907
<i>Arthrobacter nicotianae</i>	NCIMB 9458
<i>Bacillus cereus</i>	NCIMB 11925
<i>Brevibacterium linens</i>	NCIMB 8546
<i>Enterobacter aerogenes</i>	UPCC
<i>Enterococcus faecalis</i>	UPCC
<i>Listeria innocua</i>	UPCC
<i>Listeria ivanovi</i>	UPCC
<i>Listeria seeligeri</i>	UPCC
<i>Micrococcus luteus</i>	NCTC 7563
<i>Micrococcus roseus</i>	NCIMB 9149
<i>Rhodococcus equi</i>	UPCC
<i>Staphylococcus aureus</i>	UPCC
<i>Aeromonas hydrophila</i>	UPCC
<i>Aeromonas salmonicida</i>	UPCC
<i>Escherichia coli</i>	UPCC
<i>Escherichia coli</i> B	UPCC
<i>Escherichia coli</i> enteropathogenic	UPCC
<i>Klebsiella edwardii</i>	NCTC 10896
<i>Klebsiella pneumoniae</i>	NCIMB 418
<i>Pseudomonas aeruginosa</i>	ATCC 9027
<i>Pseudomonas fluorescens</i>	NCIMB 1953
<i>Serratia marcescens</i>	NCIMB 11783
<i>Vibrio anguillarum</i>	UPCC
<i>Vibrio parahaemolyticus</i>	UPCC
<i>Yersinia ruckeri</i>	UPCC

^a UPCC, University of Plymouth Culture Collection.

TABLE 3. Primers used to amplify the *R. salmoninarum* *msa*, *hly*, and *rsh* genes and the 16S-23S rDNA spacer region

Gene or region	Size of PCR product (bp)	Primer	
		Designation	Sequence (5'-3')
<i>msa</i>	487	Rs57+127 Rs57-611	TCGCAAGGTGAAGGGAATTCTTC GGTTGTCTCCAAAGGAGACTTGC
	297	Rs57+155 Rs57-449	CAACAGTACAAGGCTTCAGCAGTG CCGAAACCTACGTTTAGAGTCGTC
<i>hly</i>	542	RsMP+338 RsMP-877	ATCGGCTCAGACTAGCGCCATAAT GCTTCAAGATCGATGACCTTCGAG
	336	RsMP+487 RsMP-820	TTACTGAGGTCTTGATGGTCAGG CGATCGGTGCGGTCTATCAAGATA
<i>rsh</i>	572	RsH+231 RsH-810	TCCGGTCATCATGCTTTCTTCGCT ATTGCCACCAAGCTGAAGTACCTG
	374	RsH+401 RsH-772	TGCCCAATCTGAAGACAGCGACTA GGTCGATAATGCTCGTCATGCCTA
16S-23S spacer	751	RS+1002 ML-1329	CCGTCCAAGTCACGAAAGTTGGTA ATCGCAGATTCCACGCTCTTCTT
	895	RS+1002 ML-1469	CCGTCCAAGTCACGAAAGTTGGTA GTGGGTACTGAGATGTTTCAGTTC

of bovine serum albumin, 2.8 U of *Taq* DNA polymerase (Immunogen International), and 2.5 or 10 ng of template DNA. The reactions were performed in a Perkin-Elmer thermal cycler by using one cycle consisting of 95°C for 5 min, 39 cycles consisting of 95°C for 1 min, 50°C for 1 min, and 74°C for 1 min, and one cycle consisting of 95°C for 1 min, 50°C for 1 min, and 74°C for 10 min. The PCR products were analyzed on 1.2% agarose gels in Tris-borate-EDTA buffer.

Images of each gel were captured with a Kodak model DC40 digital camera, and the DNA profile was analyzed by using Kodak Digital Science 1D Image Analysis software.

Sequence analysis. PCR products spanning the ITS were sequenced directly by a cycle sequencing method and were aligned by workers at MWG-Biotech Ltd., Milton Keynes, United Kingdom. The *R. salmoninarum* sequences were compared with the sequences of other organisms obtained from the GenBank database by using the gapped BLAST program (1) and the GeneStream align program (IGH, Montpellier, France) (33).

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences determined in this study are shown in Table 1.

RESULTS

Amplification of specific *R. salmoninarum* genes. In order to confirm the identity of the DNA extracted from *R. salmoninarum* cultures, six sets of primers were designed to amplify known regions of three *R. salmoninarum* genes. For each of the 74 isolates of *R. salmoninarum* tested in the six PCR a single band of the appropriate size was amplified (Table 3). No amplification products were obtained from PCR mixtures containing template DNA derived from the specificity control species or from any of the interspersed negative controls.

Amplification of the 16S-23S rDNA spacer region. The ITS of 74 isolates of *R. salmoninarum* were amplified by using primers for highly conserved sequences near the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene. Primers RS+1002 and ML-1329 amplified a 751-bp fragment, while primers RS+1002 and ML-1469 amplified a 895-bp fragment. In every case only a single band was detected with the primers that were used. In addition, for each primer set no size differences were detected on 1.5 or 2% agarose gels.

SV1	ccgtccaagtcacgaaagtggtaacacccgaagccggtggcctaaccctttggggagggaacgcgcgaaggtgggactggcgattgggactaagtcgta	100
SV2	
SV3	
SV1	acaaggtagecgtaccggaaggtgcggctggatcacctcctttctaaaggagcaccacacacttgatcagtttccgcgatgtttctgggtgagtgtagg	200
SV2	
SV3	
SV1	agtttaaatcccatctgcgagaaacattcgtttctcggtgggtgctcaagggtggaatatcaacaaatttatgctgaaagtgctggttttctgggtccggagggtg	300
SV2G.....	
SV3G.....	
SV1	ttgtcgtcgttctgttctcggattgttggttgagtacgctgtttccttcggggagtggttgaaagattcgatggtttgggaggttctggtgggtacaa	400
SV2	
SV3	
SV1	aacacactgtttgggtccttgagataacaggcgcctctgctgattctggtactgacttttggttggttttgagaggtcggtgggggtgtgttgttgtttctggt	500
SV2A.....	
SV3T.....	
SV1	ttcccttcttactaatcaatccttttccgtttgttcgggtgggtgtgtgtgggagtggtgtgtgttttgagaaactgtatagtgagacccgagcatctta	600
SV2	
SV3	
SV1	agaacaaagcaatttgggttaagagcaatttttcatcacatcttttgggtggtgagagtttttagctctggtttttgtgttcaagtttttaagggcaca	700
SV2	
SV3A.....	
SV1	cggatgaatgccttggcattaggagccgaagaaggacgtaggaatctcgataagcctcggtgggtgtgataaccgaacactgatccgaggatgtccgaatg	800
SV2	
SV3	
SV1	gggaaaccccgctgcctgttatgggtagtgaaccacatctgaacacataggggtgtgtggagggaacgtggggaactgaacacatctcagtagccac	895

FIG. 1. SV1, SV2, and SV3 of the 16S-23S rRNA ITS of *R. salmoninarum*. The isolates with each sequence are identified in Table 1. The sequence of the region from nucleotide 1 to nucleotide 750 was determined for 14 isolates by using PCR-amplified products obtained with primers RS+1002 and ML-1329. The sequence for nucleotides 1 to 895 was confirmed for type strain ATCC 33209 by using PCR-amplified products obtained with primers RS+1002 and ML-1469. The uppercase letters represent the 534-bp ITS sequence. The lowercase letters for nucleotides 1 to 145 represent the 3' end of the *R. salmoninarum* 16S rRNA gene (22, 29), while the final 216 bp represents the 5' end of the *R. salmoninarum* 23S rRNA gene. The three regions that are substantially the same in members of the actinomycetes are underlined.

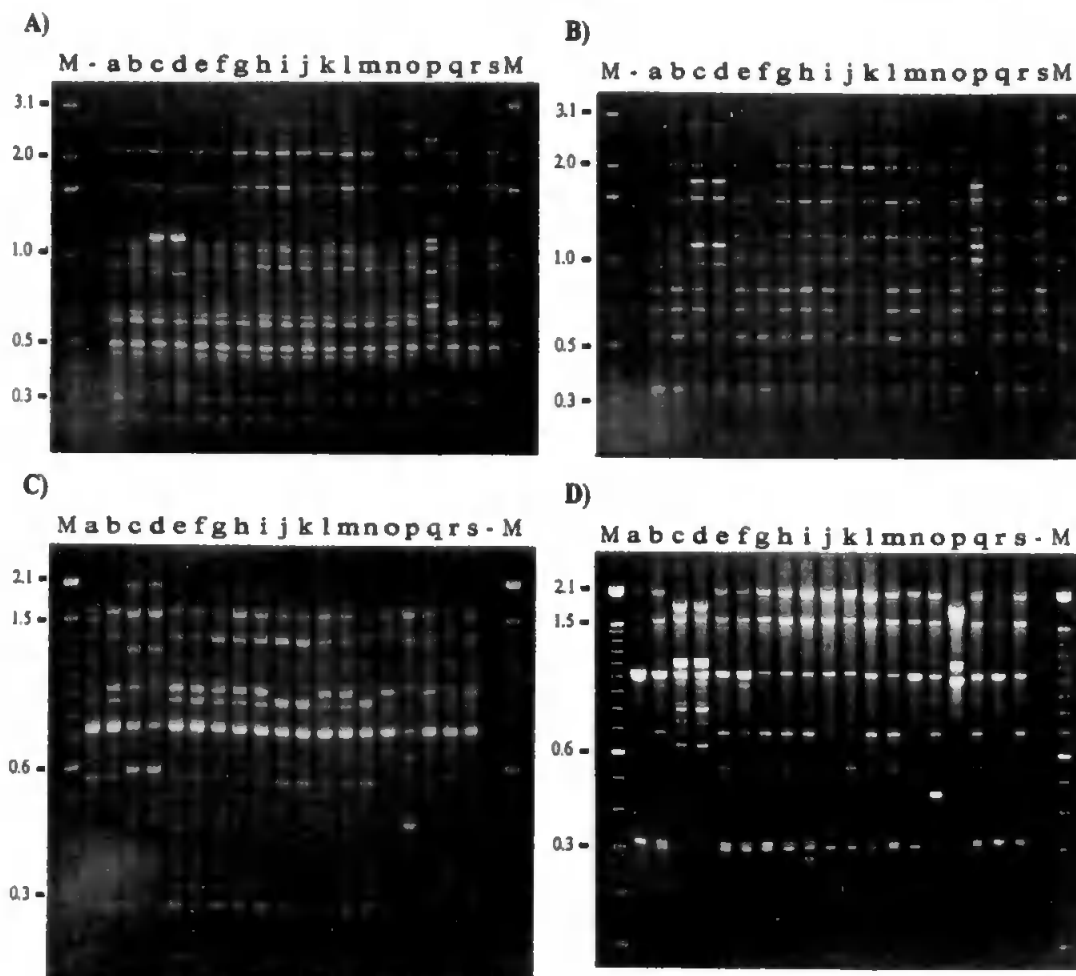


FIG. 2. RAPD fingerprints of 19 isolates of *R. salmoninarum* from a variety of geographic areas and a variety of host species. The DNA fingerprints were obtained by PCR amplification with primers OPA9 (A), OPB1 (B), P1 (C), P2 (D), P3 (E), P4 (F), P5 (G), and P6 (H). Lane a, isolate DR384; lane b, isolate DR128; lane c, isolate ATCC 33209; lane d, isolate Round Butte; lane e, isolate W2; lane f, isolate W6; lane g, isolate Little Goose; lane h, isolate MT1363; lane i, isolate BA99; lane j, isolate S-182-90; lane k, isolate F-273-87; lane l, isolate RS-TSA; lane m, isolate MT410; lane n, isolate F-138-87; lane o, isolate NCIMB2196; lane p, isolate Manon Forks; lane q, isolate MT417; lane r, isolate DR143; lane s, isolate MT420; lane —, water control. Lanes M contained markers (1-kb DNA ladder [Gibco BRL]) in panels A and B and 100-bp DNA ladder [Gibco BRL] in panels C to H. The molecular sizes (in kilobases) are indicated on the left.

Sequencing of the ITS from total PCR products and sequence analysis. The complete 16S-23S rDNA spacer region sequences of 14 *R. salmoninarum* isolates were determined by directly sequencing PCR-amplified products. PCR products were amplified with primers RS+1002 and ML-1329, which bind to highly conserved regions 2 and 5 of the 16S-23S rRNA operon (21). Only a single unambiguous sequence was obtained for each PCR product generated. We found that all of the isolates possessed ITS sequences that were the same length, 534 bp. Furthermore, 11 isolates had the same nucleotide sequence, which was designated sequevar 1 (SV1) (Fig. 1). These 11 isolates were obtained from a broad geographic area, which included the mainland United States, Alaska, Canada, Sweden, England, Scotland, and Norway, and from a variety of host salmonid fish species, including chinook salmon, Atlantic salmon, rainbow trout, brook trout, and grayling. Only three isolates possessed spacer regions whose sequences differed from this sequence. The sequences of isolates S-182-90 and Iwate, obtained from Atlantic salmon from Iceland and coho salmon from Japan, respectively, exhibited three identical

single-base differences, and the ITS sequence of these organisms was designated sequevar 2 (SV2). Sequevar 3 (SV3), the ITS sequence of isolate AcF6-1 obtained from Arctic char from the Northwest Territories of Canada, also exhibited three single-base differences, one of which was also found in the ITS sequences of S-182-90 and Iwate (Fig. 1). In order to confirm that the ITS sequences obtained by PCR amplification with primers RS+1002 and ML-1329 each represented a single homogeneous copy of the 16S-23S rRNA ITS region, we sequenced the PCR product amplified with primers RS+1002 and ML-1469 from the genome of type strain ATCC 33209. Primer ML-1469 binds deeper in the 23S rRNA gene than primer ML-1329 in highly conserved region 7 (21). The single unambiguous sequence obtained in this way exactly matched the sequences obtained for ATCC 33209 and the 10 other SV1 isolates by using primers RS+1002 and ML-1329.

The *R. salmoninarum* ITS exhibited 34 to 47% identity with the 16S-23S rDNA spacer region sequences of actinomycetes in the GenBank database. Three regions that were approximately 20, 27, and 35 bp long (Fig. 1) were found to be highly

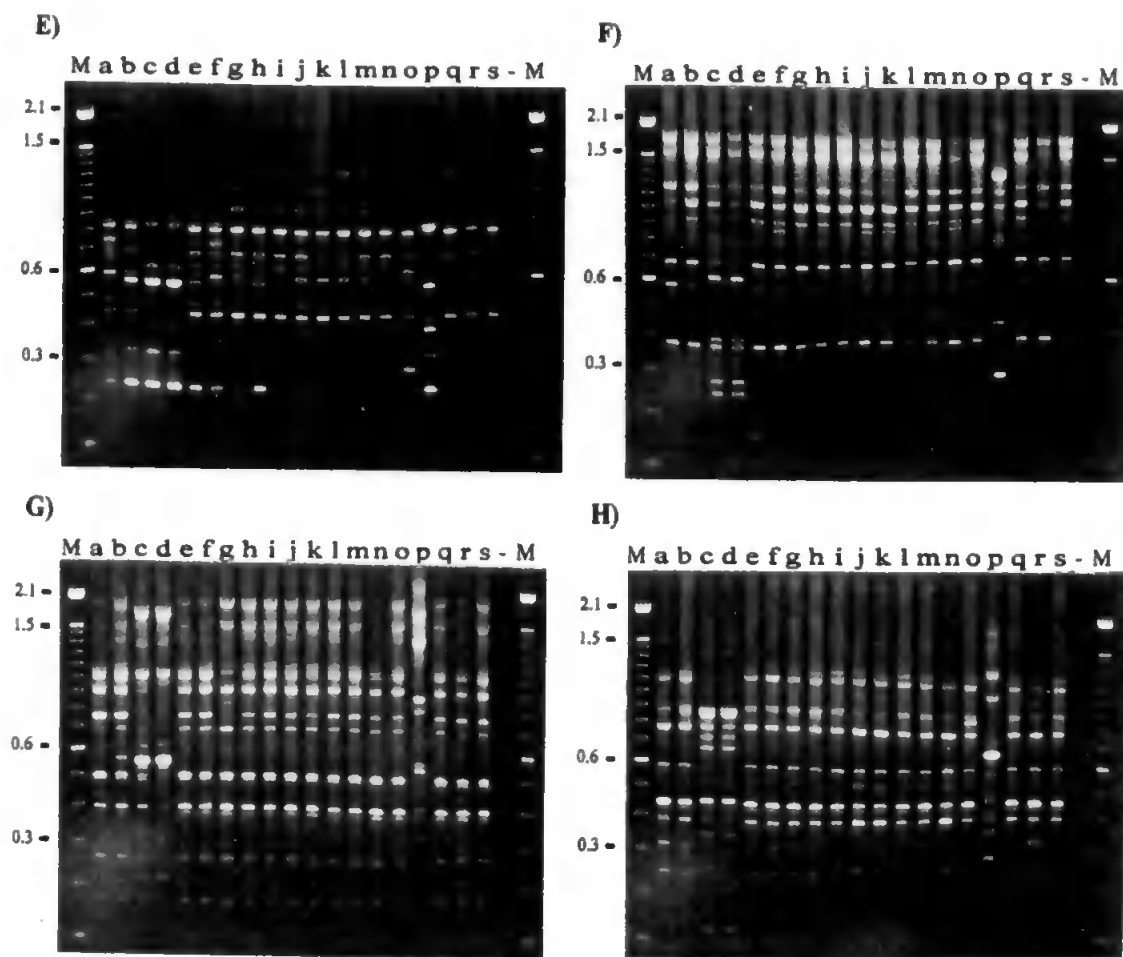


FIG. 2—Continued.

conserved in a number of other members of the actinomycetes, including *Bifidobacterium* sp., *Brevibacterium* sp., *Kitasatospora* sp., *Rhodococcus erythropolis*, *Streptomyces* sp., *Microtetraspora* sp., and *Sireptosporangium* sp. Sequences for members of the genera *Arthrobacter* and *Micrococcus*, two genera which are closely related to *R. salmoninarum*, were not available in the database and hence were not included in the comparison.

RAPD analysis as a means of differentiating isolates. We observed that with all of the primers the geographic origins of 19 isolates were reflected in the RAPD band patterns. Using eight random primers and two RAPD methods, we discerned three arbitrary groups of isolates visually (Fig. 2). Group 1 contained isolates from Canada (Fig. 2, lanes a, b, l, and r), Scotland (lanes h, i, m, q, and s), and England (lanes e and f), as well as two isolates from the United States isolates (lanes g and o); group 2 contained isolates from Iceland (lanes j, k, and n); and group 3 contained the other isolates from the United States (lanes c, d, and p). None of the isolates produced identical RAPD patterns with the eight primers, and in most cases, using two or three primers revealed differences between isolates. We chose primers which consistently gave a distinct and reproducible band pattern for each isolate tested. However, primers P2, P3, P4, P5, and P6 gave the clearest and most

discriminatory patterns for each isolate regardless of origin. When these primers were used, it was possible to identify differences between isolates from the same country; e.g., primers P2 and P6 discriminated between Icelandic isolates, while primers P2, P3, and P4 revealed differences between English isolates. Differences in RAPD fingerprints could not be attributed to the presence of plasmid DNA. We previously examined DNA extracts of more than 70 *R. salmoninarum* isolates and found no evidence of plasmid DNA (unpublished data). In order to assess the reproducibility and variation of RAPD fingerprinting, we performed PCR reamplification analyses by using all of the primers, DNA extracted from *R. salmoninarum* cultures on different occasions, and two DNA template concentrations, as recommended by Welsh et al. (44). Small differences in the quality and concentrations of two templates can lead to spurious differences in the RAPD pattern; therefore, every experiment should include at least two concentrations of genomic DNA for each individual. The results obtained with two primers, primers OPA9 and P1, are presented in Fig. 3. DNA fingerprints were very reproducible; the only discrepancies were confined to the presence or absence of faint bands. The intensities of these faint bands would render them below the limit for inclusion in any analysis of DNA fingerprints.

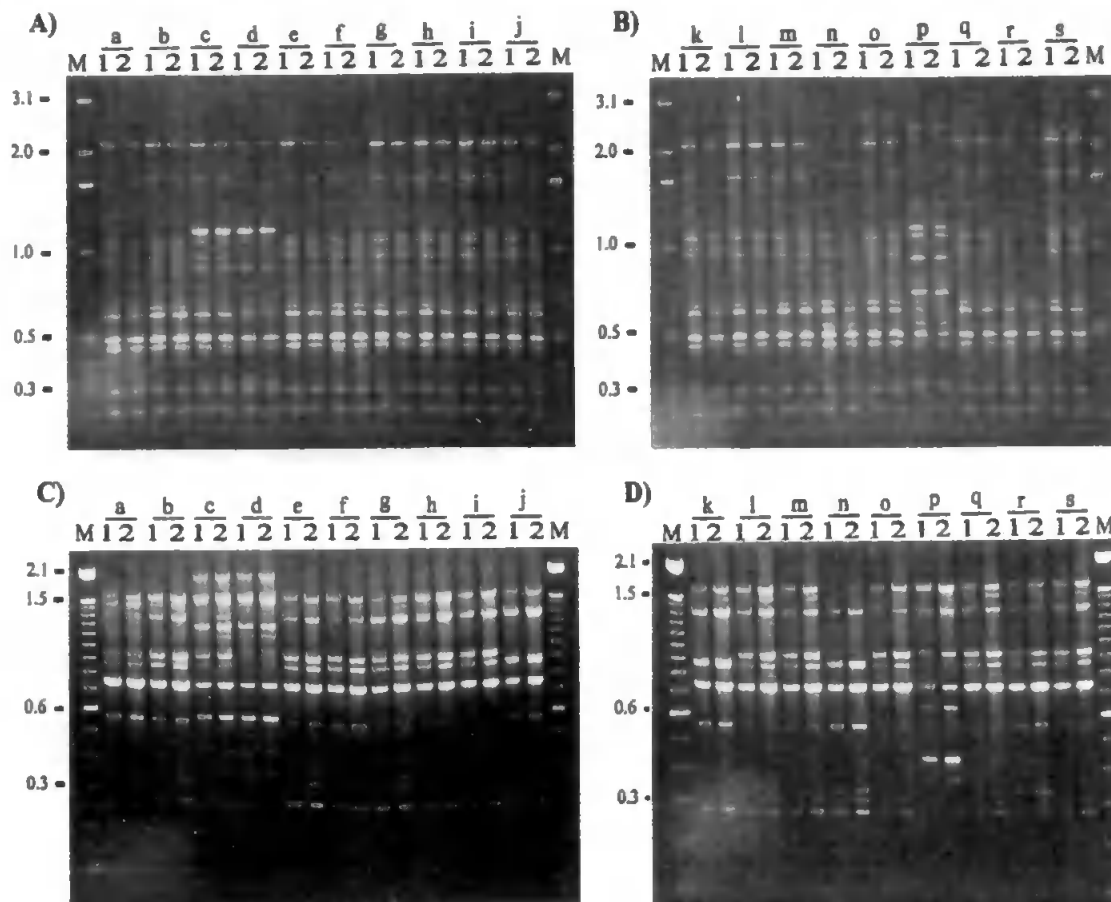


FIG. 3. Reproducibility of RAPD fingerprinting. The DNA fingerprints for DNA extracted on separate occasions with two different concentrations of template were obtained after PCR amplification with primers as described in the legend to Fig. 2. For the contents of lanes a to s see the legend to Fig. 2. Lanes 1, 2.5 ng of DNA template; lanes 2, 10 ng of DNA template. Only the results obtained with the following two primers are shown: primer OPA9 (A and B) and primer P1 (C and D). Lane M contained markers (see the legend to Fig. 2). The molecular sizes (in kilobases) are indicated on the left.

DISCUSSION

The widespread distribution of *R. salmoninarum* in the United Kingdom, many European countries, Japan, North America, and Chile and the variety of salmonid host species in these regions suggested the possibility that the genetic diversity of isolates in these areas may be reflected by the number, length, and sequence of the 16S-23S rRNA ITS region. While inter- and intragenic relationships may be elucidated by examining 16S and 23S rDNA sequences, the ITS has provided information on intraspecific relationships in other bacteria (17, 28, 30, 40). Three distinct ITS sequences (sequevars) were obtained from 14 *R. salmoninarum* isolates. Isolates from Iceland, Japan, and the Canadian Northwest Territories which had three single-base substitutions in the ITS exhibited some divergence from the highly conserved SV1 which was present in isolates from the United States, the United Kingdom, mainland Europe, and Alberta, Canada. It may be that in areas of the world which could be regarded as relatively isolated from the mainstream intensive salmonid culture areas of North America and Europe the bacterium has diverged from this pattern. It is interesting that the sole Alaskan isolate was an SV1 isolate. BKD has been reported in wild and farmed fish from a number of Alaskan river systems (11, 32), and it seems

likely that Alaskan salmon have been exposed to the sequevar of *R. salmoninarum* carried by salmon from the Pacific coast of Canada or the United States at some stage during their oceanic migrations.

This study provides no evidence that there are multiple copies of the rRNA operon in *R. salmoninarum*. A single unambiguous nucleotide sequence was obtained for all of the isolates examined, and 11 of the isolates possessed spacer regions that had the same nucleotide sequence. The presence of a nucleotide sequence generated from a highly conserved region deeper in the 23S rRNA gene confirmed these results. Typical tRNA genes were not found in the ITS region of *R. salmoninarum*. Furthermore, we found no evidence that there were multiple amplicons in PCR mixtures when we used two sets of primers for highly conserved regions of the 16S and 23S rRNA genes. However, absolute proof that there is a single rRNA operon would require direct sequencing from the genome. We concluded that *R. salmoninarum* probably has a single copy of the rRNA operon, a finding which is consistent with what has been described for a number of other slowly growing organisms (2, 7, 18, 37, 40) and is a further indication of the conservative genetic composition of this obligate pathogen. Generally, our findings suggest that the 16S-23S rDNA

spacer region is of limited use for routine discrimination between *R. salmoninarum* isolates but may offer some clues as to geographic origins.

The lack of a way to differentiate between isolates of *R. salmoninarum* has constrained epidemiological studies of BKD. In particular, development of a means of contact tracing would allow BKD outbreaks to be traced back to the source of infection and would help resolve some of the difficulties associated with investigation of the interactions between farmed and wild salmonid fish. We used two methods to do this, examination of ITS variation and RAPD analysis, which have been used successfully in studies of other bacteria. Our work shows that compared with ITS variation, RAPD analysis is a better method for discriminating between isolates of *R. salmoninarum*. In our study, *R. salmoninarum* isolates from a variety of sources, some with identical 16S-23S spacer region DNA sequences, could be distinguished on the basis of RAPD patterns generated by two different methods. RAPD analysis has provided a reliable and reproducible method for molecular typing and genetic characterization of a variety of microorganisms (23, 24, 34, 41). This method is particularly useful for examining the genomic diversity among strains of bacteria which are indistinguishable by other molecular methods. For example, RAPD analysis of strains of *Bacillus cereus* revealed a remarkable diversity which was not revealed by rRNA or tRNA ITS-targeted PCR (10). A number of factors have been identified as influencing the outcome of RAPD fingerprinting (12, 31, 35). In our studies, using eight primers and two different methods for PCR amplification of purified DNA template produced RAPD fingerprints which were reproducible with two different DNA concentrations and with DNA extracted on different occasions. In every case, RAPD fingerprints distinguished the same groups of isolates.

So far, *R. salmoninarum* has defied attempts to find a reproducible way to differentiate between isolates. This study is the first study which revealed the genetic diversity within the species by using a DNA-based method for differentiating between isolates from a wide variety of sources and therefore represents a substantial advance in our understanding of a fastidious intracellular pathogen which is capable of surviving within its host in very low numbers. We are extending our investigations of *R. salmoninarum* by using RAPD analysis in conjunction with other molecular typing methods as part of a coordinated program to examine farm and wild *R. salmoninarum* isolates from the United Kingdom and other sources. This work should result in a wide-ranging analysis of isolate differences.

In conclusion, *R. salmoninarum* is a highly conserved genotype. The molecular variation in the sequence of the 16S-23S rDNA spacer region of isolates from widely separated environments is extremely limited. RAPD analysis is a reliable and reproducible technique for discriminating between isolates of *R. salmoninarum* and should facilitate epidemiological studies of this pathogen.

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REFERENCES

1. Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
2. Amikam, D., G. Glaser, and S. Razin. 1984. *Mycoplasmas (Mollicutes)* have a low number of rRNA genes. *J. Bacteriol.* 158:376-378.
3. Allenzar, F., P. Child, A. Evenden, A. Jha, D. Savva, C. Walker, and M. Depledge. 1998. Application of the arbitrarily primed polymerase chain reaction for the detection of DNA damage. *Mar. Environ. Res.* 46:331-335.
4. Austin, B., T. M. Embley, and M. Goodfellow. 1983. Selective isolation of *Renibacterium salmoninarum*. *FEMS Microbiol. Lett.* 17:111-114.
5. Banner, C. R., J. S. Robovec, and J. L. Fryer. 1991. A new value for mol percent guanine + cytosine of DNA for the salmonid fish pathogen *Renibacterium salmoninarum*. *FEMS Microbiol. Lett.* 79:57-60.
6. Barry, T., G. Colleran, M. Glennon, L. K. Dunne, and P. Gannon. 1991. The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. *PCR Methods Appl.* 1:51-56.
7. Bercovier, H., O. Kafri, and S. Sela. 1986. *Mycobacteria* possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. *Biochem. Biophys. Res. Commun.* 136:1136-1141.
8. Bruno, D. W., and A. L. S. Munro. 1986. Uniformity in the biochemical properties of *Renibacterium salmoninarum* isolates obtained from several sources. *FEMS Microbiol. Lett.* 33:247-250.
9. Chlen, M.-S., T. L. Gilbert, C. Huang, M. L. Landolt, P. J. O'Hara, and J. R. Winton. 1992. Molecular cloning and sequence analysis of the gene coding for the 57-kDa major soluble antigen of the salmonid fish pathogen *Renibacterium salmoninarum*. *FEMS Microbiol. Lett.* 96:259-266.
10. Daffonchio, D., S. Borin, G. Frova, P. L. Manachini, and C. Sorlini. 1998. PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. *Int. J. Syst. Bacteriol.* 48:107-116.
11. Dildler, A. J. Jr. 1981. Bacterial kidney disease, p. 361-367. In R. A. Dietrich (ed.), *Alaskan wildlife diseases*. University of Alaska, Fairbanks.
12. Ellsworth, D. L., K. D. Rittenhouse, and R. L. Honeysett. 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. *Bio-Techniques* 14:214-217.
13. Engels, W. R. 1993. Contributing software to the Internet: the Amplify program. *Trends Biochem. Sci.* 18:448-450.
14. Evelyn, T. P. T. 1993. Bacterial kidney disease—BKD, p. 177-195. In V. Inglis, R. J. Roberts, and N. R. Bromage (ed.), *Bacterial diseases of fish*. Blackwell, Oxford, United Kingdom.
15. Evelyn, T. P. T., L. Prosperi-Porta, and J. E. Ketcheson. 1990. Two new techniques for obtaining consistent results when growing *Renibacterium salmoninarum* on KDM2 culture medium. *Dis. Aquat. Org.* 9:209-212.
16. Evenden, A. J. 1993. The use of gene cloning techniques in the study of the fish pathogen *Renibacterium salmoninarum*. Ph.D. thesis. University of Plymouth, Plymouth, United Kingdom.
17. Forsman, P., A. Talsala-Timijarvi, and T. Alatossava. 1997. Identification of staphylococcal and streptococcal causes of bovine mastitis using 16S-23S rRNA spacer regions. *Microbiology* 143:3491-3500.
18. Frothingham, R., and K. H. Wilson. 1993. Sequence-based differentiation of strains in the *Mycobacterium avium* complex. *J. Bacteriol.* 175:2818-2825.
19. Goodfellow, M., T. M. Embley, and B. Austin. 1985. Numerical taxonomy and emended description of *Renibacterium salmoninarum*. *J. Gen. Microbiol.* 131:2739-2752.
20. Grayson, T. H., A. J. Evenden, M. L. Gilpin, K. L. Martin, and C. B. Munn. 1995. A gene from *Renibacterium salmoninarum* encoding a product which shows homology to bacterial zinc-metalloproteases. *Microbiology* 141:1331-1341.
21. Gurtler, V., and V. A. Stanisich. 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology* 142:3-16.
22. Guttenberger, S. K., S. J. Giovannoni, K. G. Field, J. L. Fryer, and J. S. Robovec. 1991. A phylogenetic comparison of the 16S rRNA sequence of the fish pathogen, *Renibacterium salmoninarum*, to Gram-positive bacteria. *FEMS Microbiol. Lett.* 77:151-156.
23. Haase, A., H. Smith-Vaughan, A. Melder, Y. Wood, A. Janmant, J. Giffeder, D. Kemp, and B. Currie. 1995. Subdivision of *Burkholderia pseudomallei* ribotypes into multiple types by random amplified polymorphic DNA analysis provides new insights into epidemiology. *J. Clin. Microbiol.* 33:1687-1690.
24. Hansen, B. M., P. H. Damgaard, J. Eilenberg, and J. C. Pedersen. 1998. Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated

- from leaves and insects. *J. Invertebr. Pathol.* 71:106-114.
25. Jensen, M. A., J. A. Webster, and N. Straus. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl. Environ. Microbiol.* 59:945-952.
26. Kersulyte, D., J. P. Woods, E. J. Keith, W. E. Goldman, and D. E. Berg. 1992. Diversity among clinical isolates of *Histoplasma capsulatum* detected by polymerase chain reaction with arbitrary primers. *J. Bacteriol.* 174:7075-7079.
27. Koch, C., F. A. Rainey, and E. Stackebrandt. 1994. 16S rDNA studies on members of *Arthrobacter* and *Micrococcus*: an aid for their future taxonomic restructuring. *FEMS Microbiol. Lett.* 123:167-172.
28. Leblond-Bourget, N., H. Philippe, I. Mangin, and B. Decaris. 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. *Int. J. Syst. Bacteriol.* 46:102-111.
29. Magnusson, H. B., O. H. Fridjonsson, O. S. Andresson, E. Benediktsson, S. Gudmundsdottir, and V. Andressdottir. 1994. *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonid fish, detected by nested reverse transcription-PCR of 16S rRNA sequences. *Appl. Environ. Microbiol.* 60:4580-4583.
30. Mauel, M. J., S. J. Giovannoni, and J. L. Fryer. Phylogenetic analysis of *Piscirickettsia salmonis* by 16S, internal transcribed spacer (ITS) and 23S ribosomal DNA sequencing. *Dis. Aquat. Org.*, in press.
31. Meunier, J. R., and P. A. Grimont. 1993. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. *Res. Microbiol.* 144:373-379.
32. Meyers, T. R., S. Short, C. Farrington, H. J. Lipson, H. J. Gelger, and R. Gates. 1993. Establishment of a negative-positive threshold optical density value for the enzyme-linked immunosorbent assay (ELISA) to detect soluble antigen of *Renibacterium salmoninarum* in Alaskan Pacific salmon. *Dis. Aquat. Org.* 16:191-197.
33. Myers, E., and W. Miller. 1988. Optimal alignments in linear space. *CABIOS* 4:11-17.
34. Nakao, H., and T. Popovic. 1998. Use of random amplified polymorphic DNA for rapid molecular subtyping of *Corynebacterium diptheriae*. *Diagn. Microbiol. Infect. Dis.* 30:167-172.
35. Penner, G. A., A. Bush, and R. Wise. 1993. Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. *PCR Methods Appl.* 2:341-345.
36. Regensburger, A., W. Ludwig, R. Frank, H. Blocker, and K. H. Schleifer. 1988. Complete nucleotide sequence of a 23S ribosomal RNA gene from *Micrococcus luteus*. *Nucleic Acids Res.* 16:2344.
37. Sela, S., J. E. Clark-Curtiss, and H. Bercovier. 1989. Characterization and taxonomic implications of the rRNA genes of *Mycobacterium leprae*. *J. Bacteriol.* 171:70-73.
38. Stackebrandt, E., U. Wehmeyer, H. Nader, and F. Fiedler. 1988. Phylogenetic relationship of the fish pathogenic *Renibacterium salmoninarum* to *Arthrobacter*, *Micrococcus* and related taxa. *FEMS Microbiol. Lett.* 50:117-120.
39. Startipier, C. E. 1996. Genetic diversity of North American isolates of *Renibacterium salmoninarum*. *Dis. Aquat. Org.* 27:207-213.
40. Van der Giesen, J. W. B., R. M. Haring, and B. A. M. van der Zeijst. 1994. Comparison of the 23S ribosomal RNA genes and the spacer region between the 16S and 23S rRNA genes of the closely related *Mycobacterium avium* and *Mycobacterium paratuberculosis* and the fast-growing *Mycobacterium phlei*. *Microbiology* 140:1103-1108.
41. Wang, G., A. P. van Dam, L. Spanjaard, and J. Dankert. 1998. Molecular typing of *Borrelia burgdorferi* sensu lato by randomly amplified polymorphic DNA fingerprinting analysis. *J. Clin. Microbiol.* 36:768-776.
42. Wang, G., W. T. S. Wittam, C. M. Berg, and D. M. Berg. 1993. RAPD (arbitrary primer) PCR is more sensitive than multilocus enzyme electrophoresis for distinguishing related bacterial strains. *Nucleic Acids Res.* 21:5930-5933.
43. Welsh, J., and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* 18:7213-7218.
44. Welsh, J., D. Ralph, and M. McClelland. 1995. DNA and RNA fingerprinting using arbitrarily primed PCR, p. 249-275. In M. A. Innis, D. H. Gelfand, and J. J. Sninsky (ed.), *PCR strategies*. Academic Press, London, United Kingdom.
45. Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531-6535.

Benchmarks

Optimized RAPD Analysis Generates High-Quality Genomic DNA Profiles at High Annealing Temperature

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Techniques such as arbitrarily primed polymerase chain reaction (AP-PCR) (11), random amplified polymorphic DNA (RAPD) (12) and DNA amplification fingerprinting (DAF) (7) are powerful tools for genetic mapping, taxonomic and phylogenetic investigations and for the detection of various types of DNA damage and mutation (2,3). Nevertheless, the arbitrary nature of the DNA polymerization catalyzed in these methods has drawn criticism because of the low annealing temperature used with short primers. In this study, the RAPD method was optimized at an annealing temperature of 50°C for 10-mer primers. The main advantage of high stringency conditions is that nonspecific reactions are significantly reduced. Thus, protocols that use a high annealing temperature should be always preferred if the sensitivity of polymorphism detection is not jeopardized.

Although DNA profiles have been generated at a high annealing temperature for short primers (5,6,8), an optimization study using high annealing temperature has rarely been attempted, to our knowledge. Indeed, it is generally believed that repeating a PCR assay is jeopardized by annealing temperatures higher than the T_d of the oligonucleotide primer. For instance, it has been reported that annealing temperatures above 40°C prevent amplification by 10-mer primers (12). Relatively low annealing temperatures (34°C–36°C) are used in RAPD to ensure a maximal number of primer binding events and the consequent generation of many amplified DNA fragments for analytical purposes. However, the low stringency of the accompanying DNA hybridization can result in the formation of spurious amplifications (9) that affect both the reproducibility (8) and the detection of Mendelian inheritance patterns (4).

The RAPD method was developed

Table 1. Optimized Conditions to Produce High-Quality RAPD Profiles at High Annealing Temperatures

PCR Parameters	Optimized Conditions
Annealing temperature	50°C–54°C (50)
Mg ⁺⁺ concentration	3–6 mM (5.11)
dNTP concentration	0.33–0.44 µM (0.33)
Primer concentration (10-mer)	1.0–2.0 µM (2.0)
Number of native Taq DNA polymerase units	0.08–0.12 U/µL (0.112 U/µL)
Amount of DNA	0.008–4 ng/µL (0.2–0.8 ng/µL))
PCR buffer	1× (10 mM Tris-HCl pH 8.8 at 25°C, 50 mM KCl, 0.08% Nonidet P40)
Bovine serum albumin	0.1 µg/µL
Thermal cycling conditions	First cycle, denaturation at 95°C for 4 min, followed by 39 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, extension at 74°C for 1 min, except the final extension at 74°C for 10 min.

PCR reagents were obtained from Immunogen International (Sunderland, England, UK) and 10-mer primers from Operon Technologies (Southampton, England, UK). Figures in parentheses represent conditions used throughout the present study. Final reaction volume was 25 µL. For more details, refer to Reference 2 or 3.

as a result of the sequential and systematic analysis of the RAPD protocol performed by the variation of annealing temperatures, DNA purity, primer sequence and the relative concentration of each PCR reagent. The objective was to generate reproducible DNA profiles of high discrimination with a maximum number of bands, good product yield and clarity, while reducing the occurrence of spurious amplifications in the negative control reactions. Initially, the parthenogenetically produced offspring of *Daphnia magna* (clone 5) (2) was chosen to eliminate the possibility of confusing genomic changes from sexual reproduction. The optimization work was also performed using other species belonging to the bacteria, plant and animal kingdoms. Table 1 shows the results of the optimization study. Contrary to RAPD methods using low annealing temperatures, consistent genomic profiles were generated when component concentrations were sub-

jected to variation, within the predefined optimal conditions.

DNA of good purity and free from other macromolecules and inhibitory compounds produces clear and discriminatory RAPD profiles. For the bulk of our experiments, genomic DNA prepared by a standard phenol/chloroform extraction purification was sufficient to produce RAPD profiles of high quality; profiles of identical quality were obtained using either phenol chloroform or caesium chloride extracted DNA. Further experiments also confirmed that the storage buffer (analytical grade water or 1× Tris-Borate-EDTA buffer) had no influence on RAPD profiles. In addition, under optimized conditions, identical RAPD profiles were generated whenever the DNA extraction or PCR was performed. The DNA concentration was also found to be crucial in the production of reproducible genomic profiles, not only to ensure the largest number of amplified bands but also to

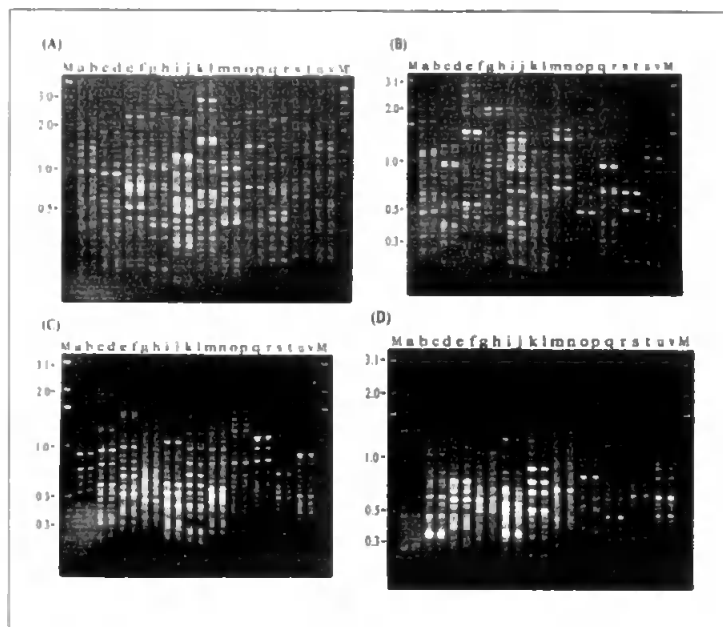


Figure 1. RAPD profiling of genomic DNA templates. (A) *Daphnia magna*, phenol-chloroform extracted DNA (2); (B) *Escherichia coli*, on (B). (C) and (D) caesium chloride DNA (Sigma, Poole, England, UK); (C) calf thymus; and (D) Human placental DNA using 10-mer primers. On (A), M' = 1 kbp ladder (Immunogen International), while on (B), (C) and (D) M = 1 kbp ladder (Life Technologies). Molecular sizes, in kilobases, of selected bands are indicated on the left of each figure. Lanes a–b, OPA9; c–d, OPB1; e–f, OPB5; g–h, OPB6; i–j, OPB7; k–l, OPB8; m–n, OPB10; o–p, OPB11; q–r, OPB12; s–t, OPB14; and u–v, OPB17. Sequences for each primer can be obtained from <http://www.operon.com>. Lanes a,c,e, etc. = 5 ng template; b,d,f, etc. = 20 ng template.

confirm the fidelity of the PCR condition. If profiles from the same genomic template or from different individuals of the same species vary, the reproducibility of the assay should be confirmed by repeating the PCR using two different template concentrations that differ by at least twofold (11). If profiles still vary, then results should always be questioned.

Although the choice of the 10-mer primers is generally crucial to the success of any RAPD protocol, our set of 11 primers generated high-quality profiles using genomic DNA extracted from *D. magna*, *E. coli* (strain B), calf thymus and human placenta (Figure 1). Profiles generated using template DNA at both 5 and 20 ng were identical, and demonstrated the high reproducibility of the methodology and the lack of template-dependent amplification artifacts. Regarding the magnesium concentration, a satisfactory reproducibility was obtained with 3–6 mM Mg⁺⁺, as already reported by earlier studies (5). Finally, one of our major goals was to minimize the primer polymerization

generated in negative controls (1,10). By varying the dNTP concentrations between otherwise identical reaction mixtures, it was shown that "dirty" negative controls could be significantly reduced. A dNTP concentration of 0.33 mM produced many bands with a complete or partial reduction in spurious amplifications in reactions without genomic DNA templates (1).

In conclusion, our RAPD protocol was found to generate high-quality genomic DNA profiles from phylogenetically different groups of organisms (bacteria, plants and animals) using the same set of primers. Since RAPD reactions are performed at a high annealing temperature, spurious amplifications are kept to a minimum and consistent profiles are generated when component concentrations are within the predefined optimal range.

REFERENCES

1. Atienzar, F., P. Child, A. Evenden, A. Jha, D. Savva, C. Walker and M. Depledge. 1998. Application of the arbitrarily primed poly-

Benchmarks

- merase chain reaction for the detection of DNA damage. *Mar. Environ. Res.* 46:331-335.
2. Atienzar, F., M. Conradi, A. Evenden, A. Jha and M. Depledge. 1999. Qualitative assessment of genotoxicity using random amplified polymorphic DNA: comparison of genomic template stability with key fitness parameters in *Daphnia magna* exposed to benzo(a)pyrene. *Environ. Toxicol. Chem.* 18:2275-2282.
3. Atienzar, F., B. Cordi, M. Donkin, A. Evenden, A. Jha and M. Depledge. Comparison of ultraviolet induced genotoxicity detected by RAPD with chlorophyll fluorescence and growth in a marine macroalgae, *Palmaria palmata*. *Aquat. Toxicol.* (In press).
4. Ayliffe, M.A., G.J. Lawrence, J.G. Ellis and A.J. Pryor. 1994. Heteroduplex molecules formed between allelic sequences cause non-parental RAPD bands. *Nucleic Acids Res.* 22:1632-1636.
5. Bentley, S. and B.J. Bassam. 1996. A robust DNA amplification fingerprinting system applied to analysis of genetic variation within *Fusarium oxysporum* f.sp. *cubense*. *J. Phytopathol.* 144:207-213.
6. Caetano-Anollés, G. 1998. DAF optimization using Taguchi methods and the effect of thermal cycling parameters on DNA amplification. *BioTechniques* 25:472-480.
7. Caetano-Anollés, G., B.J. Bassam and P.M. Gresshoff. 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Biotechnology* 9:553-557.
8. Ellsworth, D.L., K.D. Rittenhouse and R.L. Honeycutt. 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. *BioTechniques* 14:214-217.
9. Mullis, K.B. 1991. The Polymerase Chain Reaction in an anemic mode: how to avoid cold oligodeoxynucleic acid fusion. *PCR Methods and Applications* 1:1-4.
10. Ruano, G., W. Fenton and K.K. Kidd. 1989. Biphasic amplification of very dilute DNA samples via booster PCR. *Nucleic Acids Res.* 17:5407.
11. Welsh, J., D. Ralph and M. McClelland. 1995. DNA and RNA fingerprinting using arbitrarily primed PCR, p. 249-276. In M.A. Innis, D.H. Gelfand and J.J. Sninsky. (Eds.), *PCR Strategies*. Academic Press, San Diego.
12. Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531-6535.

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Molecular Diversity of *Renibacterium salmoninarum* Isolates Determined by Randomly Amplified Polymorphic DNA Analysis

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The molecular diversity among 60 isolates of *Renibacterium salmoninarum* which differ in place and date of isolation was investigated by using randomly amplified polymorphic DNA (RAPD) analysis. Isolates were grouped into 21 banding patterns which did not reflect the biological source. Four 16S-23S rRNA intergenic spacer (ITS1) sequence variations and two alleles of an exact tandem repeat locus, ETR-A, were the bases for formation of distinct groups within the RAPD clusters. This study provides evidence that the most common ITS1 sequence variant, SV1, possesses two copies of a 51-bp repeat unit at ETR-A and has been widely dispersed among countries which are associated with mainstream intensive salmonid culture.

Renibacterium salmoninarum is an important cause of clinical and subclinical infections among farmed and wild salmonid populations in North and South America, Europe, and Japan (5). The organism causes a chronic, systemic, and granulomatous infection, bacterial kidney disease (BKD), that is often fatal under conditions which are stressful to the host (11). There is no effective vaccine or chemotherapy, and the presence of subclinical infections complicates attempts to control the disease through programs of eradication. An improved understanding of the transmission and spread of BKD is of considerable importance in policy management issues relating to aquaculture and wildfisheries. There have been a number of studies investigating the presence, prevalence, and means of transmission of BKD within and between fish populations. This work has shown that *R. salmoninarum* is endemic within many wild salmonid populations as a low-level, subclinical infection; it has been isolated in up to 100% of samples (9, 12, 15). However, the epidemiology of BKD remains unclear, mainly because of the difficulty of differentiating isolates of *R. salmoninarum* by biochemical, serological, and multilocus enzyme electrophoresis techniques (1, 6, 16).

We used two approaches to assess the extent of molecular variation among *R. salmoninarum* isolates from different geographic locations. First, we investigated possible polymorphisms in specific regions within the genome, genes *msa* (3), *rsh* (4), and *hly* (8), and the rRNA genes, including the intergenic spacer (ITS) regions. PCR and DNA sequencing studies have shown that *R. salmoninarum* has only limited variation in these regions (7). Identifying specific markers of variation in the *R. salmoninarum* genome, such as insertion sequences or variable numbers of tandem repeats (TR), has been constrained by a paucity of sequence information. Second, we analyzed differences throughout the genome using randomly amplified polymorphic DNA (RAPD) analysis. RAPD analysis is a PCR-based alternative method to the use of species-specific DNA sequences for isolate or strain differentiation. The

method uses short random primers for rapidly detecting genomic polymorphisms under low-stringency conditions (18, 19). RAPD analysis is widely used for differentiating bacterial isolates (2, 10, 17) and relies on small quantities of genomic DNA, making it ideal for the study of slowly growing and fastidious organisms, such as *R. salmoninarum*. Previous studies show that, compared with other techniques, RAPD analysis is a reliable and reproducible means for differentiating isolates of *R. salmoninarum* (7). In the present study we used RAPD software to produce an objective analysis of RAPD profiles which were generated from the genomes of 60 *R. salmoninarum* isolates from a variety of sources in order to identify clusters of the isolates and determine whether there is any correlation with geographic or biological source. Furthermore, we identified the locus of a TR and showed that variation within this locus and within another specific region of the *R. salmoninarum* genome, the nucleotide sequence of the 16S-23S rRNA ITS region, is reflected in the RAPD analysis.

Generating RAPD profiles of *R. salmoninarum*. Sixty isolates of *R. salmoninarum* obtained from a variety of countries in Europe and North America, including the type strain, NCIMB2235 (ATCC 33209), were cultured in selective kidney disease medium (SKDM) broth supplemented with 5% spent broth culture at 15°C for 6 to 10 weeks. A description of the isolates, sources, and the positive identification of each as *R. salmoninarum* has been previously published (7). Genomic DNA was isolated by using the Puregene D-6000 DNA isolation kit according to the manufacturer's instructions (Gentra Systems Inc.). PCR amplification was performed in a DNA thermal cycler (Perkin-Elmer), and we used two RAPD protocols and eight random 10-mer primers which have been described elsewhere (7). PCR products were analyzed on 1.2% agarose gels in Tris-borate-EDTA buffer. The RAPD patterns were visualized by UV illumination, images of each gel were captured with a Kodak DC40 digital camera, and the DNA profile was analyzed by using the RAPDistance software package (<http://life.anu.edu.au/molecular/software/rpd.html>). The patterns were normalized with the bands that were uniformly present in all patterns, and the presence or absence of major bands was recorded in a binary matrix. Very faint bands were excluded from the analysis. A band was scored as absent only

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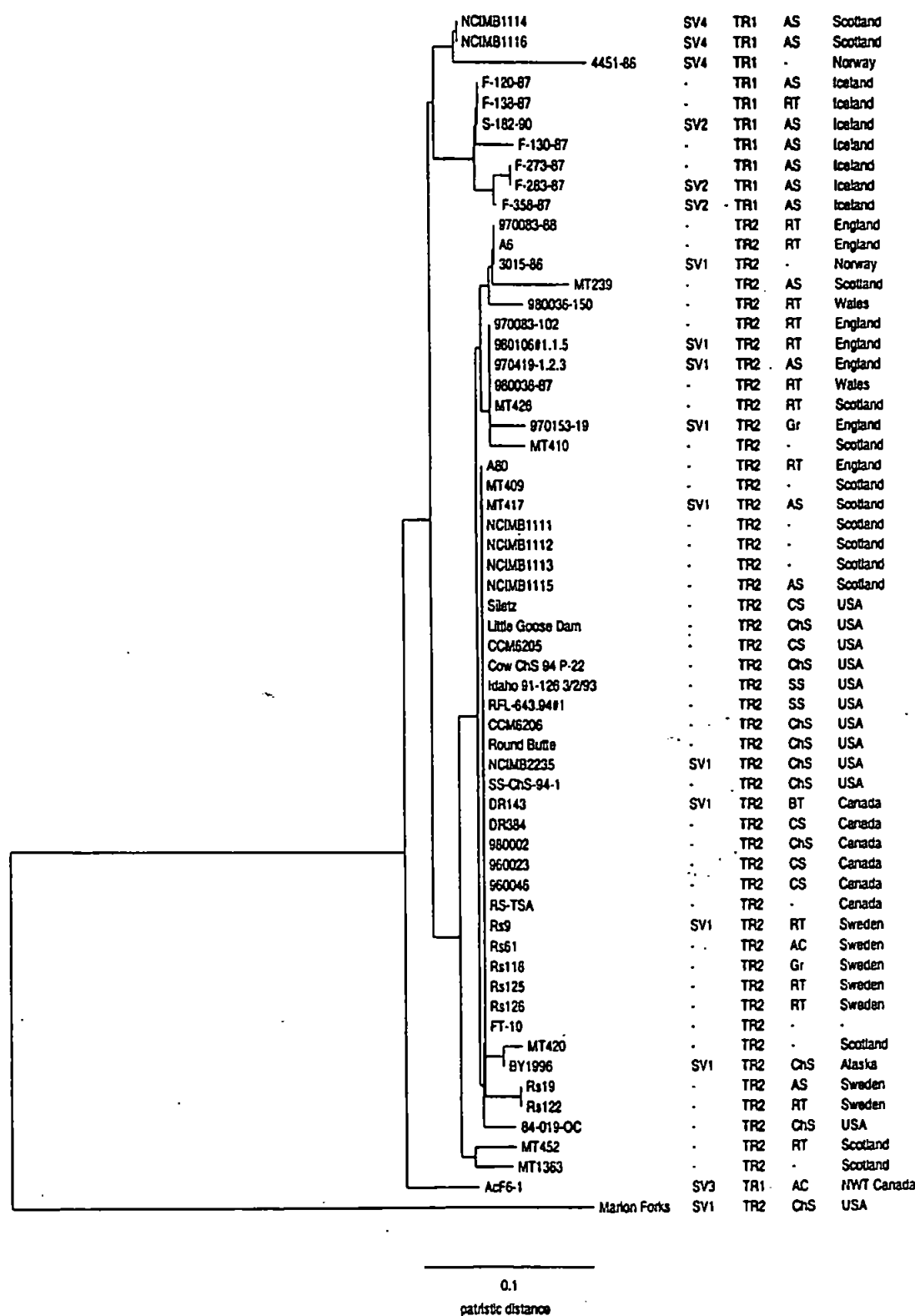


FIG. 1. Unrooted dendrogram, generated by the neighbor-joining method (15), of RAPD patterns for *R. salmoninarum* isolates ($n = 60$). Isolate designations and the respective ITS1 sequevar, number of TR at the ETR-A locus, biological source, and geographical origin are indicated. RT, rainbow trout; BT, brook trout; AS, Atlantic salmon; CS, coho salmon; ChS, chinook salmon; SS, sockeye salmon; Gr, grayling; AC, Arctic char.

if no visible band was present within a 2% size range. The patterns generated with each of the primers were combined for each isolate, and the pairwise distances for the combined band patterns were calculated by using the Dice algorithm described by Nei and Li (13). An unrooted tree was constructed based on the neighbor-joining method of Saitou and Nei (14), using NJTREE and TDRAW software (L. Jin and J. W. H. Ferguson, University of Texas Health Science Centre, Houston).

Differentiating *R. salmoninarum* isolates by RAPDistance analysis. The data for each primer were combined, and for each isolate a total of 86 bands were used to generate a distance matrix, of which 11 bands were invariant; i.e., present in all 60 isolates. By using RAPDistance software, isolates were placed in 21 clusters; 1 of these was a single major cluster which contained 29 of the 60 isolates studied (Fig. 1). The patristic distance between most paired groups was less than 0.1, reflecting the close relatedness of most isolates. Only a single isolate, Marion Forks (from the United States), was sufficiently different to exceed this value. There was no correlation of banding pattern with biological source. All Icelandic isolates were grouped in four closely associated clusters, and most of the isolates from England and Wales were grouped in four adjoining clusters. However, no strong correlation with the geographic origin of isolates was found; the single major cluster of 29 isolates contained the bulk of isolates from the United States, Canada, and Sweden and half of the isolates from Scotland.

TR allele profile of *R. salmoninarum* isolates. We identified an exact TR repeat locus (ETR-A) in the *R. salmoninarum* genome during routine sequencing of DNA fragments cloned from a number of different isolates. The repeat unit, with a length of 51 bp, was located in an open reading frame; we used PCR to examine variation in this region of the genomes of 60 *R. salmoninarum* isolates which differ in place and date of isolation. The isolate numbers are listed in Fig. 1, and the isolates are more fully described elsewhere (7). We amplified this locus using a set of specific PCR primers, 17D+95 (5'-T CGCGAATAGCTTGGCCATTTTGC-3') and 17D-344 (5'-C GTAGCACCGAAGTCAGATAAGAG-3'), complementary to flanking DNA. Both strands of selected PCR amplicons were sequenced to confirm that our PCR products corresponded to the expected region and number of TR copies. PCR amplification and sequencing were performed under conditions exactly as described for the amplification of specific *R. salmoninarum* genes (7). Most isolates yielded PCR products of an identical size, 301 bp, which contained two copies of the TR. Interestingly, all Icelandic isolates examined, as well as NCIMB1114 and NCIMB1116 (from Scotland), 4451-86 (from Norway), and AcF6-1 (from the Canadian northwest territories), yielded PCR products of 250 bp which contained only a single copy of the repeat. Furthermore, all of these isolates were clustered separately from the majority of *R. salmoninarum* isolates by RAPDistance analysis (Fig. 1).

R. salmoninarum isolates with a single TR unit are not SV1. Members of our group have previously shown that although the *R. salmoninarum* 16S-23S rRNA ITS (ITS1) is highly conserved three sequence variants which reflect the geographic origin of isolates exist (7). A majority of *R. salmoninarum* isolates from a wide variety of sources appear to belong to SV1. The other ITS1 sequence variants SV2 and SV3, are more restricted in their distribution. The DNA sequences of ITS1 are already known for isolates S-182-90 (from Iceland) and AcF6-1, and they correspond to SV2 and SV3, respectively. In order to investigate whether any relationship between ITS1 sequence variation and ETR-A exists, we sequenced ITS1 for five isolates, NCIMB1114, NCIMB1116, 4451-86,

F-283-87, and F-358-87, which possess a single copy of the TR at the ETR-A locus. The ITS1 was amplified and sequenced by the protocol previously described for PCR amplification and double-stranded sequencing of this region (7). The DNA sequences obtained in this way were found to belong to SV2 (F-283-87 and F-358-87) and a previously unknown ITS1 sequevar, SV4 (NCIMB1114, NCIMB1116, and 4451-86) (Gen Bank accession no. AF178998 to AF179002). DNA sequence data for the ITS1 region of selected isolates, including 3015-86 (from Norway) and MT417 (from Scotland), which possess two copies of the TR show that these belong to SV1 (Fig. 1). Therefore, ETR-A has a potential use as a specific marker for rapidly distinguishing ITS1 sequence variants.

The purpose of this study was to examine the molecular diversity of isolates of *R. salmoninarum* from the United Kingdom, other European countries, and North America and from a variety of salmonid host species. Previous research has shown that *R. salmoninarum* is a highly conserved genospecies with a remarkable degree of biochemical, serological, and genetic uniformity among isolates (1, 6, 16). Furthermore, studies of the *R. salmoninarum* genome have shown that isolates from diverse sources possess only limited sequence variation in the ITS of the 16S and 23S rRNA genes (7). Members of our group have previously (7) identified three ITS1 sequevars (SV1, SV2, and SV3). We found that isolates from Iceland (SV2), Japan (SV2), and the Canadian northwest territories (SV3) possessed three single-base substitutions in the ITS1 and showed some divergence from the highly conserved SV1, which was present in isolates from the United States, the United Kingdom, mainland Europe, and Canada. We proposed that in areas of the world which could be regarded as relatively isolated from the mainstream intensive salmonid culture of North America and Europe, the bacterium shows genetic divergence. The results presented here broadly support this hypothesis, although some isolates, most notably Marion Forks, vary from this pattern.

This study used an objective method based on RAPDistance software to examine the extent of molecular diversity among *R. salmoninarum* isolates from different countries around the world and related this information to specific regions of variation within the genome. We have identified four 16S-23S rRNA ITS1 sequevars and an exact TR locus (ETR-A) which are specific markers of variation within the genome of the bacterium, and furthermore, we have shown that an objective method of analysis of RAPD profiles, which can be used to differentiate *R. salmoninarum* isolates, reflects these specific markers.

Nucleotide sequence accession numbers. Sequences for DNA fragments and for ITS1 regions of isolates have been deposited in GenBank with accession numbers AF178991 to AF178997 and AF178998 to AF179002, respectively.

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REFERENCES

1. Bruno, D. W., and A. L. S. Munro. 1986. Uniformity in the biochemical properties of *Renibacterium salmoninarum* isolates obtained from several sources. *FEMS Microbiol. Lett.* 33:247-250.
2. Chakroun, C., M. C. Urdaci, D. Faure, F. Grimon, and J.-F. Bernadet. 1997. Random amplified polymorphic DNA analysis provides rapid differentiation among isolates of the fish pathogen *Flavobacterium psychrophilum* and among *Flavobacterium* species. *Dis. Aquat. Org.* 31:187-196.
3. Chien, M. S., T. L. Gilbert, C. Huang, M. L. Landolt, P. J. O'Hara, and J. R. Winton. 1992. Molecular cloning and sequence analysis of the gene coding for the 57-kDa major soluble antigen of the salmonid fish pathogen *Renibacterium salmoninarum*. *FEMS Microbiol. Lett.* 75:259-265.

4. Evenden, A. J. 1993. Gene cloning techniques in the study of the fish pathogen *Renibacterium salmoninarum*. Ph.D. thesis. University of Plymouth, Plymouth, United Kingdom.
5. Evenden, A. J., T. H. Grayson, M. L. Gilpin, and C. B. Munn. 1993. *Renibacterium salmoninarum* and bacterial kidney disease—the unfinished jigsaw. *Annu. Rev. Fish Dis.* 3:87–104.
6. Goodfellow, M., T. M. Embley, and B. Austin. 1985. Numerical taxonomy and emended description of *Renibacterium salmoninarum*. *J. Gen. Microbiol.* 131:2739–2752.
7. Grayson, T. H., L. F. Cooper, F. A. Attenzar, M. R. Knowles, and M. L. Gilpin. 1999. Molecular differentiation of *Renibacterium salmoninarum* isolates from worldwide locations. *Appl. Environ. Microbiol.* 65:961–968.
8. Grayson, T. H., A. J. Evenden, M. L. Gilpin, K. L. Martin, and C. B. Munn. 1995. A gene from *Renibacterium salmoninarum* encoding a product which shows homology to bacterial zinc-metalloproteases. *Microbiology* 141:1331–1341.
9. Jonsdottir, H., H. J. Malmquist, S. S. Snorrason, G. Gudbergsson, and S. Gudmundsdottir. 1998. Epidemiology of *Renibacterium salmoninarum* in wild Arctic charr and brown trout in Iceland. *J. Fish Biol.* 53:322–339.
10. Kersulyte, D., J. P. Woods, E. J. Keith, W. E. Goldman, and D. E. Berg. 1992. Diversity among clinical isolates of *Histoplasma capsulatum* detected by polymerase chain reaction with arbitrary primers. *J. Bacteriol.* 174:7075–7079.
11. Mesa, M. G., A. G. Maule, T. P. Poe, and C. B. Schreck. 1999. Influence of bacterial kidney disease on smoltification in salmonids: is it a case of double jeopardy? *Aquaculture* 174:25–41.
12. Meyers, T. R., S. Short, C. Farrington, K. Lipson, H. J. Geiger, and R. Gates. 1993. Comparison of the enzyme-linked immunosorbent assay (ELISA) and the fluorescent antibody test (FAT) for measuring the prevalences and levels of *Renibacterium salmoninarum* in wild and hatchery stocks of salmonid fishes in Alaska, USA. *Dis. Aquat. Org.* 16:181–189.
13. Nel, M., and W. H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* 76:5269–5273.
14. Saitou, N., and M. Nei. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–425.
15. Sanders, J. E., J. J. Long, C. K. Arakawa, J. L. Bartholomew, and J. S. Rohovec. 1992. Prevalence of *Renibacterium salmoninarum* among downstream-migrating salmonids in the Columbia River. *J. Aquat. Anim. Health* 4:72–75.
16. Startip, C. E. 1996. Genetic diversity of North American isolates of *Renibacterium salmoninarum*. *Dis. Aquat. Org.* 27:207–213.
17. Wang, G., W. T. S. Wittam, C. M. Berg, and D. M. Berg. 1993. RAPD (arbitrary primer) PCR is more sensitive than multilocus enzyme electrophoresis for distinguishing related bacterial strains. *Nucleic Acids Res.* 21:5930–5933.
18. Welsh, J., and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* 18:7213–7218.
19. Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531–6535.

Comparison of ultraviolet-induced genotoxicity detected by random amplified polymorphic DNA with chlorophyll fluorescence and growth in a marine macroalgae, *Palmaria palmata*

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Abstract

The random amplified polymorphic DNA (RAPD) technique was used to detect DNA damage in the sublittoral macroalgae *Palmaria palmata* (Rhodophyta) exposed to both ambient and elevated irradiances of UV-B (280–315 nm). To investigate the potential of this method in ecotoxicological assessments, the qualitative and quantitative modifications in RAPD profiles were compared with changes in a number of physiological and fitness parameters. RAPD detectable modifications in DNA profiles were observed in all UV exposed individuals compared with controls. Changes in chlorophyll fluorescence (F_v/F_m ratio), in vivo pigment absorbance, thallus growth and RAPD profiles, examined simultaneously, provided a sensitive measure of UV-induced toxicity. In conclusion, the application of the RAPD method in conjunction with other suitable physiological and fitness measurements, may prove to be a valuable tool for investigating the specific effects of genotoxic agents upon marine algal populations. Ultimately, this methodology may allow the ecotoxicological examination of the link between molecular alterations and measurable adverse effects at higher levels of biological organisation. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: *Palmaria palmata*; Macroalgae; UV-A and B; RAPD; Genomic template stability; Chlorophyll fluorescence; Growth

1. Introduction

The primary goal of 'genetic-ecotoxicology' or 'eco-genotoxicology' is to understand the conse-

quences of genotoxicity in individuals for population and community structures (Würgler and Kramers, 1992; Anderson et al., 1994; Depledge, 1998). Altered fertility, growth, and embryonic survival are ecologically significant because they can reduce reproductive success and thus alter population size or structure (Anderson et al.,

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1994; Jha, 1998). Despite the importance, little work has been done to explore the potential reproductive and developmental effects of exposure to genotoxic substances in natural biota.

Advances in molecular biology have led to the development of a number of selective and sensitive assays for DNA analysis. The random amplified polymorphic DNA (RAPD; Williams et al., 1990) and the arbitrarily primed polymerase chain reaction (AP-PCR; Welsh and McClelland, 1990) techniques are two semiquantitative methods which have been used in genetic mapping, taxonomy, phylogeny and for detection of various kinds of DNA damage and mutations (Kubota et al., 1992, 1995; Peinado et al., 1992; Ionov et al., 1993; Savva, 1996; Shimada and Shima, 1998; Atienzar et al., 1999; Becerril et al., 1999). In the context of detection of genotoxicity, both methods rely on the comparison of amplification products generated by control (unexposed) and exposed samples. Following extraction of the genetic material, DNA templates are amplified using one oligonucleotide primer of random sequence. Although RAPD and AP-PCR are very similar techniques, procedural differences exist between the techniques (Meunier and Grimont, 1993). In the literature, both techniques were sometimes considered as the same method (Savva, 1996; Atienzar et al., 1998). However, to avoid any confusion, the definition described by Meunier and Grimont (1993) has been adopted in this paper.

One of the potential applications of the RAPD method is the detection of UV-induced genetic damage. This is a topic of particular concern because ozone depletion in the earth's upper atmosphere and the associated higher levels of UV-B radiation, has the potential to adversely affect terrestrial and marine biota. The effect of UV-B on aquatic biota is of particular interest as it may lead to a reduction in survival and productivity (Häder et al., 1995). Investigations into the effects of enhanced UV-B radiation on phytoplankton and macroalgae are of great importance, as these vital primary producers form the basis of aquatic food webs. Although the effects of UV irradiance on the

genetic material, physiological parameters [e.g. photosynthesis, chlorophyll fluorescence (Franklin and Forster, 1997)] and decreased growth rates (Grobe and Murphy, 1994) have been studied separately, to our knowledge, there has been no attempt to elucidate the potential consequences of induced DNA damage on the physiology and Darwinian fitness parameters of exposed populations.

In the light of the aforementioned information, the objectives of this study were to (a) evaluate the suitability of the RAPD method to detect UV-induced DNA damage in the marine macroalgae *Palmaria palmata*, (b) detect links, if any, between UV-induced DNA damage (as detected by RAPD) and physiological and growth parameters, and finally (c) to compare the sensitivity of genomic template stability (a qualitative measure reflecting changes in RAPD patterns) with physiological (changes in chlorophyll fluorescence) and growth following exposure to UV radiation.

2. Materials and methods

2.1. Collection of macroalgae

The macroalga *P. palmata* (Rhodophyta) was collected at low tide from Wembury on the south-west coast of Devon, UK (50° latitude) between May and June 1997 (sea water temperature: 10°C). The thalli were harvested 24 h prior to the experiments and maintained in filtered sea water at constant temperature ($15 \pm 2^\circ\text{C}$) and in low light conditions ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$, fluorescent tubes, Phillips TLD 32W/83 HF) in growth cabinets.

2.2. UV treatments and measurements of chlorophyll fluorescence, algal pigment absorbance, and growth

Thalli were cut to a length of ~ 3 cm and covered with filtered sea water to a depth of 1.5 cm per Petri dish. Each dish was exposed to the various treatments for 3 h ($n = 4$ per treatment). Photosynthetically active radiation (PAR) and

temperature conditions were identical to those in the acclimatisation period. PAR was measured with a PAR meter (Sky Instruments). UV-B was supplied by two UV-B tubes (Philips TL' 20W/12 RS) and UV-A by two UV-A tubes (Philips 1609 15 W). A constant UV-A irradiance of 1.3 W m^{-2} , which is approximately four times less than that calculated at 50° latitude in December (Driscoll et al., 1992), was included in all UV-B treatments. The total irradiance of both UV-A and UV-B tubes was measured using UV-B (MP-229) and UV-A (MP-236) cosine sensors (MicroPulse Technology, UK). These sensors were calibrated in the experimental light field against a double monochromator spectroradiometer (model SR 9910, Macam Photometrics, UK). UV light was filtered with $35 \mu\text{m}$ cellulose diacetate foil which showed 0% transmission below 286 nm (UV-C). For the control thalli, UV tubes were covered with Mylar 125 D which showed 0% transmission below 320 nm (i.e. filtering out UV-B).

Irradiances and doses used in the UV-B treatments are listed in Table 1. The weighted UV-B doses were calculated according to the generalised plant response action spectrum normalised at 300 nm (Caldwell, 1971). The corresponding percentage ozone depletion was calculated for 15th July 1997 at Plymouth (UK) during clear sky conditions using the computer model of Björn and Murphy (1985). The relatively high UV-B irradiance values were chosen to investigate the entire range of the exposure–response curves.

Measurements of chlorophyll-fluorescence, algal pigment absorbance, and growth have been described elsewhere in details (Cordi et al., 1997).

2.3. DNA isolation and RAPD reactions

Approximately 0.10 g of thalli from individual samples were frozen in liquid nitrogen, ground with a mortar and pestle, and homogenised in 400 μl of sperm lysis buffer (100 mM Tris-HCl pH 8, 500 mM NaCl, 10 mM EDTA pH 8, 1% SDS, 2% mercaptoethanol). The DNA was treated with RNase (40 μg , 1.5 h at 37°C) and extracted twice with phenol (pH 8), followed by an extraction with chloroform:isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase with 0.10 volumes of sodium acetate (pH 4.8) and two volumes of cold ethanol at -80°C overnight. Precipitated DNA was harvested by centrifugation, air-dried, with the final pellet dissolved in sterile analytical grade water. The amount and quality of DNA was estimated using agarose gel electrophoresis. All chemicals used throughout the described procedure were obtained from Sigma (Poole, UK).

DNA profiles were generated in RAPD reactions performed in a reaction volume of 25 μl as described previously (Atienzar et al., 1999). The decamer oligonucleotides OPA9 (GGGTAA-CGCC) or OPB1 (GTTTCGCTCC), or OPB14 (TCCGCTCTGG), or OPB 17 (AGGGAAC-GAG) (sequences given from 5' to 3') were obtained from Operon Technologies (Southampton, UK). Approximately 10 ng of genomic DNA was subjected to RAPD amplification with a primer concentration of 2 μM , deoxy-trinucleotide phosphate (dNTP) concentration of 0.33 mM, MgCl_2 concentration of 5.11 mM. This was performed in the presence of 2.8 U of *Thermus aquaticus* (Taq) DNA polymerase and 1 \times reaction buffer (10 mM Tris-HCl, pH 8.8 at

Table 1
Exposure irradiances and doses used in the experiment^a

UV-B irradiance (W m^{-2})	0	1.4	2.6	9.0	12.7
Unweighted UV-B dose (kJ m^{-2})	0	15.1	28.1	97.2	137.2
Weighted UV-B dose (kJ m^{-2})	0	4.3	7.9	27.4	38.7
Calculated ozone depletion (%)	0	Ambient August	17.0	41.0	100.0

^a The doses were calculated for the 3-h exposure and the weighted UV-B doses were calculated according to the generalised plant response action spectrum normalised at 300 nm (Caldwell, 1971). The corresponding percentage ozone depletion was calculated on the daily dose using the computer model of Björn and Murphy (1985).

25°C; 50 mM KCl, 0.08% Nonidet P40, 2.5 µg bovine serum albumin). PCR chemicals were obtained from Immunogen International (Sunderland, UK) except when otherwise notified. Thermal cycling parameters consisted of 4 min denaturation (95°C) followed by 40 cycles of 1 min denaturation (95°C), 1 min annealing at 50°C, and 1 min extension [(74°C); with the final extension period adjusted to 10 min]. Reaction mixtures were stored at 4°C prior to use.

2.4. Agarose gel electrophoresis and analysis of DNA profiles

Electrophoresis of RAPD reaction products was performed in 1.2% (w/v) agarose, using a Tris–Borate–EDTA buffer system (1 × TBE = 90 mM Tris-base, 90 mM Boric acid and 2 mM EDTA). Amplified DNA was mixed with 1/5th volume of gel loading buffer (analytical grade water containing 25% ficoll, 0.25% bromophenol blue, and 0.25% xylene cyanol) with 15 µl of this solution loaded onto the agarose gel. A DNA molecular size marker [M = 1 kb marker, Gibco BRL (Paisley, UK)] was run for each agarose gel. Bands visualised were from top to bottom 3054, 2036, 1636, 1018, 517, 506, 396, 344 and 298 bp. DNA samples were subjected to electrophoresis at 80 V for 7 h, after which, the gels were stained in a 1 × TBE solution containing ethidium bromide (0.015% v/v) for a period of not less than 40 min. Gels were photographed under UV illumination using a Polaroid camera (CU-5, Eastman Kodak, New York, NY). Images of each gel were also captured using a Kodak DC40 digital camera (Eastman Kodak, New York, NY). Band intensity was subsequently analysed using Kodak Digital Science™ 1 D Image Analysis Software (Eastman Kodak, New York, NY).

2.5. Estimation of genomic template stability

Each change observed in RAPD profiles (disappearance, appearance of bands and variation in band intensity in comparison to control RAPD profiles) was given the arbitrary score of +1. The average was then calculated for each experimental group exposed to varying UV irradiances. The

template genomic stability (%) was calculated as $100 - (100a/n)$ where 'a' is the average number of changes in DNA profiles and 'n' the number of bands selected in control DNA profiles.

2.6. Transformation of the data and statistical analysis

To compare the sensitivity of each parameter [genomic template stability, chlorophyll fluorescence (F_v/F_m), and growth rate], changes in these values were calculated as a percentage of their control value (set to 100%).

Statistical analyses were performed using the software package STATGRAPHICS (Statgraphics plus for Windows version 3.1, Statistical Graphics, USA). Changes in RAPD profiles were tested statistically by performing one-way analysis of variance (ANOVA). The least significant differences (LSD) test was used to reveal statistical differences. The statistical analyses applied to physiological responses and fitness parameters have been previously described by Cordi et al. (1997).

3. Results

3.1. Measurements of physiological and growth parameters

The results dealing with chlorophyll fluorescence, in vivo spectrophotometric pigment absorbance and growth of the UV-irradiated macroalgae have been reported previously by Cordi et al. (1997). The effects of UV exposure on F_v/F_m ratio, in vivo absorbance spectra, and growth rates of *P. palmata* are recorded in Table 2. Briefly, reduced levels of in vivo absorbance and chlorophyll fluorescence were measured with increasing UV-B irradiance. After a recovery period of 45 h, only thalli exposed to UV-A or the lowest UV-B irradiance (1.4 W m^{-2}) exhibited increases in chlorophyll fluorescence. Thalli exposed to 1.3 W m^{-2} UV-A and 1.4 W m^{-2} unweighted UV-B suffered 25 and 58% reduction in growth rate compared to controls, respectively. Thalli exposed to higher UV-B levels were unable

Table 2
Physiological and fitness parameters measured after exposure of *P. palmata* to varying UV irradiances

Time after UV exposure (h)	Physiological and fitness parameters	UV-B irradiance (W m^{-2})					
		0	1.3 ^a	1.4	2.6	9.0	12.7
0	Chlorophyll fluorescence, F_v/F_m ratio (%)	100	80	42	47	2	2
21	Recovery experiment, chlorophyll fluorescence, F_v/F_m ratio (%)	100	88	54	32	1	1
45	Recovery experiment, chlorophyll fluorescence, F_v/F_m ratio (%)	100	91	58	5	0	0
45	In vivo absorbance spectrum	100	100	↓ ^b	↓↓ ^c	↓↓↓ ^d	
–	Growth rate (%) until day 11	100	75	42	0	0	0

^a Indicates that the algae was irradiated with UV-A only (in W m^{-2}).

^b ↓, little decrease.

^c ↓↓, medium decrease.

^d ↓↓↓, high decrease. For details, please see Cordi et al. (1997).

to grow. Thus, these physiological data suggested irreversible damage following exposure to 2.6 W m^{-2} (equivalent to 17% reduction in atmospheric ozone levels in July). However, these results may be influenced by the sampling time, due to seasonal variations in the sensitivity of algae to UV exposure.

3.2. RAPD DNA profiling

In total five 10-mer priming oligonucleotides were used to analyse the results. In all cases, RAPD patterns generated by UV-exposed algae were different from those obtained using control DNA. The results obtained from 4 primers are presented in Fig. 1. DNA patterns generated by each treatment group were reproducible, although each RAPD profile was obtained from individual alga. The principal events observed following the UV exposure were a variation in the band intensity, as well as the disappearance and appearance of new bands (Fig. 1). Selected changes in comparison to control profiles are indicated in Fig. 1. Table 3 and Fig. 2 present a summary of all RAPD profile modifications and the analysis of band intensity, respectively. The decrease in band intensity was particularly obvious for algae exposed to the two highest UV-B irradiances (9.0 and 12.7 W m^{-2}). In contrast, an increase in band intensity occurred for the three lowest UV irradiances (UV-A and UV-B irradiances of 1.4 and 2.6 W m^{-2}). Further experiments confirmed

that the changes in band intensities were not due to a variation in template DNA concentration (data not shown) or in PCR reagent concentration (e.g. *Taq* DNA polymerase, since a master mix was performed). The number of disappearing RAPD bands which occurred for all UV irradiances tested, was greater at higher UV-B irradiances. Only bands of molecular size greater than 1 kb were shown to disappear (Fig. 1). The sensitivity of detection of disappearing bands by the PCR method was dependent upon the size of the target sequence. Amplification of the longer fragments is expected to be inhibited more than that of shorter fragments [e.g. when DNA adducts block the polymerisation of the DNA (see Section 4)]. Finally, extra bands appeared for the three lowest UV irradiances; this event occurred, however, very rarely for 9.0 W m^{-2} and never for 12.4 W m^{-2} .

3.3. Comparison of chlorophyll fluorescence, growth and RAPD profiles

In Fig. 3, the genomic template stability, a qualitative measure reflecting changes in RAPD patterns, was used to compare the modifications in RAPD profiles with reductions in chlorophyll fluorescence (F_v/F_m ratio) and growth rates. Following exposure to increasing UV-B irradiance, chlorophyll fluorescence and growth rates decreased gradually to zero. In contrast, the genomic template stability decreased after exposure

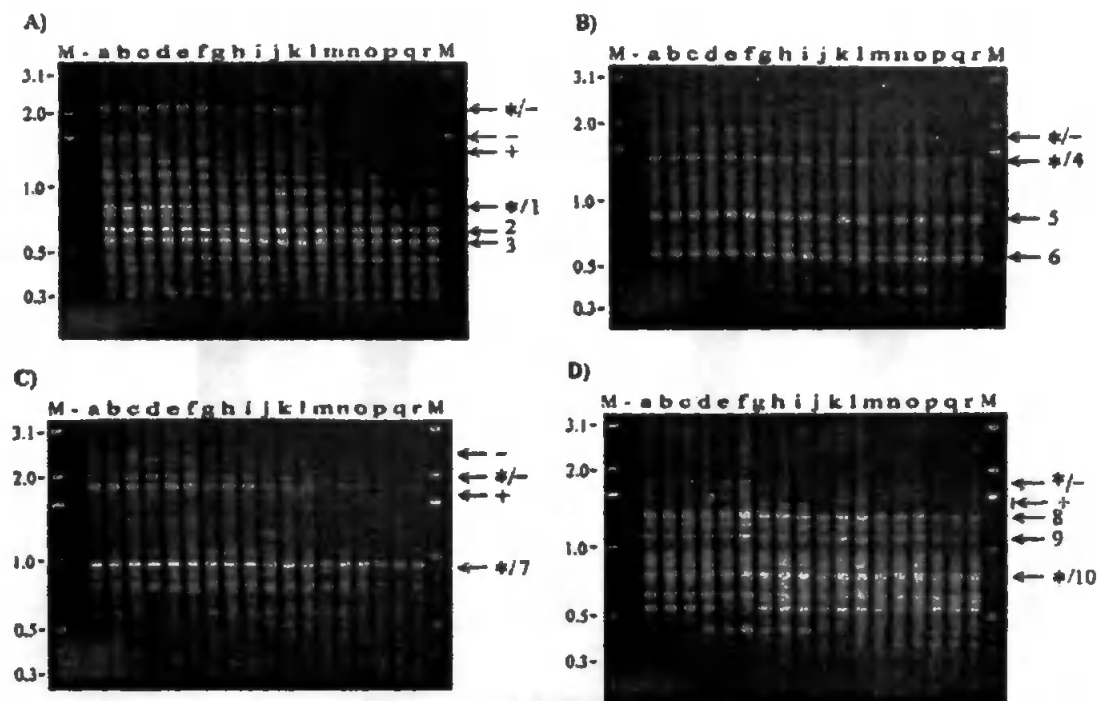


Fig. 1. RAPD profiles of genomic DNA from *P. palmata* exposed to varying UV irradiances. M, DNA molecular size marker (1 kb ladder, BRL; the molecular sizes (in kilobases) are indicated on the left). –, no DNA control. RAPD reactions were performed using oligonucleotide primers OPA9 (A), OPB1 (B), OPB14 (C) and OPB17 (D). Each small letter represents an individual algae. Lanes a–r: algae exposed to varying UV irradiances; a–c: ambient UV levels (1) (control algae collected directly from the field site), d–f: 1 + 1.3 W m⁻² UV-A (2), g–i: 1 + 2 + UV-B (1.4 W m⁻²), j–l: 1 + 2 + UV-B (2.6 W m⁻²), m–o: 1 + 2 + UV-B (9.0 W m⁻²), p–r: 1 + 2 + UV-B (12.7 W m⁻²). Selected changes are indicated by arrows in comparison to control patterns; *, variation in band intensities, +, appearance of a new band, and –, disappearance of a band. Numbers (from 1 to 10) show the ten bands selected to calculate the variation in band intensity (for more details please see Fig. 2). The RAPD and thermocycling conditions are described in Section 2.

Table 3
RAPD profile changes after exposure of *P. palmata* to UV radiations^a

Changes in RAPD profiles compared with the control	UV-B irradiance (W m ⁻²)				
	1.3 ^b	1.4	2.6	9.0	12.7
Appearance of bands	5	10	5	5	0
Disappearance of bands	10	10	15	30	40
Decrease in band intensities	5	15	15	50	60
Increase in band intensities	80	65	65	15	0

^a Numbers indicate the frequency in percent of each event described in the table. Results were generated using approximately 18 bands for each UV irradiance.

^b Algae was irradiated with UV-A only (in W m⁻²).

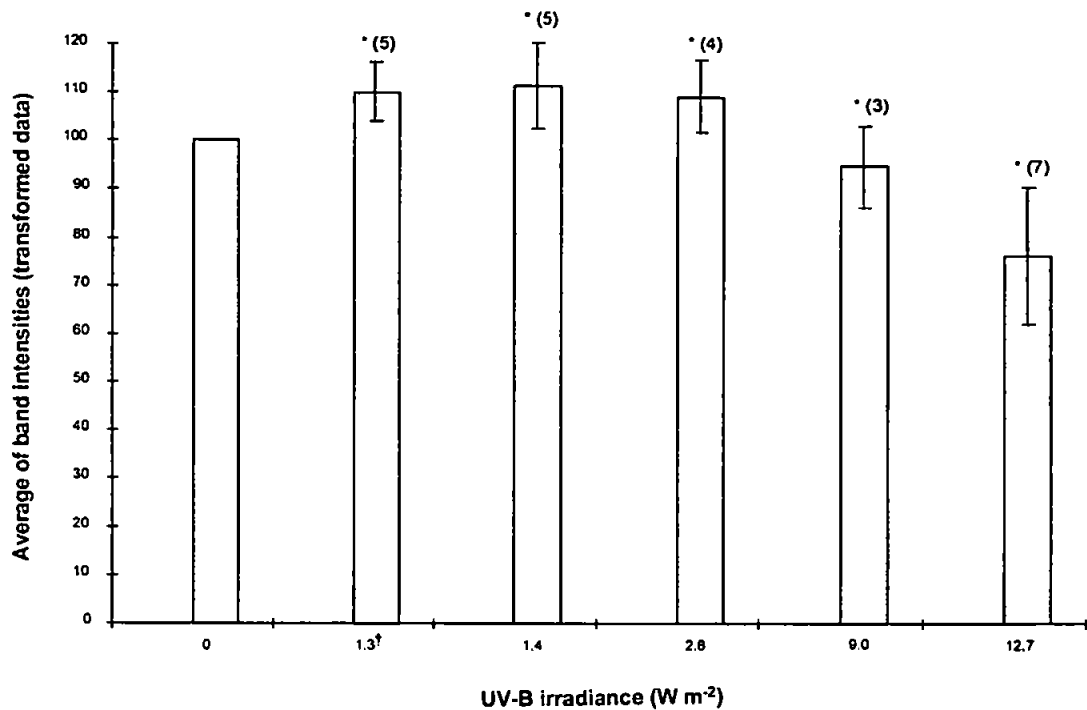


Fig. 2. Variation of band intensities selected from RAPD profiles of genomic DNA from *P. palmata* exposed to varying UV irradiances. A total of ten relatively intense bands (shown in Fig. 1) appearing across the width of the gels were arbitrarily selected. The band intensities were calculated as a percentage of its own control value (set to 100%). Average and standard deviation (error bars) were calculated with the ten selected bands. The figures in parentheses above the standard deviation indicate the number of bands (out of ten) being significantly different from control (* $P < 0.05$). †, indicates that the algae was irradiated with UV-A only (in $W m^{-2}$).

to UV-A, but stabilised after UV-B exposure, varying from 52–60%. The plateau effect is ascribed to multiple changes in RAPD profiles (appearance, disappearance of bands, etc.) which tend to counterbalance each other (Table 3). In other words, for the highest UV irradiances, the high frequency of disappearing bands was compensated by the low frequency of newly appearing bands.

4. Discussion

After suitable optimisation of the PCR conditions (Atienzar et al., 1998, 2000), and the judicious choice of oligonucleotide primers for each species-specific DNA template, the RAPD assay performs

well in terms of number of bands, product yield and clarity of the profiles. Although the generation of RAPD profiles has often been criticised as unreliable (Ellsworth et al., 1993), reproducible DNA profiles have been generated from a range of aquatic invertebrates, plants and bacterial species (Grayson et al., 1999; Atienzar et al., 2000) and successfully used to detect genotoxins induced DNA damage (Savva, 1996; Atienzar et al., 1999; Becerril et al., 1999). Once optimised, the use of RAPD for the detection of DNA damage presents a number of advantages. The assay, which is suitable for any extracted DNA (of sufficient quality), allows rapid analysis of a large number of samples. As arbitrary primers are used, specific details of the DNA damage or the

sequence of the genome are not needed. Furthermore, no radioactivity or chemical or enzymatic degradation of the DNA is required before analysis.

Previous studies have shown that changes in band patterns observed in DNA fingerprint analyses reflect DNA alterations from single base changes (point mutations) to complex chromosomal rearrangements (White et al., 1990; Welsh et al., 1991; Atienzar et al., 1999). Similarly, in the present study, DNA damage induced by UV-radiation was reflected by changes in RAPD profiles; variation in band intensity, disappearance of bands, and appearance of new PCR products occurred in the profiles generated by exposed organisms. The variation in band intensities and

the disappearance of bands may be attributed to the presence of DNA photoproducts (e.g. pyrimidine dimers, 6-4 photoproducts), which can act to block or reduce (bypass event) the polymerisation of DNA in the PCR reaction (Donahue et al., 1994; Nelson et al., 1996). The bypass event is a complicated process which depends on the enzymatic properties of the DNA polymerase, the structure of the lesion, and the sequence context of its location (Ide et al., 1991). Increase in band intensity occurred for the three lowest UV irradiances (UV-A and UV-B irradiances of 1.4 and 2.6 W m^{-2}) whereas decrease in band intensity was particularly obvious for algae exposed to the two highest UV-B irradiances (9.0 and 12.7 W m^{-2}). Both changes can be ascribed to the structural

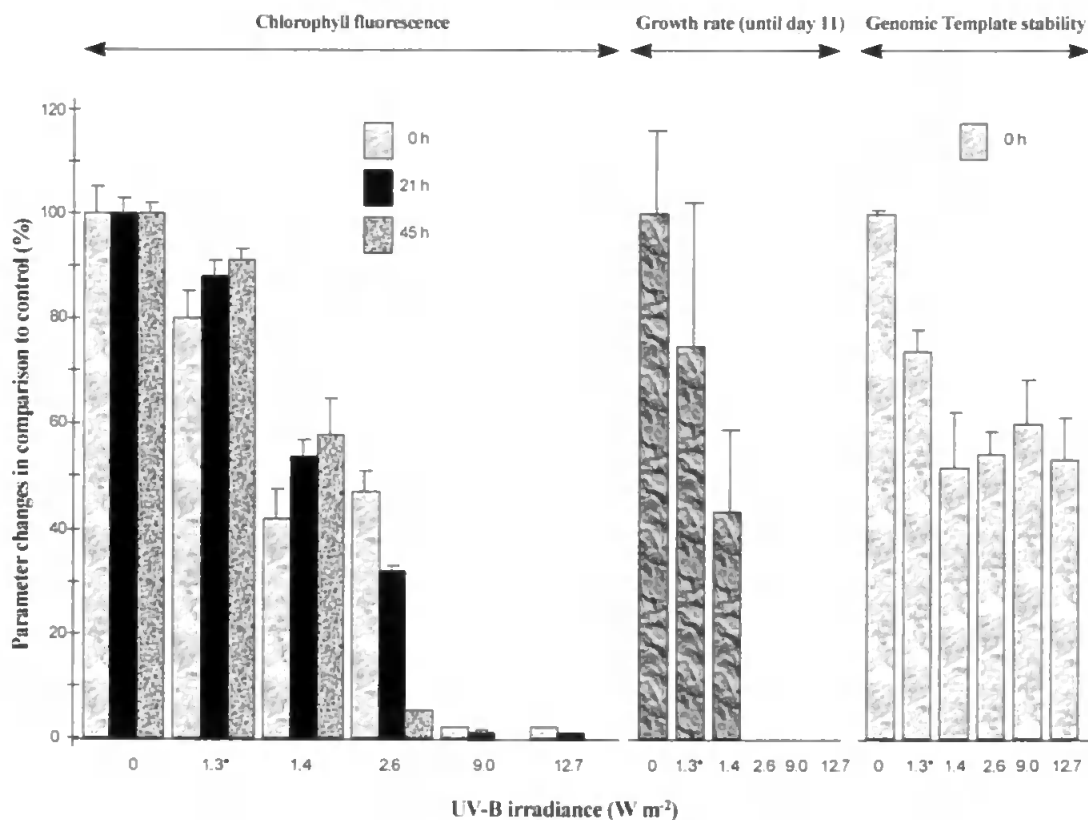


Fig. 3. Comparison among chlorophyll fluorescence, growth, and genomic template stability in populations of *P. palmata* exposed to UV radiation. The different times represent the length of time (h) following the 3 h of UV exposure. Error bars represent standard deviation. †, indicates that the algae was irradiated with UV-A only (in W m^{-2}). All the measured parameters were significantly different from control values ($P < 0.05$) apart from growth at 1.3 W m^{-2} .

alterations induced by the DNA photoproducts (Wang and Taylor, 1993), which influence the availability of the *Taq* DNA polymerase and primers. For instance, the level of DNA photoproducts induced by UV-A and UV-B irradiances of 1.4 and 2.6 W m⁻² may block the PCR enzyme at certain sites and allow a more efficient amplification of non-damaged genomic DNA. Alternatively, the increase in band intensity could be also due to a better availability of 10-mer primers. At higher level of UV-B radiations (i.e. 9.0 and 12.7 W m⁻²), it seems that the extent of DNA lesion is so important that the *Taq* DNA polymerase is more often blocked which implies a decrease in band intensity and possibly a disappearance of band (depending on the extent of DNA damage). The disappearance of PCR products mainly affected the high molecular weight bands because the odds of obtaining DNA photoproducts increase with the length of the amplified fragment. In summary, the data suggest that the frequency of DNA photoproducts increases with increasing UV-B irradiance.

Extra bands were also detected in RAPD profiles (Fig. 1). New PCR amplification products may reveal a change in the DNA sequence due to mutations [resulting in (a) new annealing event(s)], and/or large deletions (bringing two pre-existing annealing sites closer), and/or homologous recombination (juxtaposing two sequences that match the sequence of the primer). It is noteworthy that the RAPD assay has the potential to detect mutations outside the priming site (Bowditch et al., 1993). Following exposure to mutagens, DNA replication and error-prone DNA repair are generally involved in generating mutations (Livneh et al., 1993). Unlike mammals, plant cells enter meiosis only after significant vegetative growth. In this context, the induction of mutations, which accumulate over time in the somatic tissue, may therefore be passed to the gametophytes (Walbot and Cullis, 1985). This could have implications for the short- and long-term survival of the species and consequently for the ecosystem (Würgler and Kramers, 1992; Anderson et al., 1994). Mutations can only be responsible for the appearance of new bands if they occur at the same locus in a sufficient number of

cells (a minimum of 10% of mutations may be required to get a new PCR product visible in agarose gel) to be amplified by PCR. For example, 'hot spot' interactions between DNA and metabolised B(a)P products have been reported in the literature (Boles and Hogan, 1984). A similar outcome can occur with UV radiation. Appearing bands may also be the result of structural changes induced by DNA photoproducts and/or by non genotoxic events such as transposition, DNA amplification, etc. In the present study, the appearance of extra bands occurred principally for the three lowest UV irradiances used because extremely high irradiances of UV inhibit DNA repair and replication in plant tissues (Vornarx et al., 1998). Genomic template stability is related to the level of DNA damage, the efficiency of DNA repair and replication. Therefore, a high level of DNA damage does not necessarily decrease the genomic template stability (in comparison to a low level of DNA alterations) because DNA repair and replication are inhibited by the high frequency of DNA damage. In addition, since the time of exposure was very short (3 h) it is likely that extra bands were the results of DNA photoproducts (inducing some structural modifications and possibly new annealing events) rather than mutations.

In earlier studies, a non-mammalian test system for germ-cell mutagenesis was developed for detecting DNA alterations in F₁ progeny descended from the γ -irradiated male medaka fish using the AP-PCR technique. In these studies, DNA alterations were detected as changes in patterns, i.e. band loss and/or band gain (Kubota et al., 1995) and the frequency of band loss was shown to increase with increasing radiation doses (Kubota et al., 1992). In an unrelated work, RAPD profiles generated from rats exposed to benzo(a)pyrene revealed appearing and disappearing bands in comparison to control RAPD patterns (Savva, 1996). In the present study, the results of UV exposure of an algal species are consistent with these earlier studies on vertebrates.

In this study, although the two highest UV irradiances selected (i.e. 12.7 and 9.0 W m⁻²) are not environmentally realistic, they were chosen to investigate the entire range of the expo-

interpretation of the changes occurring in gels, principally if no other methods of detection of DNA lesions are used. For instance, in the present study, although it seems obvious that the frequency of DNA photoproducts increased with increasing UV-B irradiance, it is not known if mutations occurred. In addition, it seems fundamental not only to understand the effect of each category of DNA damage (e.g. DNA adducts, DNA breakages) and mutations on RAPD profiles, but also to determine the sensitivity and selectivity of the RAPD technique by comparison with pre-existing DNA analysis methodologies (e.g. 32 P-post-labelling assay, chromosome aberrations, DNA strand breakage). In conclusion, the RAPD method has been successfully used as a sensitive means of detecting UV-induced DNA damage and shows potential as a reliable and reproducible assay for genotoxicity. Furthermore, the present study suggests that the RAPD assay applied in conjunction with other biomarkers from higher levels of biological organisation would prove a powerful ecotoxicological tool.

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References

Andersson, S.L., Sadinski, W., Shugart, L., et al., 1994. Genetic and molecular ecotoxicology — a research framework. *Environ. Health Perspect.* 102 (Suppl. 12), 3-8.

Alenxar, F., Child, P., Evenden, A., Jha, A., Sava, D., Walker, C., Depledge, M., 1998. Application of the aryl-uracil primed polymerase chain reaction for the detection of DNA damage. *Mar. Environ. Res.* 46, 331-335.

Alenxar, F., Conrad, M., Evenden, A., Jha, A., Depledge, M., 1999. Qualitative assessment of genotoxicity using RAPD: comparison of genomic template stability with key fitness parameters in *Daphnia magna* exposed to benzo(a)pyrene. *Environ. Toxicol. Chem.* 18, 2275-2282.

Alenxar, F., Evenden, A., Jha, A., Sava, D., Depledge, M., 2000. Optimized RAPD analysis generates high-quality

sure-response curves. Non-chlorophyll fluorescence and growth rates decreased gradually to zero following exposure to increasing UV-B irradiance (Fig. 3) because a toxic effect can completely inhibit a biological response (e.g. when the survival of a population is affected). In contrast, the genomic template stability exhibited the same degree of inhibition at all UV-B irradiances tested. Therefore, the genomic template stability can be misleading. However, to better understand the effect of UV on *P. palmata*, the changes in RAPD profiles must be interpreted carefully. Indeed, the frequency of appearance and disappearance of bands, which have specific significance, may allow a better understanding of the results if parameters at higher levels of biological organisation are measured. For instance, the relatively high frequency of disappearance of bands may reveal that the survival of the individuals was greatly affected. The chlorophyll fluorescence and growth experiments support this observation.

The simultaneous use of more than one biomarker enhances the detection of toxic effects since different biomarker responses are induced at different stages of the organism's health status curve (Depledge, 1994). Chlorophyll fluorescence, an important indicator of reversible and irreversible changes in photosystem II (within the photosynthetic process) in plants and algae, is known to respond to a wide variety of stressors, both natural and xenobiotic (Bohar-Nordenkamp et al., 1989). Chlorophyll fluorescence has been characterised as a sensitive general biomarker to UV exposure (Cordi et al., 1997). Likewise, changes in *in vivo* absorbance spectra exhibited an inverse relationship with UV-B irradiation indicating its potential use as a biomarker. In this study, changes in RAPD patterns, reductions in optimal quantum yield and growth rates, indicated that UV-B radiation (equivalent to 17% ozone depletion) caused irreversible damage in *P. palmata*.

Whilst the RAPD technique clearly shows promise for the detection of pollutant-induced DNA effects, a great deal of further experimentation and validation is required. In the context of detection of genotoxicity using the RAPD technique, the most difficult exercise is certainly the

- generic DNA profiles at high annealing temperature. *Bio Techniques* 28, 51–54.
- Becerril, C., Ferrero, M., Sanz, F., Castano, A., 1999. Detection of mitomycin C-induced genetic damage in fish cells by use of RAPD. *Mutagenesis* 14, 449–456.
- Björn, L.O., Murphy, T.M., 1985. Computer calculation of solar ultraviolet radiation at ground level. *Physiol. Veg.* 23, 555–561.
- Boles, T.C., Hogan, M.E., 1984. Site-specific carcinogen binding to DNA. *Proc. Natl. Acad. Sci. USA* 81, 5623–5627.
- Bolhar-Nordenkamp, H.R., Long, S.P., Baker, N.R., Oquist, G., Schreiber, U., Lechner, E.G., 1989. Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. *Funct. Ecol.* 3, 497–514.
- Bowditch, B.M., Albright, D.G., Williams, J.G.K., Braun, M.J., 1993. Use of Randomly amplified polymorphic DNA markers in comparative genome studies. *Methods Enzymol.* 224, 294–309.
- Caldwell, M.M., 1971. Solar UV irradiation and the growth and development of higher plants. In: Giese, A.C. (Ed.), *Photophysiology*, vol. 6. Academic Press, New York.
- Cordi, B., Depledge, M.H., Price, D.N., Salter, L.F., Donkin, M.E., 1997. Evaluation of chlorophyll fluorescence, in vivo spectrophotometric pigment absorption and ion leakage as biomarkers of UV-B exposure in marine macroalgae. *Mar. Biol.* 130, 41–49.
- Depledge, M.H., 1994. The rational basis for the use of biomarkers as ecotoxicological tools. In: Fossi, M.C., Leonzio, C. (Eds.), *Non-Destructive Biomarkers in Vertebrates*. CRC Press, Boca Raton, FL, pp. 271–295.
- Depledge, M.H., 1998. The ecotoxicological significance of genotoxicity in marine invertebrates. *Mutat. Res.* 399, 109–122.
- Donahue, B.A., Yin, S., Taylor, J.S., Reines, D., Hanawalt, P.C., 1994. Transcript cleavage by RNA polymerase II arrested by a cyclobutane pyrimidine dimer in the DNA template. *Proc. Natl. Acad. Sci. USA* 91, 8502–8506.
- Driscoll, C.M.H., Whillock, M.J., Pearson, A.J., McKinlay, A.F., 1992. Three years of solar radiation measurement at NRPB. *Radiol. Prot. Bull.* 128, 11–16.
- Ellsworth, D.L., Rittenhouse, K.D., Honeycutt, R.L., 1993. Artefactual variation in randomly amplified polymorphic DNA banding patterns. *Biotechniques* 14, 214–217.
- Franklin, L.A., Forster, R.M., 1997. The changing irradiance environment: consequences for marine macrophyte physiology, productivity and ecology. *Eur. J. Phycol.* 32, 207–232.
- Grayson, T.H., Cooper, L.F., Atienzar, F.A., Knowles, M.R., Gilpin, M.L., 1999. Molecular differentiation of *Renibacterium salmoninarum* isolates from world-wide locations. *Appl. Environ. Microbiol.* 65, 961–968.
- Grobe, C.W., Murphy, T.M., 1994. Inhibition of growth of *Ulva expansa* (Chlorophyta) by ultraviolet-B radiation. *J. Phycol.* 30, 783–790.
- Häder, D.P., Worrest, R.C., Kumar, H.D., Smith, R.C., 1995. Effects of increased solar ultraviolet radiation on aquatic ecosystems. *Ambio* 24, 174–180.
- Ide, H., Petruccio, L.A., Hataet, Z., Wallace, S.S., 1991. Processing of DNA base damage by DNA polymerases — dihydrothymine and β -ureidoisobutyric acid as models for instructive and noninstructive lesions. *J. Biol. Chem.* 266, 1469–1477.
- Ionov, Y., Peinado, M.A., Malkhosyan, S., Shibata, D., Perucho, M., 1993. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 363, 558–561.
- Jha, A.N., 1998. Use of aquatic invertebrates in genotoxicological studies. *Mutat. Res.* 399, 1–2.
- Kubota, Y., Shimada, A., Shima, A., 1992. Detection of γ -ray-induced DNA damage in malformed dominant lethal embryos of the Japanese medaka (*Oryzias latipes*) using AP-PCR fingerprinting. *Mutat. Res.* 283, 263–270.
- Kubota, Y., Shimada, A., Shima, A., 1995. DNA alterations detected in the progeny of paternally irradiated Japanese medaka fish (*Oryzias latipes*). *Proc. Natl. Acad. Sci. USA* 92, 330–334.
- Livneh, Z., Cohen-Fix, O., Skaliter, R., Elizur, T., 1993. Replication of damaged DNA and the molecular mechanism of ultraviolet light mutagenesis. *Crit. Rev. Biochem. Mol. Biol.* 28, 465–513.
- Meunier, J.R., Grumont, P.A.D., 1993. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. *Res. Microbiol.* 144, 373–379.
- Nelson, J.R., Lawrence, C.W., Hinkle, D.C., 1996. Thymine-thymine dimer bypass by yeast DNA-polymerase-zeta. *Science* 272, 1646–1649.
- Peinado, M.A., Malkhosyan, S., Velaquez, A., Perucho, M., 1992. Isolation and characterization of allelic losses and gains in colorectal tumors by arbitrarily primed polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* 89, 10065–10069.
- Savva, D., 1996. DNA fingerprinting as a biomarker assay in ecotoxicology. *Toxicol. Ecotoxicol. News Rev.* 3, 110–114.
- Shimada, A., Shima, A., 1998. Combination of genomic DNA fingerprinting into the medaka specific-locus test system for studying environmental germ-line mutagenesis. *Mutat. Res.* 399, 149–165.
- Vornara, E.J., Mitchell, H.L., Karthikeyan, R., Chatterjee, I., Kunz, B.A., 1998. DNA repair in higher plants. *Mutat. Res.* 400, 187–200.
- Walbot, V., Cullis, C.A., 1985. Rapid genomic change in plants. *Annu. Rev. Plant Physiol.* 36, 367–396.
- Wang, C.I., Taylor, J.S., 1993. The *trans-syn-I* thymine dimer bends DNA by approximately 22 degrees and unwinds DNA by approximately 15 degrees. *Chem. Res. Toxicol.* 6, 519–523.
- Welsh, J., McClelland, M., 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* 18, 7213–7218.
- Welsh, J., Petersen, C., McClelland, M., 1991. Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. *Nucleic Acid Res.* 19, 303–306.

- White, J.W., Neuwirth, H., Miller, C.D., Schneider, E.L., 1990. DNA alterations in prostatic adenocarcinoma and benign prostatic hyperplasia: detection by DNA fingerprint analyses. *Mutat. Res.* 237, 37–43.
- Williams, J.G.K., Kubelic, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Res.* 18, 6531–6535.
- Würdler, F.E., Kramers, P.G.N., 1992. Environmental-effects of genotoxins (ecogenotoxicology). *Mutagenesis* 7, 321–327.

REFERENCES

Abercrombie M, Hickman M, Johnson ML, Thain M. 1980. *Dictionary of biology*, eight edition, Penguin, England.

Abruzzo MA, Hassold TJ. 1995. Etiology of nondisjunction in humans. *Environmental and Molecular Mutagenesis* 25:38-47.

Adams JM, Gerondakis S, Webb E, Corcoran LM, Cory S. 1983. Cellular *myc* oncogene is altered by chromosome translocation to an immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. *Proceedings of The National Academy of Sciences of The United States of America* 80:1982-1986.

Adema DMM. 1978. *Daphnia magna* as a test animal in acute and chronic toxicity tests. *Hydrobiologia* 59:125-134.

Aguilar F, Hussain SP, Cerutti P. 1993. Aflatoxin B₁ induces the transversion of G→T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proceedings of The National Academy of Sciences of The United States of America* 90:8586-8590.

Ahel M, Giger W, Koch M. 1994. Behavior of alkylphenol polyethoxylate surfactants in the aquatic environment. 1. occurrence and transformation in sewage treatment. *Water Research* 28:1131-1142.

Akiyama N, Alexander D, Aoki Y, Noda M. 1996. Characterization of mutations induced by 300 and 320 nm UV radiation in a rat fibroblast cell line. *Mutation Research* 372:119-131.

Akopyanz N, Bukanov NO, Westblom TU, Kresovich S, Berg DE. 1992. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Research* 20:5137-5142.

Alapetite C, Wachter T, Sage E, Moustachi E. 1996. Use of the alkaline comet assay to detect DNA repair deficiencies in human fibroblasts exposed to UVC, UVB, UVA and γ -rays. *International Journal of Radiation Biology* 69:359-369.

Albertini RJ, Nicklas JA, Oneill JP, Robison SH. 1990. *In vivo* somatic mutations in humans, measurement and analysis. *Annual Review of Genetics* 24:305-326.

Albertini RJ, Nicklas JA, Oneill JP. 1996. Future research directions for evaluating human genetic and cancer risk from environmental exposures. *Environmental Health Perspectives* 104:503-510.

Al-Sabti K, Kurelec B. 1985. Induction of chromosomal aberrations in the mussel *Mytilus galloprovincialis* watch. *Bulletin of Environmental Contamination and Toxicology* 35:660-665.

Al-Sabti K. 1989. Chromosomal investigation of the mussel *Mytilus galloprovincialis* from the Adriatic sea. *Cytobios* 58:149-153.

Ames BN, Durston WE, Yamasaki E, Lee FD. 1973. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proceedings of The National Academy of Sciences of The United States of America* 70:2281-2285.

Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutation Research* 31:347-364.

Anathaswamy HN, Pierceall WE. 1990. Molecular mechanisms of ultraviolet radiation carcinogenesis. *Photochemistry and Photobiology* 52: 1119-1136.

Anderson SL, Harrison FL, Chan G, Moore DH. 1990. Comparison of cellular and whole animal bioassays for estimation of radiation effects in the polychaete worm *Neanthes arenaceodentata* (Polychaeta). *Archives of Environmental Contamination and Toxicology* 19:164-174.

Anderson SL, Hose JE, Knezovich JP. 1994a. Genotoxic and developmental effects in sea urchins are sensitive indicators of effects of genotoxic chemicals. *Environmental Toxicology and Chemistry* 13:1033-1041.

Anderson SL, Sadinski W, Shugart L, Brussard P, Depledge M, Ford T, Hose J, Stegeman J, Suk W, Wirgin I, et al. 1994b. Genetic and molecular ecotoxicology: a research framework. *Environmental Health Perspectives* 102:3-8.

Anderson SL, Wild GC. 1994. Linking genotoxic responses and reproductive success in ecotoxicology. *Environmental Health Perspectives* 102:9-12.

Anderson WF, Killos L, Sanders-Haigh L, Kretschmer PJ, Diacumakos EG. 1980. Replication and expression of thymidine kinase and human globin genes microinjected into mouse fibroblasts. *Proceedings of The National Academy of Sciences of The United States of America* 77:5399-5403.

'Anonymous'. 1984. India chemical tragedy: death toll at Bhopal still rising. *Chemical and Engineering News* 62:6-7.

Arambasic MB, Bjelic S, Subakov G. 1995. Acute toxicity of heavy metals (copper, lead, zinc), phenol and sodium on *Allium cepa* L., *Lepidium sativum* L. and *Daphnia magna* St.: comparative investigations and the practical applications. *Water Research* 29:497-503.

Arribas R, Capella G, Tortola S, Masramon L, Grizzle WE, Perucho M, Peinado MA. 1997. Assessment of genomic damage in colorectal cancer by DNA fingerprinting: prognostic applications. *Journal of Clinical Oncology* 15:3230-3240.

Aruoma OI. 1994. Free radicals and antioxidant strategies in sports. *Journal of Nutritional Biochemistry* 5:370-381.

Aruwke A, Goksoyr A. 1996. Vitellogenin, zona radiata proteins, P450 and conjugating enzyme levels of juvenile Atlantic salmon treated with 4-nonylphenol. In *17th Annual meeting of SETAC*, Society of Environmental Toxicology and Chemistry, Washington.

Ashby J. 1995. Druckrey's definition of 'genotoxic'. *Mutation Research* 329:225.

ASTM. 1975. Method for acute toxicity tests with fish, macro-invertebrates and amphibians. EPA-660/3-75-009, American Society for Testing Materials, Springfield.

- Atienzar F, Child P, Evenden A, Jha A, Savva D, Walker C, Depledge M. 1998a. Application of the arbitrarily primed polymerase chain reaction for the detection of DNA damage. *Marine Environmental Research* 46:331-335.
- Atienzar F, Conradi M, Evenden AJ, Jha AN, Depledge MH. 1999. Qualitative assessment of genotoxicity using random amplified polymorphic DNA: comparison of genomic template stability with key fitness parameters in *Daphnia magna* exposed to benzo[a]pyrene. *Environmental Toxicology and Chemistry* 18:2275-2282.
- Atienzar F, Cordi B, Donkin ME, Evenden AJ, Jha AN, Depledge MH. 2000a. Comparison of ultraviolet-induced genotoxicity detected by random amplified polymorphic DNA with chlorophyll fluorescence and growth in a marine macroalgae, *Palmaria palmata*. *Aquatic Toxicology* (in press).
- Atienzar F, Desor D, Burnel D, Keller JM, Lehr P, Vasseur P. 1998b. Effect of aluminum on superoxide dismutase activity in the adult rat brain. *Biological Trace Element Research* 65:19-30.
- Atienzar F, Evenden A, Jha A, Savva D, Depledge M. 2000b. Optimized RAPD analysis generates high-quality genomic DNA profiles at high annealing temperature. *BioTechniques* 28:52-54.
- Austin B, Embley TM, Goodfellow M. 1983. Selective isolation of *Renibacterium salmoninarum*. *Fems Microbiology Letters* 17:111-114.
- Axelrod JD, Majors J. 1989. An improved method for photofootprinting yeast genes *in vivo* using *Taq* polymerase. *Nucleic Acids Research* 17:171-183.
- Ayliffe MA, Lawrence GJ, Ellis JG, Pryor AJ. 1994. Heteroduplex molecules formed between allelic sequences cause nonparental RAPD bands. *Nucleic Acids Research* 22:1632-1636.
- Baan RA, Fichtinger-Schepman AMJ, Van Loon AAWM, Van Delft JHM. 1991. Methods for detection of DNA lesions induced by genotoxic agents; developments and applications in monitoring human exposure. In *New horizons in biological dosimetry*, Gledhill BL, Mauro F (eds), Wiley-Liss, New York, USA.
- Bacon CE, Jarman WM, Costa DP. 1992. Organochlorine and polychlorinated biphenyl levels in pinniped milk from the Arctic, the Antarctic, California and Australia. *Chemosphere* 24:779-791.
- Baird DJ, Barber I, Bradley M, Calow P, Soares AMVM. 1989. The *Daphnia* bioassay: a critique. *Hydrobiologia* 188:403-406.
- Baker RJ, Van Denbussche RA, Wright AJ, Wiggins LE, Hamilton MJ, Reat EP, Smith MH, Lomakin MD, Chesser RK. 1996. High levels of genetic change in rodents of Chernobyl. *Nature* 380:707-708. (Retracted).
- Baldwin JD, Pillai MC, Cherr GN. 1992. Response of embryos of the sea urchin *Strongylocentrotus purpuratus* to aqueous petroleum waste includes the expression of a high molecular weight glycoprotein. *Marine Biology* 114:21-30.

- Banerjee SK, Banerjee S, Li SA, Li JJ.** 1994. Induction of chromosome aberrations in Syrian hamster renal cortical cells by various estrogens. *Mutation Research* 311:191-197.
- Barbacid M.** 1986. Mutagens, oncogenes and cancer. *Trends in Genetics* 2:188-192.
- Barrett JC, McLachlan JA, Elmore E.** 1983. Inability of diethylstilbestrol to induce 6-thioguanine resistant mutants and to inhibit metabolic cooperation of V79 Chinese hamster cells. *Mutation Research* 107:427-432.
- Barrett JC, Wong A, McLachlan JA.** 1981. Diethylstilbestrol induces neoplastic transformation without measurable gene mutation at two loci. *Science* 191:1402-1404.
- Barrie LA, Gregor D, Hargrave B, Lake R, Muir D, Shearer R, Tracey B, Bidleman T.** 1992. Arctic contaminants: sources, occurrence and pathways. *Science of The Total Environment* 122:1-74.
- Bassam BJ, Bentley S.** 1994. DNA Fingerprinting using arbitrary primer technology (APT): a tool or a torment. *Australian Biotechnology* 4:232-236.
- Bassam BJ, Caetano-Anolles G, Gresshoff PM.** 1992. DNA amplification fingerprinting of bacteria. *Applied Microbiology and Biotechnology* 38:70-76.
- Bast A.** 1986. Is formation of reactive oxygen by cytochrome P-450 perilous and predictable? *Trends in Pharmacological Sciences* 7:266-270.
- Bayne BL.** 1980. Physiological measurement of stress. *Rapport et Proces-Verbaux des Reunions. Conseil International pour l'Exploration de la Mer* 179:56-61.
- Beaton MJ, Hebert PDN.** 1989. Miniature genomes and endopolyploidy in cladoceran crustaceans. *Genome* 32:1048-1053.
- Becerril C, Ferrero M, Sanz F, Castano A.** 1999. Detection of mitomycin C-induced genetic damage in fish cells by use of RAPD. *Mutagenesis* 14:449-456.
- Benigni R.** 1992. Relationships between *in vitro* mutagenicity assays. *Mutagenesis* 7:335-341.
- Benter T, Papadopoulos S, Pape M, Manns M, Poliwoda H.** 1995. Optimization and reproducibility of random amplified polymorphic DNA in human. *Analytical Biochemistry* 230:92-100.
- Bentley S, Bassam BJ.** 1996. A robust DNA amplification fingerprinting system applied to analysis of genetic variation within *Fusarium oxysporum* f.sp. *cubense*. *Journal of Phytopathology* 144:207-213.
- Bhat HK, Han X, Gladek A, Liehr JG.** 1994. Regulation of the formation of the major diethylstilbestrol-DNA adduct and some evidence of its structure. *Carcinogenesis* 15:2137-2142.
- Bhattacharyya NP, Maher VM, McCormick JJ.** 1990. Effect of nucleotide excision repair in human cells on intrachromosomal homologous recombination induced by UV and 1-nitrosopyrene. *Molecular and Cellular Biology* 10:3945-3951.

Bickham JW, Smolen MJ. 1994. Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. *Environmental Health Perspectives* 102:25-28.

Bickham JW. 1990. Flow cytometry as a technique to monitor the effects of environmental genotoxins on wildlife populations. In *In situ evaluations of biological hazards of environmental pollutants*, Sandhu SS, Lower WR, De Serres FJ, Suk WA, Tice RR, (eds), Plenum Press, New York, USA.

Bielawski JP, Noack K, Pumo DE. 1995. Reproducible amplification of RAPD markers from vertebrate DNA. *BioTechniques* 18:856-860.

Bijlsma R, Bundgaard J, Boerema AC, Van Putten WF. 1997. Genetic and environmental stress and the persistence of populations. *Environmental Stress, Adaptation and Evolution* 83:193-207.

Bijlsma R, Loeschcke V. 1997. Introductory remarks: environmental stress, adaptation and evolution. *Environmental Stress, Adaptation and Evolution* 83:XIII-XVII.

Billinghurst Z, Clare AS, Fileman T, McEvoy J, Readman J, Depledge MH. 1998. Inhibition of barnacle settlement by the environmental oestrogen 4-nonylphenol and the natural oestrogen 17- β oestradiol. *Marine Pollution Bulletin* 36:833-839.

Binelli G, Bucci G. 1994. A genetic linkage map of *Picea abies* karst, based on RAPD markers, as a tool in population genetics. *Theoretical and Applied Genetics* 88:283-288.

Bingham JP, Hartley JA, Souhami RL, Grimaldi KA. 1996. Strand specific measurement of cisplatin induced DNA damage and repair using quantitative PCR. *Nucleic Acids Research* 24:987-989.

Bjerregaard P, Vislie T. 1986. Effect of copper on ion regulation and osmoregulation in the shore crab *Carcinus maenas*. *Marine Biology* 91:69-76.

Bjorn LO, Murphy TM. 1985. Computer calculation of solar ultraviolet radiation at ground level. *Physiologie Vegetale* 23:555-561.

Bodell WJ, Banerjee MR. 1976. Reduced DNA repair in mouse satellite DNA after treatment with methyl-methane sulfonate, and *N*-methyl-*N*-nitrosurea. *Nucleic Acids Research* 3:1689-1701.

Boehm TLJ, Drahovsky D. 1983. Alteration of enzymatic methylation of DNA cytosines by chemical carcinogens: a mechanism involved in the initiation of carcinogenesis. *Journal of The National Cancer Institute* 71:429-433.

Boerrigter METI, Dolle MET, Martus HJ, Gossen JA, Vijg J. 1995. Plasmid based transgenic mouse model for studying *in vivo* mutations. *Nature* 377:657-659.

Bohr VA, Okumoto DS, Hanawalt PC. 1986. Survival of UV-irradiated mammalian cells correlates with efficient DNA repair in an essential gene. *Proceedings of The National Academy of Sciences of The United States of America* 83:3830-3833.

- Bohr VA, Phillips DH, Hanawalt PC.** 1987. Heterogeneous DNA damage and repair in the mammalian genome. *Cancer Research* 47:6426-6436.
- Bohr VA, Taffe BG, Larminat F.** 1995. DNA repair, oxidative stress and aging. In *Oxidative stress and aging*, Cutler RG, Packer J, Bertram J, Mori A (eds), Birkhauser Verlag Basel, Switzerland.
- Bokern M, Harms HH.** 1997. Toxicity and metabolism of 4-n-nonylphenol in cell suspension cultures of different plant species. *Environmental Science and Technology* 31:1849-1854.
- Boles TC, Hogan ME.** 1984. Site-specific carcinogen binding to DNA. *Proceedings of The National Academy of Sciences of The United States of America* 81:5623-5627.
- Boles TC, Hogan ME.** 1986. High-resolution mapping of carcinogen binding-sites on DNA. *Biochemistry* 25:3039-3043.
- Bolhar-Nordenkamp HR, Long SP, Baker NR, Oquist G, Schreiber U, Lechner EG.** 1989. Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. *Functional Ecology* 3:497-514.
- Bond JA, Gonzalez CRM, Bradley BP.** 1993. Age dependent expression of proteins in the cladoceran *Daphnia magna* under normal and heat stress conditions. *Comparative Biochemistry and Physiology* 106:913-917.
- Borck K, Beggs JD, Brammar WJ, Hopkins AS, Murray NE.** 1976. The construction *in vitro* of transducing derivatives of phage lambda. *Molecular and General Genetics* 146:199.
- Bottger EC.** 1990. Frequent contamination of *Taq* polymerase with DNA. *Clinical Chemistry* 36:1258-1259.
- Bowditch BM, Albright DG, Williams JGK, Braun MJ.** 1993. Use of randomly amplified polymorphic DNA markers in comparative genome studies. *Methods in Enzymology* 224:294-309.
- Box HC, Budzinski EE, Dawidzik JB, Gobey JS, Freund HG.** 1997. Free radical induced tandem base damage in DNA oligomers. *Free Radical Biology and Medicine* 23:1021-1030.
- Box HC, Freund HG, Budzinski EE, Wallace JC, MacCubbin AE.** 1995. Free radical induced double base lesions. *Radiation Research* 141:91-94.
- Braithwaite E, Wu XH, Wang ZG.** 1999. Repair of DNA lesions: mechanisms and relative repair efficiencies. *Mutation Research* 424:207-219.
- Brandt TY, Ali-Osman F.** 1997. Detection of DNA damage in transcriptionally active genes by RT-PCR and assessment of repair of cisplatin induced damage in the glutathione S-transferase- π gene in human glioblastoma cells. *Toxicology and Applied Pharmacology* 143:22-29.

- Brash DE.** 1988. UV mutagenic photoproducts in *Escherichia coli* and human cells: a molecular-genetics perspective on human skin cancer. *Photochemistry and Photobiology* 48:59-66.
- Bray EA.** 1993. Molecular responses to water deficit. *Plant Physiology* 103:1035-1040.
- Breimer LH.** 1990. Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: the role of DNA base damage. *Molecular Carcinogenesis* 3:188-197.
- Bressac B, Kew M, Wands J, Ozturk M.** 1991. Selective G mutation to T mutation of p53 gene in hepatocellular carcinoma from southern Africa. *Nature* 350:429-431.
- Bridges BA.** 1997. Microbial genetics: hypermutation under stress. *Nature* 387:557-558.
- Britt AB.** 1995. Repair of DNA damage induced by ultraviolet radiation. *Plant Physiology* 108:891-896.
- Broschard TH, Wiessler M, Schmeiser HH.** 1995. Effect of site specifically located aristolochic acid DNA adducts on *in vitro* DNA synthesis by human DNA polymerase α . *Cancer Letters* 98:47-56.
- Brown RJ, Conradi M, Depledge MH.** 1999. Long term exposure to 4-nonylphenol affects sexual differentiation and growth of the amphipod *Corophium volutator* (Pallas, 1766). *Science of The Total Environment* 233:77-88.
- Brown WC, Romano LJ.** 1991. Effects of benzo[a]pyrene DNA adducts on a reconstituted replication system. *Biochemistry* 30:1342-1350.
- Brumley CM, Haritos VS, Ahokas JT, Holdway DA.** 1995. Validation of biomarkers of marine pollution exposure in sand flathead using aroclor 1254. *Aquatic Toxicology* 31:249-262.
- Brunetti R, Gola I, Majone F.** 1986. Sister chromatid exchange in developing eggs of *Mytilus galloprovincialis* Lmk. (Bivalvia). *Mutation Research* 174:207-211.
- Buening MK, Wislocki PG, Levin W, Yagi H, Thakker DR, Akagi H, Koreeda M, Jerina DM, Conney AH.** 1978. Tumorigenicity of the optical enantiomers of the diastereomeric benzo[a]pyrene 7,8-diol-9,10-epoxides in newborn mice: exceptional activity of (+)-7 β ,8 α -dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. *Proceedings of The National Academy of Sciences of The United States of America* 75:5358-5361.
- Burke JF, Heywood LA, Moorwood K, Mogg AE.** 1990. Repair of DNA mismatches in mammalian cells: evidence for short patch excisions. *Mutagenesis* 5:79.
- Butterworth BE, Ashby J, Bermudez E, Casciano D, Mirsalis J, Probst G, Williams G.** 1987. A protocol and guide for the *in vivo* rat hepatocyte DNA repair assay. *Mutation Research* 189:123-133.
- Cadenas E.** 1989. Biochemistry of oxygen toxicity. *Annual Review of Biochemistry* 58:79-110.

- Cadet J, Delatour T, Douki T, Gasparutto D, Pouget JP, Ravanat JL, Sauvaigo S.** 1999. Hydroxyl radicals and DNA base damage. *Mutation Research* 424:9-21.
- Caetano-Anolles G, Bassam BJ, Gresshoff PM.** 1992. Primer template interactions during DNA amplification fingerprinting with single arbitrary oligonucleotides. *Molecular and General Genetics* 235:157-165.
- Caetano-Anolles G.** 1998. DAF optimization using Taguchi methods and the effect of thermal cycling parameters on DNA amplification. *BioTechniques* 25:472-480.
- Cairns J, Mount DI.** 1990. Aquatic toxicology. 2. *Environmental Science and Technology* 24:154-161.
- Cairns J.** 1988. Origin of mutants disputed: reply. *Nature* 336:527-528.
- Cairns MJ, Murray V.** 1994. Comparison of the sequence specificity of *cis*-diaminedichloroplatinum(II) damage in guanine containing and 7-deazaguanine-containing DNA. *Biochimica et Biophysica Acta* 1218:315-321.
- Caldwell MM.** 1971. Solar UV irradiation and the growth and development of higher plants. In *Photophysiology*, Giese AC (ed), Academic Press, New York, USA.
- Calow P, Silby RM, Forbes V.** 1997. Risk assessment on the basis of simplified life-history scenarios. *Environmental Toxicology and Chemistry* 16:1983-1989.
- Campos LP, Raelson JV, Grant WF.** 1994. Genome relationships among lotus species based on random amplified polymorphic DNA (RAPD). *Theoretical and Applied Genetics* 88:417-422.
- Canova S, Degan P, Peters LD, Livingstone DR, Voltan R, Venier P.** 1998. Tissue dose, DNA adducts, oxidative DNA damage and CYP1A-immunopositive proteins in mussels exposed to waterborne benzo[a]pyrene. *Mutation Research* 399:17-30.
- Carmichael PL, Hewer A, Osborne MR, Strain AJ, Phillips DH.** 1995. Detection of bulky DNA lesions in the liver of patients with Wilsons disease and primary hemochromatosis. *Mutation Research* 326:235-243.
- Carr AM, Hoekstra MF.** 1995. The cellular responses to DNA damage. *Trends in Cell Biology* 5:32-40.
- Carr RS, Hillman RE, Neff JM.** 1991. Field assessment of biomarkers for winter flounder. *Marine Pollution Bulletin* 22:61-67.
- Carrano AV, Thompson LH, Lindl PA, Minkler JL.** 1978. Sister chromatid exchange as an indicator of mutagenesis. *Nature* 271:551-553.
- Cavalieri EL, Rogan EG.** 1995. Central role of radical cations in metabolic activation of polycyclic aromatic hydrocarbons. *Xenobiotica* 25:677-688.
- Cebula TA, Koch WH.** 1991. Polymerase chain reaction (PCR) and its application to mutational analysis. In *New horizons in Biology Dosimetry*, Gledhill BL, Mauro F (eds), Wiley-Liss, New York, USA.

- Cerda S, Weitzman SA.** 1997. Influence of oxygen radical injury on DNA methylation. *Mutation Research* 386:141-152.
- Cerutti PA, Trump BF.** 1991. Inflammation and oxidative stress in carcinogenesis. *Cancer Cells* 3:1-7.
- Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT.** 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Research* 16:11141-11156.
- Chan GL, Doetsch PW, Haseltine WA.** 1985. Cyclobutane pyrimidine dimers and (6-4) photoproducts block polymerization by DNA polymerase I. *Biochemistry* 24:5723-5728.
- Chary P, Lloyd RS.** 1995. *In vitro* replication by prokaryotic and eukaryotic polymerases on DNA templates containing site specific and stereospecific benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide adducts. *Nucleic Acids Research* 23:1398-1405.
- Cheeseman KH, Beavis A, Esterbauer H.** 1988. Hydroxyl radical induced iron catalysed degradation of 2-deoxyribose quantitative determination of malondialdehyde. *Biochemical Journal* 252:649-653.
- Chen RH, Maher VM, McCormick JJ.** 1990. Effect of excision repair by diploid human fibroblasts on the kinds and locations of mutations induced by (\pm)-7 β ,8 α -dihydroxy-9- α ,10- α -epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene in the coding region of the *HPRT* gene. *Proceedings of The National Academy of Sciences of The United States of America* 87:8680-8684.
- Chiocca SM, Sandy MS, Cerutti PA.** 1992. Genotypic analysis of *N*-ethyl-*N*-nitrosourea induced mutations by *Taq* I restriction fragment length polymorphism polymerase chain reaction in the c-H-*ras*1 gene. *Proceedings of The National Academy of Sciences of The United States of America* 89:5331-5335.
- Chipman JK, Marsh JW.** 1991. Bio-techniques for the detection of genetic toxicity in the aquatic environment. *Journal of Biotechnology* 17:199-208.
- Choi DJ, Marinoalelessandri DJ, Geacintov NE, Scicchitano DA.** 1994. Site specific benzo[a]pyrene diol epoxide DNA adducts inhibit transcription elongation by bacteriophage T7 RNA polymerase. *Biochemistry* 33:780-787.
- Chorazy M.** 1985. Sequence rearrangements and genome instability: a possible step in carcinogenesis. *Journal of Cancer Research and Clinical Oncology* 109:159-172.
- Christman JK, Sheikhejad G, Marasco CJ, Sufrin JR.** 1995. 5-methyl-2'-deoxycytidine in single-stranded DNA can act *in cis* to signal *de novo* DNA methylation. *Proceedings of The National Academy of Sciences of The United States of America* 92:7347-7351.
- Christner DF, Lakshman MK, Sayer JM, Jerina DM, Dipple A.** 1994. Primer extension by various polymerases using oligonucleotide templates containing stereoisomeric benzo[a]pyrene deoxyadenosine adducts. *Biochemistry* 33:14297-14305.

- Ciarrocchi G, Pedrini AM.** 1982. Determination of pyrimidine dimer unwinding angle by measurement of DNA electrophoretic mobility. *Journal of Molecular Biology* 155:177-183.
- Clark AG, Lanigan CMS.** 1993. Prospects for estimating nucleotide divergence with RAPDs. *Molecular Biology and Evolution* 10:1096-1111.
- Claxton LD, Houk VS, Hughes TJ.** 1998. Genotoxicity of industrial wastes and effluents. *Mutation Research* 410:237-243.
- Clive D, Caspary W, Kirby PE, Krehl R, Moore M, Mayo J, Oberly TJ.** 1987. Guide for performing the mouse lymphoma assay for mammalian cell mutagenicity. *Mutation Research* 189:143-156.
- Cockerham LG, Shane BS.** 1994. *Basic environmental toxicology*, CRC Press, Boca Raton, Florida.
- Colborn T, Clement C.** 1992. *Chemically induced alterations in sexual and functional development: the wildlife/human connection*, Princeton Scientific Publishing, Princeton, New Jersey.
- Cole J, Fox M, Garner RC, McGregor DB, Thacker J.** 1990. Gene mutation assays in cultured mammalian cells. In *Basic mutagenicity tests: UKEMS recommended procedures*, Kirland DJ (ed), The Bath Press, Avon, UK.
- Cole RJ, Taylor NA, Cole J, Arlett CF.** 1979. Transplacental effects of chemical mutagens detected by the micronucleus test. *Nature* 277:317-318.
- Colerangle JB, Roy D.** 1996. Exposure of environmental estrogenic compound nonylphenol to noble rats alters cell cycle kinetics in the mammary gland. *Endocrine* 4:115-122.
- Coller HA, Thilly WG.** 1994. Development and applications of mutational spectra technology. *Environmental Science and Technology* 28:A478-A487.
- Concordet JP, Ferry A.** 1993. Physiological programmed cell death in thymocytes is induced by physical stress (exercise). *American Journal of Physiology* 265:C626-C629.
- Conradi M, Depledge MH.** 1998. Population responses of the marine amphipod *Corophium volutator* (Pallas, 1766) to copper. *Aquatic Toxicology* 44:31-45.
- Cordi B, Depledge MH, Price DN, Salter LF, Donkin ME.** 1997. Evaluation of chlorophyll fluorescence, *in vivo* spectrophotometric pigment absorption and ion leakage as biomarkers of UV-B exposure in marine macroalgae. *Marine Biology* 130:41-49.
- Cordi B, Hyde P, Donkin ME, Price DN, Depledge MH.** 1999. Evaluation of *in vivo* thallus absorbance and chlorophyll fluorescence as biomarkers of UV-B exposure and effects in marine macroalgae from different tidal levels. *Marine Environmental Research* 48:193-212.
- Cortopassi GA, Arnheim N.** 1992. Using the polymerase chain reaction to estimate mutation frequencies and rates in human cells. *Mutation Research* 277:239-249.

- Cosman M, Delossantos C, Fiala R, Hingerty BE, Singh SB, Ibanez V, Margulis LA, Live D, Geacintov NE, Broyde S, et al.** 1992. Solution conformation of the major adduct between the carcinogen (+)-anti-benzo[a]pyrene diol epoxide and DNA. *Proceedings of The National Academy of Sciences of The United States of America* 89:1914-1918.
- Counts JL, Goodman JI.** 1994. Hypomethylation of DNA: an epigenetic mechanism involved in tumor promotion. *Molecular Carcinogenesis* 11:185-188.
- Crawford DR, Edbauer-Nechamen N, Schools GP, Salmon SL, Davies JMS, Davies KJA.** 1995. Oxidant-modulated gene expression. In *The oxygen paradox*, Davies KJA, Ursini F (eds), Kleup University Press, Padova, Italy.
- Cui X, Brenneman M, Meyne J, Oshimura M, Goodwin EH, Chen DJ.** 1999. The XRCC2 and XRCC3 repair genes are required for chromosome stability in mammalian cells. *Mutation Research* 434:75-88.
- Daffonchio D, Borin S, Frova G, Manachini PL, Sorlini C.** 1998. PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. *International Journal of Systematic Bacteriology* 48:107-116.
- Darras BT, Francke U.** 1987. A partial deletion of the muscular dystrophy gene transmitted twice by an unaffected male. *Nature* 329:556-558.
- Das RK, Nanda NK.** 1986. Induction of micronuclei in peripheral erythrocytes of fish *Heteropneustes fossilis* by mitomycin C and paper mill effluent. *Mutation Research* 175:67-71.
- Dave G.** 1984. Effects of copper on growth, reproduction, survival and hemoglobin in *Daphnia magna*. *Comparative Biochemistry and Physiology* 78:439-443.
- Dax E, Livneh O, Edelbaum O, Kedar N, Gavish N, Karchi H, Milo J, Sela I, Rabinowitch HD.** 1994. A random amplified polymorphic DNA (RAPD) molecular marker for the TM-2^a gene in tomato. *Euphytica* 74:159-163.
- De Boer JG.** 1995. Software package for the management of sequencing projects using *lacI* transgenic animals. *Environmental and Molecular Mutagenesis* 25:256-262.
- De Flora S, Bagnasco M, Zanicchi P.** 1991. Genotoxic, carcinogenic, and teratogenic hazards in the marine environment, with special reference to the Mediterranean sea. *Mutation Research* 258:285-320.
- De Raat WK, Hanstveit AO, Dekreuk JF.** 1985. The role of mutagenicity testing in the ecotoxicological evaluation of industrial discharges into the aquatic environment. *Food and Chemical Toxicology* 23:33-41.
- Degrassi F, Rizzoni M.** 1982. Micronucleus test in *Vicia faba* root tips to detect mutagen damage in fresh water pollution. *Mutation Research* 97:19-33.

- De Jong D, Voetdijk BMH, Kluinnelemans JC, Van Ommen GJB, Kluin PM.** 1988. Somatic changes in B lymphoproliferative disorders (B-LPD) detected by DNA fingerprinting. *British Journal of Cancer* 58:773-775.
- Demarini DM.** 1994. Induction of mutation spectra by complex mixtures: approaches, problems, and possibilities. *Environmental Health Perspectives* 102:127-130.
- Depledge MH, Amaral-Mendes JJ, Daniel B, Halbrook RS, Kloepper-Sams P, Moore MN, Peakall DB.** 1993. The conceptual basis of the biomarker approach. In *Biomarkers*, Peakall DB, Shugart LR (eds), Springer Verlag, Berlin, Germany.
- Depledge MH, Billingham Z.** 1999. Ecological significance of endocrine disruption in marine invertebrates. *Marine Pollution Bulletin* 39:32-38.
- Depledge MH.** 1994a. Genotypic toxicity: implications for individuals and populations. *Environmental Health Perspectives* 102:101-104.
- Depledge MH.** 1994b. The rational basis for the use of biomarkers as ecotoxicological tools. In *Nondestructive biomarkers in vertebrates*, Fossi MC, Leonzio C (eds), Lewis Publishers, Boca Raton, Florida, USA.
- Depledge MH.** 1998. The ecotoxicological significance of genotoxicity in marine invertebrates. *Mutation Research* 399:109-122.
- Devanesan PD, Higginbotham S, Ariese F, Jankowiak R, Suh M, Small GJ, Cavalieri EL, Rogan EG.** 1996. Depurinating and stable benzo[a]pyrene DNA adducts formed in isolated rat liver nuclei. *Chemical Research in Toxicology* 9:1113-1116.
- Dixon DR, Clarke KR.** 1982. Sister chromatid exchange: a sensitive method for detecting damage caused by exposure to environmental mutagens in the chromosomes of adult *Mytilus edulis*. *Marine Biology Letters* 3:163-172.
- Dixon DR.** 1982. Aneuploidy in mussel embryos *Mytilus edulis* originating from a polluted dock. *Marine Biology Letters* 3:155-161.
- Dizdaroglu M, Laval J, Boiteux S.** 1993. Substrate specificity of the *Escherichia coli* endonuclease III excision of thymine derived and cytosine derived lesions in DNA produced by radiation generated free radicals. *Biochemistry* 32:12105-12111.
- Djordjevic B, Tolmach LJ.** 1967. Response of synchronous populations of Hela cells to ultraviolet irradiation at selected stages of the generation cycle. *Radiation Research* 32:327-346.
- Dolle MET, Martus HJ, Novak M, VanOrsouw NJ, Vijg J.** 1999. Characterization of color mutants in *lacZ* plasmid-based transgenic mice, as detected by positive selection. *Mutagenesis* 14:287-293.
- Doma S.** 1979. Ephippia of *Daphnia Magna* Straus: a technique for their mass production and quick revival. *Hydrobiologia* 67:183-188.
- Donahue BA, Yin S, Taylor JS, Reines D, Hanawalt PC.** 1994. Transcript cleavage by RNA polymerase II arrested by a cyclobutane pyrimidine dimer in the DNA template.

Douglas GR, Jiao JL, Gingerich JD, Gossen JA, Soper LM. 1995. Temporal and molecular characteristics of mutations induced by ethylnitrosourea in germ cells isolated from seminiferous tubules and in spermatozoa of *lacZ* transgenic mice. *Proceedings of The National Academy of Sciences of The United States of America* 92:7485-7489.

Downes CS, Wilkins AS. 1994. Cell cycle checkpoints, DNA repair and DNA replication strategies. *Bioessays* 16:75-79.

Drevon C, Piccoli C, Montesano R. 1981. Mutagenicity assays of estrogenic hormones in mammalian cells. *Mutation Research* 89:83-90.

Driscoll CMH, Whillock MJ, Pearson AJ, Mckinlay AF. 1992. Three years of solar radiation measurement at NRPB. *Radiological Protection Bulletin* 128:11-16.

Drouin R, Gao S, Holmquist GP. 1996a. Agarose gel electrophoresis for DNA damage analysis. In *Technologies for detection of DNA damage and mutations*, Pfeifer GP (ed), Plenum Press, New York, USA.

Drouin R, Rodriguez H, Gao SW, Gebreyes Z, Oconnor TR, Holmquist GP, Akman SA. 1996b. Cupric ion ascorbate hydrogen peroxide induced DNA damage: DNA bound copper ion primarily induces base modifications. *Free Radical Biology and Medicine* 21:261-273.

Druckrey H. 1973. Specific carcinogenic and teretogenic effects of 'indirect' alkylating methyl and ethyl compounds and their dependency on stages of ontogenic developments. *Xenobiotica* 3:271-303.

Dubrova YE, Nesterov VN, Krouchinsky NG, Ostapenko VA, Neumann R, Neil DL, Jeffreys AJ. 1996. Human minisatellite mutation rate after the Chernobyl accident. *Nature* 380:683-686.

Dugle DL, Gillespie CJ, Chapman JD. 1976. DNA strand breaks, repair, and survival in x-irradiated mammalian cells. *Proceedings of The National Academy of Sciences of The United States of America* 73:809-812.

Eberhardt MK, Ramirez G, Ayala E. 1989. Does the reaction of Cu^+ with H_2O_2 give OH^\cdot radicals: a study of aromatic hydroxylation. *Journal of Organic Chemistry* 54:5922-5926.

Eckert KA, Opresko PL. 1999. DNA polymerase mutagenic bypass and proofreading of endogenous DNA lesions. *Mutation Research* 424:221-236.

Eichhorn GL, Shin YA. 1968. Interaction of metal ions with polynucleotides and related compounds. XII. The relative effect of various metal ions on DNA helicity. *Journal of the American Chemical Society* 90:7323-7328.

Elendt BP, Bias WR. 1990. Trace nutrient deficiency in *Daphnia magna* cultured in standard medium for toxicity testing: effects of the optimization of culture conditions on life history parameters of *Daphnia magna*. *Water Research* 24:1157-1167.

Ellis D. 1989. *Environments at risks. Case histories of impact assessment*, Springer-Verlag, Berlin.

Ellsworth DL, Rittenhouse KD, Honeycutt RL. 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. *BioTechniques* 14:214-217.

Enserink L, Delahaye M, Maas H. 1993. Reproductive strategy of *Daphnia magna*: implications for chronic toxicity tests. *Aquatic Toxicology* 25:111-123.

Epe B, Metzler M. 1985. Nature of the macromolecular binding of diethylstilbestrol to DNA and protein following oxidation by peroxidase hydrogen peroxidase. *Chemico Biological Interactions* 56:351-361.

Erlich HA, Gelfand D, Sninsky JJ. 1991. Recent advances in the polymerase chain reaction. *Science* 252:1643-1651.

Evenden AJ, Grayson TH, Gilpin ML, Munn CB. 1993. *Reinbacterium salmoninarum* and bacterial kidney disease: the unfinished jigsaw. *Annual Review of Fish Diseases* 3:87-104.

Evers EHG, Dulfer JW, Schobben HPM, Van Hattum B, Scholten MC, Frintrop PCM, Van Steenwijk JM, Van Der Heijdt LM. 1997. *Oil and oil constituents*, RWS report RIKZ 97.032.

Fahrig R, Neuhauser-Klaus A. 1985. Similar pigmentation characteristics in the specific locus and the mammalian spot test: a way to distinguish between induced mutation and recombination. *Journal of Heredity* 76:421-426.

Fairbairn DW, Olive PL, Oneill KL. 1995. The comet assay: a comprehensive review. *Mutation Research* 339:37-59.

Farmer PB. 1994. Carcinogen adducts: use in diagnosis and risk assessment. *Clinical Chemistry* 40:1438-1443.

Farmer PB. 1999. Studies using specific biomarkers for human exposure assessment to exogenous and endogenous chemical agents. *Mutation Research* 428:69-81.

Feig DI, Reid TM, Loeb LA. 1994a. Reactive oxygen species in tumorigenesis. *Cancer Research* 54:S1890-S1894.

Feig DI, Sowers LC, Loeb LA. 1994b. Reverse chemical mutagenesis: identification of the mutagenic lesions resulting from reactive oxygen species mediated damage to DNA. *Proceedings of The National Academy of Sciences of The United States of America* 91:6609-6613.

Feldman G, Remsen J, Wang TV, Cerutti P. 1980. Formation and excision of covalent deoxyribonucleic acid adducts of benzo[a]pyrene 4,5-epoxide and benzo[a]pyrenediol epoxide I in human lung cells A549. *Biochemistry* 19:1095-1101.

Fenton HJH. 1894. Oxidation of tartaric acid in presence of iron. *Journal of the Chemical Society* 65:899-910.

- Fetterman BA, Kim BS, Margolin BH, Schildcrout JS, Smith MG, Wagner SM, Zeiger E. 1997. Predicting rodent carcinogenicity from mutagenic potency measured in the Ames *Salmonella* assay. *Environmental and Molecular Mutagenesis* 29:312-322.
- Fey MF, Wells RA, Wainscoat JS, Thein SL. 1988. Assessment of clonality in gastrointestinal cancer by DNA fingerprinting. *Journal of Clinical Investigation* 82:1532-1537.
- Fielder RJ, Allen JA, Boobis AR, Botham PA, Doe J, Esdaile DJ, Gatehouse DG, Hodsonwalker G, Morton DB, Kirkland DJ, et al. 1992. Dose setting in *in vivo* mutagenicity assays. *Mutagenesis* 7:313-319.
- Finney DJ. 1971. *Probit Analysis*. London Cambridge University Press, London, UK.
- Fischer SG, Lerman LS. 1983. DNA fragments differing by single base pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. *Proceedings of The National Academy of Sciences of The United States of America* 80:1579-1583.
- Flowers L, Bleczinski WF, Burczynski ME, Harvey RG, Penning TM. 1996. Disposition and biological activity of benzo[a]pyrene-7,8-dione: a genotoxic metabolite generated by dihydrodiol dehydrogenase. *Biochemistry* 35:13664-13672.
- Forbes VE, Calow P. 1997. Responses of aquatic organisms to pollutant stress: theoretical and practical implications. *Environmental Stress, Adaptation and Evolution* 83:25-41.
- Forbes VE, Calow P. 1999. Is the *per capita* rate of increase a good measure of population level effects in ecotoxicology? *Environmental Toxicology and Chemistry* 18:1544-1556.
- Forbes VE, Depledge MH. 1992. Cadmium effects on the carbon and energy balance of mudsnails. *Marine Biology* 113:263-269.
- Forbes VE, Forbes TL. 1994. *Ecotoxicology in Theory and Practice*, Chapman and Hall, London, UK.
- Fossi MC, Leonzio C, Peakall DB. 1994. The use of nondestructive biomarkers in the hazard assessments of vertebrate populations. In *Nondestructive biomarkers in vertebrates*, Fossi MC, Leonzio C (eds), Boca Raton, Lewis publishers, Florida, USA.
- Fossi MC, Leonzio C. 1994. *Nondestructive biomarkers in vertebrates*, Boca Raton, Lewis publishers, Florida, USA.
- Foster PL. 1998. Adaptive mutation: has the unicorn landed? *Genetics* 148:1453-1459.
- Foulds L. 1975. *Neoplastic development*, Academic Press, New York, USA.
- Fox HM, Phear EA. 1953. Factors influencing haemoglobin synthesis. *Proceedings of the Royal Society of London* B141:179-189.
- Fox M. 1985. Assay of colony forming ability in established cell lines. In *Cell clones: manual of mammalian cell technique*, Potten CS, Hendry JH (eds), Churchill Livingstone, New York, USA.

- Franklin LA, Forster RM.** 1997. The changing irradiance environment: consequences for marine macrophyte physiology, productivity and ecology. *European Journal of Phycology* 32:207-232.
- Fridovich I.** 1995. Superoxide radical and superoxide dismutases. *Annual Review of Biochemistry* 64:97-112.
- Fry DM, Toone CK, Speich SM, Peard RJ.** 1987. Sex ratio skew and breeding patterns of gulls: demographic and toxicological considerations. *Studies in Avian Biology* 10:26-43.
- Ganguly A, Prockop DJ.** 1990. Detection of single-base mutations by reaction of DNA heteroduplexes with a water-soluble carbodiimide followed by primer extension: application to products from the polymerase chain reaction. *Nucleic Acids Research* 18:3933-3939.
- Ganguly T, Duker NJ.** 1992. Inhibition of DNA polymerase activity by thymine hydrates. *Mutation Research* 293:71-77.
- Gao SW, Drouin R, Holmquist GP.** 1994. DNA repair rates mapped along the human *PGK1* gene at nucleotide resolution. *Science* 263:1438-1440.
- Gardner GR, Yevich PP, Hurst J, Thayer P, Benyi S, Harshbarger JC, Pruell RJ.** 1991. Germinomas and teratoid siphon anomalies in softshell clams, *Mya arenaria*, environmentally exposed to herbicides. *Environmental Health Perspectives* 90:43-51.
- George AM, Sabovljevic SA, Hart LE, Cramp WA, Harris G, Hornsey S.** 1987. DNA quaternary structure in the radiation sensitivity of human lymphocytes: a proposed role of copper. *British Journal of Cancer* 55:141-144.
- Giam CS, Holliday TL, Williams JL.** 1989. DNA adducts in medaka exposed to diethylnitrosamine. *Biochemistry* 28:1949.
- Giattina JD, Garton RR.** 1983. A review of the preference avoidance responses of fishes to aquatic contaminants. *Residue Reviews* 87:43-90.
- Gibbs RA, Nguyen PN, Edwards A, Civitello AB, Caskey CT.** 1990. Multiplex DNA deletion detection and exon sequencing of the hypoxanthine phosphoribosyltransferase gene in *Lesch nyhan* families. *Genomics* 7:235-244.
- Gillespie RB, Guttman SI.** 1993. Correlations between water quality and frequencies of allozyme genotypes in spotfin shiner *Notropis spilopterus* populations. *Environmental Pollution* 81:147-150.
- Glatt HR, Metzler M, Oesch F.** 1979. Diethylstilbestrol and 11 derivatives: a mutagenicity study with *Salmonella typhimurium*. *Mutation Research* 67:113-121.
- Godet F, Vasseur P, Babut M.** 1993. *In vitro* and *in vivo* genotoxicity tests for studying contaminated aquatic environmental samples. *Revue des Sciences de L'eau* 6:285-314.
- Goksoyr A, Forlin L.** 1992. The cytochrome P450 system in fish, aquatic toxicology and environmental monitoring. *Aquatic Toxicology* 22:287-311.

- Gomez-Arroyo S, Armienta MA, CortesEslava J, VillalobosPietrini R.** 1997. Sister chromatid exchanges in *Vicia faba* induced by arsenic: contaminated drinking water from Zimapan, Hidalgo, Mexico. *Mutation Research* 394:1-7.
- Gonzalgo ML, Jones PA.** 1997. Mutagenic and epigenetic effects of DNA methylation. *Mutation Research* 386:107-118.
- Goodfellow M, Embley TM, Austin B.** 1985. Numerical taxonomy and emended description of *Renibacterium salmoninarum*. *Journal of General Microbiology* 131:2739-2752.
- Gossen JA, Deleeuw WJF, Tan CHT, Zwarthoff EC, Berends F, Lohman PHM, Knook DL, Vijg J.** 1989. Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutations *in vivo*. *Proceedings of The National Academy of Sciences of The United States of America* 86:7971-7975.
- Govan HL, Vallesayoub Y, Braun J.** 1990. Fine mapping of DNA damage and repair in specific genomic segments. *Nucleic Acids Research* 18:3823-3830.
- Govers H, Ruepert C, Aiking H.** 1984. Quantitative structure activity relationships for polycyclic aromatic hydrocarbons: correlation between molecular connectivity, physicochemical properties, bioconcentration and toxicity in *Daphnia pulex*. *Chemosphere* 13:227-236.
- Gray RH.** 1990. Fish behavior and environmental assessment. *Environmental Toxicology and Chemistry* 9:53-67.
- Grayson TH, Atienzar FA, Alexander SM, Cooper LF, Gilpin ML.** 2000. Molecular diversity of *Renibacterium salmoninarum* isolates determined by randomly amplified polymorphic DNA analysis. *Applied and Environmental Microbiology* 66:435-438.
- Grayson TH, Cooper LF, Atienzar FA, Knowles MR, Gilpin ML.** 1999. Molecular differentiation of *Renibacterium salmoninarum* isolates from worldwide locations. *Applied and Environmental Microbiology* 65:961-968.
- Grinfeld S, Jaylet A, Siboulet R, Deparis P, Chouroulinkov I.** 1986. Micronuclei in red blood cells of the newt *Pleurodeles waltl* after treatment with benzo(a)pyrene: dependence on dose, length of exposure, posttreatment time, and uptake of the drug. *Environmental Mutagenesis* 8:41-51.
- Grobe CW, Murphy TM.** 1994. Inhibition of growth of *Ulva expansa* (Chlorophyta) by ultraviolet-B radiation. *Journal of Phycology* 30:783-790.
- Grunstein M.** 1997. Histone acetylation in chromatin structure and transcription. *Nature* 389:349-352.
- Guillette LJ, Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR.** 1994. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environmental Health Perspectives* 102:680-688.

- Gupta RC, Reddy MV, Randerath K.** 1982. ^{32}P post-labeling analysis of nonradioactive aromatic carcinogen DNA adducts. *Carcinogenesis* 3:1081-1092.
- Gupta RC.** 1984. Nonrandom binding of the carcinogen *N*-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA *in vivo*. *Proceedings of The National Academy of Sciences of The United States of America* 81:6943-6947.
- Guttman SI.** 1994. Population genetic structure and ecotoxicology. *Environmental Health Perspectives* 102:97-100.
- Gwakisa PS, Kemp SJ, Teale AJ.** 1994. Characterization of zebu cattle breeds in Tanzania using random amplified polymorphic DNA markers. *Animal Genetics* 25:89-94.
- Hader DP, Worrest RC, Kumar HD, Smith RC.** 1995. Effects of increased solar ultraviolet radiation on aquatic ecosystems. *Ambio* 24:174-180.
- Hall BG.** 1990. Spontaneous point mutations that occur more often when advantageous than when neutral. *Genetics* 126:5-16.
- Hall BG.** 1991. Adaptive evolution that requires multiple spontaneous mutations: mutations involving base substitutions. *Proceedings of The National Academy of Sciences of The United States of America* 88:5882-5886.
- Halliwell B, Aruoma OI.** 1991. DNA damage by oxygen derived species: its mechanism and measurement in mammalian systems. *Febs Letters* 281:9-19.
- Halliwell B, Gutteridge JMC.** 1985. The importance of free radicals and catalytic metal ions in human diseases. *Molecular Aspects of Medicine* 8:89-193.
- Hamer DH.** 1986. Metallothionein. *Annual Review of Biochemistry* 55:913-951.
- Han X, Liehr JG.** 1994a. DNA single strand breaks in kidneys of Syrian hamsters treated with steroidal estrogens. Hormone-induced free radical damage preceding renal malignancy. *Carcinogenesis* 15:977-1000.
- Han X, Liehr JG.** 1994b. 8-Hydroxylation of guanine bases in kidney and liver DNA of hamsters treated with estradiol: role of free radicals in estrogen-induced carcinogenesis. *Carcinogenesis* 15:5515-5517.
- Hanawalt PC.** 1998. Genomic instability: environmental invasion and the enemies within. *Mutation Research* 400:117-125.
- Hando JC, Nath J, Tucker JD.** 1994. Sex chromosomes, micronuclei and aging in women. *Chromosoma* 103:186-192.
- Hansen BM, Damgaard PH, Eilenberg J, Pedersen JC.** 1998. Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated from leaves and insects. *Journal of Invertebrate Pathology* 71:106-114.
- Hargrave BT, Muir DCG, Bidleman TF.** 1993. Toxaphene in amphipods and zooplankton from the Arctic ocean. *Chemosphere* 27:1949-1963.

- Harrison FL, Anderson SL.** 1989. Reproductive success as an indicator of genotoxicity in the polychaete worm, *Neanthes arenaceodentata*. *Marine Environmental Research* 28:313-316.
- Harrison FL, Jones IM.** 1982. An *in vivo* sister chromatid exchange assay in the larvae of the mussel *Mytilus edulis*: response to three mutagens. *Mutation Research* 105:235-242.
- Harshbarger JC.** 1969. The registry of tumors in lower animals. *National Cancer Institute Monograph* 31:xi-xvi.
- Harshbarger JC.** 1977. Role of the registry of tumors in lower animals in the study of environmental carcinogenesis in aquatic animals. *Annals of the New York Academy of Sciences* 298:280-289.
- Hartmann A, Niess AM, Grunertfuchs M, Poch B, Speit G.** 1995. Vitamin E prevents exercise induced DNA damage. *Mutation Research* 346:195-202.
- Hartmann A, Plappert U, Raddatz K, Grunertfuchs M, Speit G.** 1994. Does physical activity induce DNA damage? *Mutagenesis* 9:269-272.
- Hartmann A, Speit G.** 1995. Genotoxic effects of chemicals in the single cell gel (SCG) test with human blood cells in relation to the induction of sister chromatid exchanges (SCE). *Mutation Research* 346:49-56.
- Harvey JS, Lyons BP, Page TS, Stewart C, Parry JM.** 1999. An assessment of the genotoxic impact of the sea empress oil spill by the measurement of DNA adduct levels in selected invertebrate and vertebrate species. *Mutation Research* 441:103-114.
- Harvey JS, Parry JM.** 1998. The analysis of DNA adduct formation, removal and persistence in the common mussel *Mytilus edulis* exposed to 4-nitroquinoline 1-oxide. *Mutation Research* 399:31-42.
- Hecker E.** 1987. Three stage carcinogenesis in mouse skin: recent results and present status of an advanced model system of chemical carcinogenesis. *Toxicologic Pathology* 15:245-258.
- Heddle JA, Hite M, Kirkhart B, Mavournin K, MacGregor JT, Newell GW, Salamone MF.** 1983. The induction of micronuclei as a measure of genotoxicity: a report of the United States Environmental Protection Agency Gene Tox Program. *Mutation Research* 123:61-118.
- Hedrick P.** 1992. Population genetics: shooting the RAPDs. *Nature* 355:679-680.
- Hesselman DM, Blake NJ, Peters EC.** 1988. Gonadal neoplasms in hard shell clams *Mercenaria* spp., from the indian river, Florida: occurrence, prevalence, and histopathology. *Journal of Invertebrate Pathology* 52:436-446.
- Hessen DO.** 1994. *Daphnia* responses to UV-light. *Archiv Hydrobiologie beih Ergebn Limnologie* 43:185-195.
- Hinton DE, Baumann PC, Gardner GR, Hawkins WE, Hendricks JD, Murchelano RA, Okihiro MS.** 1992. Histopathological biomarkers. In *Biomarkers: biochemical,*

physiological, and histological markers of anthropogenic stress, Huggett RJ, Kimerle RA, Mehrle PM, Bergman HL (eds), Lewis Publishers, USA.

Hinton DE, Lauren DJ. 1990. Integrative histopathological approaches to detecting effects of environmental stressors on fishes. *American Fisheries Society Symposium* 8:51-66.

Hoffmann GR. 1992. Bacterial assays for recombinagens. *Mutation Research* 284:125-146.

Hoffmann JS, Pillaire MJ, Maga G, Podust V, Hubscher U, Villani G. 1995. DNA polymerase β bypasses *in vitro* a single d(GpG)-cisplatin adduct placed on codon 13 of the *HRAS* gene. *Proceedings of The National Academy of Sciences of The United States of America* 92:5356-5360.

Holler E, Bauer R, Bernges F. 1992. Monofunctional DNA platinum(II) adducts block frequently DNA polymerases. *Nucleic Acids Research* 20:2307-2312.

Holliday R. 1987. The inheritance of epigenetic defects. *Science* 238:163-170.

Holloway GJ, Sibly RM, Povey SR. 1990. Evolution in toxin stressed environments. *Functional Ecology* 4:289-294.

Hormaza JI, Dollo L, Polito VS. 1994. Identification of a RAPD marker linked to sex determination in *Pistacia vera* using bulked segregant analysis. *Theoretical and Applied Genetics* 89:9-13.

Houghton RA, Woodwell GM. 1989. Global climatic change. *Scientific American* 260:36-44.

Houk VS. 1992. The genotoxicity of industrial wastes and effluents. *Mutation Research* 277:91-138.

Howland GP. 1975. Dark repair of ultraviolet induced pyrimidine dimers in the DNA of wild carrot protoplasts. *Nature* 254:160-161.

Hruszkewycz A, Canella KA, Peltonen K, Kotrappa L, Dipple A. 1992. DNA polymerase action on benzo[a]pyrene DNA adducts. *Carcinogenesis* 13:2347-2352.

Hruszkewycz AM, Canella KA, Dipple A. 1991. DNA polymerase mediated nucleotide incorporation adjacent to hydrocarbon deoxyadenosine and hydrocarbon deoxyguanosine adducts. *Carcinogenesis* 12:1659-1663.

Hruszkewycz AM, Dipple A. 1991. Bypass of a hydrocarbon adduct in an oligonucleotide template mediated by mispairing adjacent to the adduct. *Carcinogenesis* 12:2185-2187.

Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. 1991. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature* 350:427-428.

Huang L, Turchi JJ, Wahl AF, Bambara RA. 1993. Effects of the anticancer drug *cis*-diamminedichloroplatinum(II) on the activities of calf thymus DNA polymerase I. *Biochemistry* 32:841-848.

- Hutcheson M, Miller DC, White AQ.** 1985. Respiratory and behavioral responses of the grass shrimp *Palaemonetes pugio* to cadmium and reduced dissolved oxygen. *Marine Biology* 88:59-66.
- Hutchinson F.** 1987. A review of some topics concerning mutagenesis by ultraviolet light. *Photochemistry and Photobiology* 45:897-903.
- Hutchinson TH, Jha AN, MacKay JM, Elliott BM, Dixon DR.** 1998. Assessment of developmental effects, cytotoxicity and genotoxicity in the marine polychaete *Platynereis dumerilii* exposed to disinfected municipal sewage effluent. *Mutation Research* 399:97-108.
- ICPEMC.** 1983. International Commission for Protection against Environmental Mutagens and Carcinogens. Committee 4 final report: estimation of genetic risks and increased incidence of genetic disease due to environmental mutagens. *Mutation Research* 115:255-291.
- Ide H, Petrullo LA, Hatahet Z, Wallace SS.** 1991. Processing of DNA base damage by DNA polymerases: dihydrothymine and β -ureidoisobutyric acid as models for instructive and noninstructive lesions. *Journal of Biological Chemistry* 266:1469-1477.
- Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M.** 1993. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 363:558-561.
- Jaenisch R, Mintz B.** 1974. Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. *Proceedings of The National Academy of Sciences of The United States of America* 71:1250-1254.
- Jagoe CH.** 1996. Responses at the tissue level: quantitative methods in histopathology applied to ecotoxicology. In *Ecotoxicology. A hierarchical treatment*, Newman MC, Jagoe CH (eds), CRC Press, Boca Raton, Florida, USA.
- James A.** 1990a. The treatment of toxic wastes. In *Pollution: causes, effects, and control*, Harrison RM (ed), The Royal Society of Chemistry, Thomas Graham House, Cambridge, UK.
- James MO, Altman AH, Li CLJ, Boyle SM.** 1992. Dose dependent and time dependent formation of benzo[a]pyrene metabolite DNA adducts in the spiny lobster, *Panulirus argus*. *Marine Environmental Research* 34:299-302.
- James MO.** 1990b. Isolation of cytochrome P450 from hepatopancreas microsomes of the spiny lobster, *Panulirus argus*, and determination of catalytic activity with NADPH cytochrome P450 reductase from vertebrate liver. *Archives of Biochemistry and Biophysics* 282:8-17.
- Jeffreys AJ, Neumann R, Wilson V.** 1990. Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis. *Cell* 60:473-485.

- Jeffreys AJ, Royle NJ, Wilson V, Wong Z.** 1988. Spontaneous mutation rates to new length alleles at tandem repetitive hypervariable loci in human DNA. *Nature* 332:278-281.
- Jeffreys AJ, Tamaki K, MacLeod A, Monckton DG, Neil DL, Armour JAL.** 1994. Complex gene conversion events in germline mutation at human minisatellites. *Nature Genetics* 6:136-145.
- Jeffreys AJ.** 1987. Highly variable minisatellites and DNA fingerprints. *Biochemical Society Transactions* 15:309-317.
- Jenkins GJS, Suzen HS, Sueiro RA, Parry JM.** 1999. The restriction site mutation assay: a review of the methodology development and the current status of the technique. *Mutagenesis* 14:439-448.
- Jennerwein MM, Eastman A.** 1991. A polymerase chain reaction based method to detect cisplatin adducts in specific genes. *Nucleic Acids Research* 19:6209-6214.
- Jha AN, Cheung VV, Foulkes ME, Hill SJ, Depledge MH.** 2000. Detection of genotoxins in the marine environment: adoption and evaluation of an integrated approach using the embryo-larval stages of the marine mussel, *Mytilus edulis*. *Mutation Research* 464:213-228.
- Jha AN, Hutchinson TH, MacKay JM, Elliott BM, Dixon DR.** 1996. Development of an *in vivo* genotoxicity assay using the marine worm *Platynereis dumerilii* (Polychaeta, Nereidae). *Mutation Research* 359:141-150.
- Jha AN, Hutchinson TH, Mackay JM, Elliott BM, Dixons DR.** 1997. Evaluation of the genotoxicity of municipal sewage effluent using the marine worm *Platynereis dumerilii* (Polychaeta, Nereidae). *Mutation Research* 391:179-188.
- Jha AN, Hutchinson TH, Mackay JM, Elliott BM, Pascoe PL, Dixon DR.** 1995. The chromosomes of *Platynereis dumerilii* (Polychaeta, Nereidae). *Journal of The Marine Biological Association of The United Kingdom* 75:551-562.
- Jha AN.** 1998. Use of aquatic invertebrates in genotoxicological studies. *Mutation Research* 399:1-2.
- Jobling S, Sumpter JP.** 1993. Detergent components in sewage effluent are weakly estrogenic to fish: an *in vitro* study using rainbow trout *Oncorhynchus mykiss* hepatocytes. *Aquatic Toxicology* 27:361-372.
- Jolly C, Morimoto RI.** 1999. Stress and the cell nucleus: dynamics of gene expression and structural reorganization. *Gene Expression* 7:261-270.
- Jones NJ, Parry JM.** 1992. The detection of DNA adducts, DNA base changes and chromosome damage for the assessment of exposure to genotoxic pollutants. *Aquatic Toxicology* 22:323-344.
- Jones PA.** 1986. DNA methylation and cancer. *Cancer Research* 46:461-466.

- Kaiser RE, Cooks RG, Syka JEP, Stafford GC.** 1990. Collisionally activated dissociation of peptides using a quadrupole ion trap mass spectrometer. *Rapid Communications in Mass Spectrometry* 4:30-33.
- Kamiya H, Murata N, Murata T, Iwai S, Matsukage A, Masutani C, Hanaoka F, Ohtsuka E.** 1993. Cyclobutane thymine dimers in a *ras* proto-oncogene hot spot activate the gene by point mutation. *Nucleic Acids Research* 21:2355-2361.
- Karin M, Herschman HR.** 1981. Induction of metallothionein in hela cells by dexamethasone and zinc. *European Journal of Biochemistry* 113:267-272.
- Kelly JJ, Maddock MB.** 1985. *In vitro* induction of unscheduled DNA synthesis by genotoxic carcinogens in the hepatocytes of the oyster toadfish *Opsanus tau*. *Archives of Environmental Contamination and Toxicology* 14:555-563.
- Kemp SJ, Teale AJ.** 1994. Randomly primed PCR amplification of pooled DNA reveals polymorphism in a ruminant repetitive DNA sequence which differentiates *Bos indicus* and *B. taurus*. *Animal Genetics* 25:83-88.
- Kennish MJ.** 1992. Polynuclear aromatic hydrocarbons. In *Ecology of estuaries: anthropogenic effects*, Kennish MJ (ed), CRC Press, Boca Raton, Florida, USA.
- Khandka DK, Tuna M, Tal M, Nejdat A, GolanGoldhirsh A.** 1997. Variability in the pattern of random amplified polymorphic DNA. *Electrophoresis* 18:2852-2856.
- Khangarot BS, Ray PK, Chandra H.** 1987. Preventive effects of amino acids on the toxicity of copper to *Daphnia magna*. *Water Air and Soil Pollution* 32:379-387.
- Kidd KK, Ruano G.** 1995. Optimizing PCR. In *PCR 2 a Practical approach*, McPherson MJ, Hames BD, Taylor GR (eds.), Oxford University Press, New York, USA.
- Kille P, Kay J, Leaver M, George S.** 1992. Induction of piscine metallothionein as a primary response to heavy metal pollutants: applicability of new sensitive molecular probes. *Aquatic Toxicology* 22:279-286.
- Kinne O.** 1980. 14th European Marine Biology Symposium 'Protection of life in the sea': summary of symposium papers and conclusions. *Hegolander Meeresunters* 33:732-761.
- Kirkwood TBL.** 1989. DNA, mutations and aging. *Mutation Research* 219:1-7.
- Kitchin PA, Szotyori Z, Fromholz C, Almond N.** 1990. Avoidance of false positives. *Nature* 344:201.
- Kittredge JS.** 1980. Behavioral bioassays: the range of response thresholds. *Rapport et Proces-Verbaux des Reunions. Conseil International pour l'Exploration de la Mer* 179:152-153.
- Klaude M, Eriksson S, Nygren J, Ahnstrom G.** 1996. The comet assay: mechanisms and technical considerations. *Mutation Research* 363:89-96.
- Klein CB, Costa M.** 1997a. DNA methylation and gene expression: introduction and overview. *Mutation Research* 386:103-105.

- Klein CB, Costa M.** 1997b. DNA methylation, heterochromatin and epigenetic carcinogens. *Mutation Research* 386:163-180.
- Klein G.** 1981. The role of gene dosage and genetic transpositions in carcinogenesis. *Nature* 294:313-318.
- Knopp D, Schedl M, Achatz S, Kettrup A, Niessner R.** 1999. Immunochemical test to monitor human exposure to polycyclic aromatic hydrocarbons: urine as sample source. *Analytica Chimica Acta* 399:115-126.
- Kobayashi M, Hoshi T.** 1982. Relationship between the hemoglobin concentration of *Daphnia magna* and the ambient oxygen concentration. *Comparative Biochemistry and Physiology* 72:247-249.
- Kobayashi M, Nezu T.** 1986. Variation of hemoglobin content in *Daphnia magna*. *Physiological Zoology* 59:35-42.
- Kocal TE, Crane TL, Quinn BA, Ferguson HW, Hayes MA.** 1988. Degradation of extracellular thymidine by cultured hepatocytes from rainbow trout (*Salmo gairdneri*). *Comparative Biochemistry and Physiology* 91:557-561.
- Kohno T, Morishita K, Takano H, Shapiro DN, Yokota J.** 1994. Homozygous deletion at chromosome 2q33 in human small-cell lung carcinoma identified by arbitrarily primed PCR genomic fingerprinting. *Oncogene* 9:103-108.
- Kohwi Y, Kohwi-Shigematsu T.** 1991. Altered gene expression correlates with DNA structure. *Genes and Development* 5:2547-2554.
- Koivisto S, Ketola M, Walls M.** 1992. Comparison of five cladoceran species in short term and long term copper exposure. *Hydrobiologia* 248:125-136.
- Koivisto S, Ketola M.** 1995. Effects of copper on life history traits of *Daphnia pulex* and *Bosmina longirostris*. *Aquatic Toxicology* 32:255-269.
- Koji T.** 1996. Nonradioactive *in situ* nick translation: a useful molecular histochemical tool to detect single-stranded DNA breaks. *Acta Histochemica et Cytochemica* 29:71-79.
- Koller B, Lehmann A, McDermott JM, Gessler C.** 1993. Identification of apple cultivars using RAPD markers. *Theoretical and Applied Genetics* 85:901-904.
- Kramers PGN, Knaap AGAC, Van Derheijden CA, Taalman RDFM, Mohn GR.** 1991. Role of genotoxicity assays in the regulation of chemicals in the Netherlands: considerations and experiences. *Mutagenesis* 6:487-493.
- Krane DE, Sternberg DC, Burton GA.** 1999. Randomly amplified polymorphic DNA profile based measures of genetic diversity in crayfish correlated with environmental impacts. *Environmental Toxicology and Chemistry* 18:504-508.
- Krebs CJ.** 1978. *Ecology*. Harper and Row, New York, USA.

- Kubota Y, Shimada A, Shima A.** 1992. Detection of γ ray induced DNA damages in malformed dominant lethal embryos of the Japanese medaka (*Oryzias latipes*) using AP-PCR fingerprinting. *Mutation Research* 283:263-270.
- Kubota Y, Shimada A, Shima A.** 1995. DNA alterations detected in the progeny of paternally irradiated japanese medaka fish *Oryzias latipes*. *Proceedings of The National Academy of Sciences of The United States of America* 92:330-334.
- Kurelec B, Garg A, Krca S, Chacko M, Gupta RC.** 1989a. Natural environment surpasses polluted environment in inducing DNA damage in fish. *Carcinogenesis* 10:1337-1339.
- Kurelec B, Garg A, Krca S, Gupta RC.** 1989b. DNA adducts as biomarkers in genotoxic risk assessment in the aquatic environment. *Marine Environmental Research* 28:317-321.
- Kurelec B.** 1992. The multixenobiotic resistance mechanism in aquatic organisms. *Critical Reviews in Toxicology* 22:23-43.
- Kurelec B.** 1993. The genotoxic disease syndrome. *Marine Environmental Research* 35:341-348.
- Kwok S, Higuchi R.** 1989. Avoiding false positives with PCR. *Nature* 339:237-238.
- Kwok S, Kellogg DE, Mckinney N, Spasic D, Goda L, Levenson C, Sninsky JJ.** 1990. Effects of primer template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. *Nucleic Acids Research* 18:999-1005.
- La DK, Swenberg JA.** 1996. DNA adducts: biological markers of exposure and potential applications to risk assessment. *Mutation Research* 365:129-146.
- Lambert B, Andersson B, Bastlova T, Hou SM, Hellgren D, Kolman A.** 1994. Mutations induced in the hypoxanthine phosphoribosyl transferase gene by three urban air pollutants: acetaldehyde, benzo[a]pyrene diolepoxide, and ethylene-oxide. *Environmental Health Perspectives* 102:135-138.
- Lampert W, Trubetskova I.** 1996. Juvenile growth rate as a measure of fitness in *Daphnia*. *Functional Ecology* 10:631-635.
- Lande R.** 1994. Risk of population extinction from fixation of new deleterious mutations. *Evolution* 48:1460-1469.
- Lande R.** 1998. Risk of population extinction from fixation of deleterious and reverse mutations. *Genetica* 103:21-27.
- Landre PA, Gelfand DH, Watson RM.** 1995. The use of cosolvents to enhance amplification by the polymerase chain reaction. In *PCR Strategies*, Innis MA, Gelfand DH, Sninsky JJ (eds), Academic Press, San Diego, USA.
- Larkum AWD, Wood WF.** 1993. The effect of UV-B radiation on photosynthesis and respiration of phytoplankton, benthic macroalgae and seagrasses. *Photosynthesis Research* 36:17-23.

- Lawrence CW, Hinkle DC.** 1996. DNA polymerase ξ and the control of DNA damage induced mutagenesis in eukaryotes. *Cancer Surveys* 28:21-31.
- Leblanc GA.** 1985. Effects of copper on the competitive interactions of two species of cladocera. *Environmental Pollution* 37:13-25.
- Lee YW, Klein CB, Kargacin B, Salnikow K, Kitahara J, Dowjat K, Zhitkovich A, Christie NT, Costa M.** 1995. Carcinogenic nickel silences gene expression by chromatin condensation and DNA methylation: a new model for epigenetic carcinogens. *Molecular and Cellular Biology* 15:2547-2557.
- Leonzio C, Fossi MC.** 1994. Nondestructive biomarker strategy: perspectives and applications. In *Nondestructive biomarkers in vertebrates*, Fossi MC, Leonzio C (eds), Lewis publishers, Boca Raton, Florida, USA.
- Lepage F, Gentil A, Sarasin A.** 1999. Repair and mutagenesis survey of 8-hydroxyguanine in bacteria and human cells. *Biochimie* 81:147-153.
- Lepp NW, Salmon D.** 1999. A field study of the ecotoxicology of copper to bryophytes. *Environmental Pollution* 106:153-156.
- Leroy T, Van Hummelen P, Anard D, Castelain P, Kirschvolders M, Lauwerys R, Lison D.** 1996. Evaluation of three methods for the detection of DNA single strand breaks in human lymphocytes: alkaline elution, nick translation, and single-cell gel-electrophoresis. *Journal of Toxicology and Environmental Health* 47:409-422.
- Lewis MA, Valentine LC.** 1981. Acute and chronic toxicities of boric acid to *Daphnia magna* Straus. *Bulletin of Environmental Contamination and Toxicology* 27:309-315.
- Lewis MA.** 1995. Use of fresh water plants for phytotoxicity testing: a review. *Environmental Pollution* 87:319-336.
- Li AP, Carver JH, Choy WN, Hsie AW, Gupta RS, Loveday KS, Oneill JP, Riddle JC, Stankowski LF, Yang LL.** 1987. A guide for the performance of the Chinese hamster ovary cell hypoxanthine guanine phosphoribosyl transferase gene mutation assay. *Mutation Research* 189:135-141.
- Li K, Chen RL, Zhao BT, Liu M, Karu AE, Roberts VA, Li QX.** 1999. Monoclonal antibody based ELISAs for part-per-billion determination of polycyclic aromatic hydrocarbons: effects of haptens and formats on sensitivity and specificity. *Analytical Chemistry* 71:302-309.
- Liang P, Averboukh L, Pardee AB.** 1993. Distribution and cloning of eukaryotic messenger RNAs by means of differential display: refinements and optimization. *Nucleic Acids Research* 21:3269-3275.
- Liang P, Pardee AB.** 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967-971.
- Liehr JG, Avitts TA, Randerath E, Randerath K.** 1986. Estrogen-induced endogenous DNA adduction: possible mechanism of hormonal cancer. *Proceedings of The National Academy of Sciences of The United States of America* 83:5301-5305.

Liehr JG. 1997. Dual role of oestrogens as hormones and pro-carcinogens: tumour initiation by metabolic activation of oestrogens. *European Journal of Cancer Prevention* 6:3-10.

Liepelt A, Karbe L, Westendorf J. 1995. Induction of DNA strand breaks in rainbow trout *Oncorhynchus mykiss* under hypoxic and hyperoxic conditions. *Aquatic Toxicology* 33:177-181.

Lilius H, Hastbacka T, Isomaa B. 1995. A comparison of the toxicity of 30 reference chemicals to *Daphnia magna* and *Daphnia pulex*. *Environmental Toxicology and Chemistry* 14:2085-2088.

Lindahl T. 1993. Instability and decay of the primary structure of DNA. *Nature* 362:709-715.

Linder MC. 1991. *Biochemistry of copper*. Frieden E (ed), Plenum Press, New York, USA.

Liu XQ, Smerdon MJ. 1995. A slot blot method for detection of ultraviolet photoproducts in DNA. *Analytical Biochemistry* 229:323-328.

Liu YS, Thomas RJS, Phillips WA. 1995. Single-step direct PCR amplification from solid tissues. *Nucleic Acids Research* 23:1640.

Liu ZJ, Li P, Argue BJ, Dunham RA. 1999. Random amplified polymorphic DNA markers: usefulness for gene mapping and analysis of genetic variation of catfish. *Aquaculture* 174:59-68.

Livingstone DR. 1993. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. *Journal of Chemical Technology and Biotechnology* 57:195-211.

Livneh Z, Cohenfix O, Skaliter R, Elizur T. 1993. Replication of damaged DNA and the molecular mechanism of ultraviolet light mutagenesis. *Critical Reviews in Biochemistry and Molecular Biology* 28:465-513.

Lloyd DR, Phillips DH, Carmichael PL. 1997. Generation of putative intrastrand cross links and strand breaks in DNA by transition metal ion mediated oxygen radical attack. *Chemical Research in Toxicology* 10:393-400.

Lloyd DR, Phillips DH. 1999. Oxidative DNA damage mediated by copper(II), iron(II) and nickel(II) Fenton reactions: evidence for site specific mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links. *Mutation Research* 424:23-36.

Loechler EL. 1995. How are potent bulky carcinogens able to induce such a diverse array of mutations? *Molecular Carcinogenesis* 13:213-219.

Longerich S, Galloway AM, Harris RS, Wong C, Rosenberg SM. 1995. Adaptive mutation sequences reproduced by mismatch repair deficiency. *Proceedings of The National Academy of Sciences of The United States of America* 92:12017-12020.

- Lopez A, Xamena N, Cabre O, Creus A, Marcos R, Velazquez A.** 1999. Analysis of genomic damage in the mutagen sensitive mus-201 mutant of *Drosophila melanogaster* by arbitrarily primed PCR (AP-PCR) fingerprinting. *Mutation Research* 435:63-75.
- Lotka AJ.** 1925. *Elements of Physical Biology*. Williams and Wilkins, Baltimore, USA.
- Luckey TD.** 1975. Hormology with inorganic compounds. In *Environmental quality and safety*, Cowston F, Korte F (eds), Georg Thieme, Stuttgart, Germany.
- Lumsden P.** 1997. *Plants and UV-B responses to environmental change*. Cambridge university Press, Cambridge, UK.
- Lutz WK, Jaggi W, Schlatter C.** 1982. Covalent binding of diethylstilbestrol to DNA in rat and hamster liver and kidney. *Chemico Biological Interactions* 42:251-257.
- Lynch M, Conery J, Burger R.** 1995. Mutation accumulation and the extinction of small populations. *American Naturalist* 146:489-518.
- Lynch M, Milligan BG.** 1994. Analysis of population genetic structure with RAPD markers. *Molecular Ecology* 3:91-99.
- Lynch M.** 1985. Elements of mechanistic theory for the life history consequences of food limitation. *Archiv fur Hydrobiologie Ergebnisse der Limnologie* 21:351-362.
- Lynch M.** 1990. The similarity index and DNA fingerprinting. *Molecular Biology and Evolution* 7:478-484.
- Lyons BP, Harvey JS, Parry JM.** 1997. An initial assessment of the genotoxic impact of the sea empress oil spill by the measurement of DNA adduct levels in the intertidal teleost *Lipophrys pholis*. *Mutation Research* 390:263-268.
- Ma TH, Xu C, Liao S, McConnell H, Jeong BS, Won CD.** 1996. *In situ* monitoring with the *Tradescantia* bioassays on the genotoxicity of gaseous emissions from a closed landfill site and an incinerator. *Mutation Research* 359:39-52.
- MacLean N.** 1998. Regulation and exploitation of transgenes in fish. *Mutation Research* 399:255-266.
- MacPhee DG, Ambrose M.** 1996. Spontaneous mutations in bacteria: chance or necessity. *Genetica* 97:87-101.
- MacPherson JM, Eckstein PE, Scoles GJ, Gajadhar AA.** 1993. Variability of the random amplified polymorphic DNA assay among thermal cyclers, and effects of primer and DNA concentration. *Molecular and Cellular Probes* 7:293-299.
- Mager WH, Ferreira PM.** 1993. Stress response of yeast. *Biochemical Journal* 290:1-13.
- Majone F, Brunetti R, Gola I, Levis AG.** 1987. Persistence of micronuclei in the marine mussel, *Mytilus galloprovincialis*, after treatment with mitomycin C. *Mutation Research* 191:157-161.

- Malia SA, Basu AK.** 1995. Mutagenic specificity of reductively activated 1-nitropyrene in *Escherichia coli*. *Biochemistry* 34:96-104.
- Mallya SM, Sikpi MO.** 1999. Requirement for p53 in ionizing radiation inhibition of double strand break rejoining by human lymphoblasts. *Mutation Research* 434:119-132.
- Manchester DK, Weston A, Choi JS, Trivers GE, Fennessey PV, Quintana E, Farmer PB, Mann DL, Harris CC.** 1988. Detection of benzo[a]pyrene diol epoxide DNA adducts in human placenta. *Proceedings of The National Academy of Sciences of The United States of America* 85:9243-9247.
- Maniatis T, Fritsch EF, Sambrook J.** 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, New York, USA.
- Margoshes M, Vallee BL.** 1957. A cadmium protein from equine kidney cortex. *Journal of The American Chemistry Society* 79:4813-4814.
- Marmur J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *Journal of Molecular Biology* 3:208.
- Marselos M, Tomatis L.** 1992. Diethylstilbestrol: I. Pharmacology, toxicology, and carcinogenicity in humans. *European Journal of Cancer* 28:1182-1189.
- Martin AP.** 1995. Metabolic rate and directional nucleotide substitution in animal mitochondrial DNA. *Molecular Biology and Evolution* 12:1124-1131.
- Marx JL.** 1987. Oxygen free radicals linked to many diseases. *Science* 235:529-531.
- Matsuoka A, Matsuura K, Sakamoto H, Hayashi M, Sofuni T.** 1998. Spindle disturbances induced by benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene in a Chinese hamster cell line (V79-MZ) and the stability of the numerical chromosome aberrations that follow. *Mutation Research* 419:1-12.
- Mazurier S, Vandegiessen A, Heuvelman K, Wernars K.** 1992. Rapid analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. *Letters in Applied Microbiology* 14:260-262.
- McMahon G.** 1994. The genetics of human cancer: implications for ecotoxicology. *Environmental Health Perspectives* 102:75-80.
- McCarthy JF, Shugart LR.** 1990. Biological markers of environmental contamination. In *Biomarkers of Environmental Contamination*, McCarthy JF, Shugart LR (eds), Lewis Publishers, Boca Raton, Florida, USA.
- McEwan NR, Wheeler CT.** 1995. PCR on the gram positive organism *Frankia* without prior DNA extraction. *Trends in Genetics* 11:168.
- McGregor WG, Maher VM, McCormick JJ.** 1990. Kinds of mutations induced by ultraviolet (UV) light in the hypoxanthine phosphoribosyltransferase (HPRT) gene. *Proceedings of The American Association of Cancer Research* 31:132.

McKelvey VJ, McKerr G, Joyner D, Browne M. 1991. The DNA comet as an indicator of DNA damage. *Applications* ?, 30.

McKenney CL, Hamaker DB. 1984. Effects of fenvalerate on larval development of *Palaemonetes pugio* (Holthuis) and on larval metabolism during osmotic stress. *Aquatic Toxicology* 5:343-355.

McKenney CL, Matthews E. 1990. Alterations in the energy metabolism of an estuarine mysid (*Mysidopsis bahia*) as indicators of stress from chronic pesticide exposure. *Marine Environmental Research* 30:1-19.

McLennan AG. 1987. DNA damage, repair, and mutagenesis. In *DNA replication in plants*, Bryant JA, Dunham VE (eds), CRC Press, Boca Raton, Florida, USA.

McMahon G. 1994. The genetics of human cancer: implications for ecotoxicology. *Environmental Health Perspectives* 102:75-80.

Meador JP. 1991. The interaction of pH, dissolved organic carbon, and total copper in the determination of ionic copper and toxicity. *Aquatic Toxicology* 19:13-31.

Mellon I, Bohr VA, Smith CA, Hanawalt PC. 1986. Preferential DNA repair of an active gene in human cells. *Proceedings of The National Academy of Sciences of The United States of America* 83:8878-8882.

Mercier B, Gaucher C, Feugeas O, Mazurier C. 1990. Direct PCR from whole blood, without DNA extraction. *Nucleic Acids Research* 18:5908.

Meunier JR, Grimont PAD. 1993. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. *Research in Microbiology* 144:373-379.

Meuth M. 1990. The structure of mutation in mammalian cells. *Biochimica et Biophysica Acta* 1032:1-17.

Michel XR, Cassand PM, Narbonne JF. 1993. Activation of benzo[a]pyrene and 2-aminoanthracene to bacteria mutagens by mussel digestive gland postmitochondrial fraction. *Mutation Research* 301:113-119.

Michel XR, Cassand PM, Ribera DG, Narbonne JF. 1992. Metabolism and mutagenic activation of benzo(a)pyrene by subcellular fractions from mussel (*Mytilus galloprovincialis*) digestive gland and sea bass (*Discenthrarcus labrax*) liver. *Comparative Biochemistry and Physiology* 103:43-51.

Milliken GA, Johnson DE. 1984. *Analysis of messy data*, Van Nostrand Reinhold, New York, USA.

Minissi S, Caccese D, Passafiume F, Grella A, Ciccotti E, Rizzoni M. 1998. Mutagenicity (micronucleus test in *Vicia faba* root tips), polycyclic aromatic hydrocarbons and heavy metal content of sediments collected in Tiber river and its tributaries within the urban area of Rome. *Mutation Research* 420:77-84.

Minissi S, Lombi E. 1997. Heavy metal content and mutagenic activity, evaluated by *Vicia faba* micronucleus test, of Tiber river sediments. *Mutation Research* 393:17-21.

- Miralles VJ, Serrano R.** 1995. A genomic locus in *Saccharomyces cerevisiae* with four genes up regulated by osmotic stress. *Molecular Microbiology* 17:653-662.
- Mirsalis JC, Tyson CK, Butterworth BE.** 1982. Detection of genotoxic carcinogens in the *in vivo in vitro* hepatocyte DNA repair assay. *Environmental Mutagenesis* 4:553-562.
- Mitchell AD, Casciano DA, Meltz ML, Robinson DE, San RHC, Williams GM, Vonhalle ES.** 1983. Unscheduled DNA synthesis tests: a report of the United States Environmental Protection Agency Gene Tox Program. *Mutation Research* 123:363-410.
- Mitchell DL, Karentz D.** 1993. The induction and repair of DNA photodamage in the environment. In *Environmental UV photobiology*, Young AR (ed), Plenum Press, New York, USA.
- Mitchell DL, Nairn RS.** 1989. The biology of the (6-4) photoproduct. *Photochemistry and Photobiology* 49:805-819.
- Mitchelmore CL, Birmelin C, Chipman JK, Livingstone DR.** 1998a. Evidence for cytochrome P450 catalysis and free radical involvement in the production of DNA strand breaks by benzo[a]pyrene and nitroaromatics in mussel (*Mytilus edulis*) digestive gland cells. *Aquatic Toxicology* 41:193-212.
- Mitchelmore CL, Birmelin C, Livingstone DR, Chipman JK.** 1998b. Detection of DNA strand breaks in isolated mussel (*Mytilus edulis*) digestive gland cells using the comet assay. *Ecotoxicology and Environmental Safety* 41:51-58.
- Mitchelmore CL, Chipman JK.** 1998a. Detection of DNA strand breaks in brown trout (*Salmo trutta*) hepatocytes and blood cells using the single cell gel electrophoresis (comet) assay. *Aquatic Toxicology* 41:161-182.
- Mitchelmore CL, Chipman JK.** 1998b. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. *Mutation Research* 399:135-147.
- Modrich P, Lahue R.** 1996. Mismatch repair in replication fidelity, genetic-recombination, and cancer biology. *Annual Review of Biochemistry* 65:101-133.
- Mohn GR, De Raat WK.** 1993. Ecological significance of mutagens. *Science of the Total Environment* supplement (part 2):1771-1778.
- Monteith DK, Vanstone J.** 1995. Comparison of the microgel electrophoresis assay and other assays for genotoxicity in the detection of DNA damage. *Mutation Research* 345:97-103.
- Moore MN, Simpson MG.** 1992. Molecular and cellular pathology in environmental impact assessment. *Aquatic Toxicology* 22:313-322.
- Moore P, Strauss BS.** 1979. Sites of inhibition of *in vitro* DNA synthesis in carcinogen and UV-treated ϕ X174 DNA. *Nature* 278:664-666.

- Moore PD, Bose KK, Rabkin SD, Strauss BS.** 1981. Sites of termination of *in vitro* DNA synthesis on ultraviolet treated and *N*-acetylaminofluorene-treated ϕ X174 templates by prokaryotic and eukaryotic DNA polymerases. *Proceedings of The National Academy of Sciences of The United States of America* 78:110-114.
- Mori T, Matsunaga T, Chang CC, Trosko JE, Nikaido O.** 1990. *In situ* (6-4) photoproduct determination by laser cytometry and autoradiography. *Mutation Research* 236:99-105.
- Mullis KB.** 1991. The polymerase chain reaction in an anemic mode: how to avoid cold oligodeoxyribonuclear fusion. *PCR Methods and Applications* 1:1-4.
- Murray V, Motyka H, England PR, Wickham G, Lee HH, Denny WA, McFadyen WD.** 1992. The use of *Taq* DNA polymerase to determine the sequence specificity of DNA damage caused by *cis*-diamminedichloroplatinum(II), acridine tethered platinum(II) diammine complexes or two analogs. *Journal of Biological Chemistry* 267:18805-18809.
- Musarrat J, Arezina Wilson J, Wani AA.** 1996. Prognostic and aetiological relevance of 8-hydroxyguanosine in human breast carcinogenesis. *European Journal of Cancer* 32A:1209-1214.
- Nadig SG, Lee KL, Adams SM.** 1998. Evaluating alterations of genetic diversity in sunfish populations exposed to contaminants using RAPD assay. *Aquatic Toxicology* 43:163-178.
- Nagao M, Sugimura T.** 1978. Environmental mutagens and carcinogens. *Annual Review in Genetics* 12:117-159.
- Natarajan AT, Boei JJWA, Darroudi F, Van Diemen PCM, Dulout F, Hande MP, Ramalho AT.** 1996. Current cytogenetic methods for detecting exposure and effects of mutagens and carcinogens. *Environmental Health Perspectives* 104:445-448.
- Nath J, Tucker JD, Hando JC.** 1995. Y-chromosome aneuploidy, micronuclei, kinetochores and aging in men. *Chromosoma* 103:725-731.
- Nei M.** 1987. *Molecular evolutionary genetics*, Columbia, University Press, New York, USA.
- Nelson JR, Lawrence CW, Hinkle DC.** 1996a. Thymine-thymine dimer bypass by yeast DNA polymerase ξ . *Science* 272:1646-1649.
- Nelson SL, Parks KK, Grosovsky AJ.** 1996b. Ionizing radiation signature mutations in human cell mutants induced by low dose exposures. *Mutagenesis* 11:275-279.
- Nera EA, Iverson F, Lok E, Armstrong CL, Karpinski K, Clayson DB.** 1988. A carcinogenesis reversibility study of the effects of butylated hydroxyanisole on the forestomach and urinary bladder in male fischer 344 rats. *Toxicology* 53:251-268.
- Nevo E, Noy R, Lavie B, Beiles A, Muchtar S.** 1986. Genetic diversity and resistance to marine pollution. *Biological Journal of The Linnean Society* 29:139-144.

- Newcomb TG, Allen KJ, Tkeshelashvili L, Loeb LA. 1999. Detection of tandem CC→TT mutations induced by oxygen radicals using mutation specific PCR. *Mutation Research* 427:21-30.
- Newman MC. 1998. *Fundamentals of Ecotoxicology*, Ann Arbor press, USA.
- Newton CR, Graham A. 1997. *PCR*, second edition, Springer-Verlag, New York, USA.
- Niess AM, Baumann M, Roecker K, Horstmann T, Mayer F, Dickhuth HH. 1998. Effects of intensive endurance exercise on DNA damage in leucocytes. *Journal of Sports Medicine and Physical Fitness* 38:111-115.
- Nowell PC. 1976. The clonal evolution of tumor cell populations. *Science* 194:23-28.
- Nyce JW. 1997. Drug induced DNA hypermethylation: a potential mediator of acquired drug resistance during cancer chemotherapy. *Mutation Research* 386:153-161.
- O'Connor TR, Laval J. 1989. Physical association of the 2,6-diamino-4-hydroxy-5N-formamidopyrimidine-DNA glycosylase of *Escherichia coli* and an activity nicking DNA at apurinic apyrimidinic sites. *Proceedings of The National Academy of Sciences of The United States of America* 86:5222-5226.
- Oda Y, Nakamura S, Oki I, Kato T, Shinagawa H. 1985. Evaluation of the new system (*umu*-test) for the detection of environmental mutagens and carcinogens. *Mutation Research* 147:219-229.
- Oda Y, Yamazaki H, Watanabe M, Nohmi T, Shimada T. 1993. Highly sensitive *umu* test system for the detection of mutagenic nitroarenes in *Salmonella typhimurium* NM3009 having high O-acetyltransferase and nitroreductase activities. *Environmental and Molecular Mutagenesis* 21:357-364.
- Olive PL, Banath JP, Durand RE. 1990. Heterogeneity in radiation induced DNA damage and repair in tumor and normal cells measured using the comet assay. *Radiation Research* 122:86-94.
- Orita M, Suzuki Y, Sekiya T, Hayashi K. 1989. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 5:874-879.
- Oshimura M, Barrett JC. 1986. Chemically induced aneuploidy in mammalian cells: mechanisms and biological significance in cancer. *Environmental Mutagenesis* 8:129-159.
- Ostling O, Johanson KJ. 1984. Microelectrophoretic study of radiation induced DNA damages in individual mammalian cells. *Biochemical and Biophysical Research Communications* 123:291-298.
- Park JW, Cundy KC, Ames BN. 1989. Detection of DNA adducts by high performance liquid chromatography with electrochemical detection. *Carcinogenesis* 10:827-832.
- Parry JM, Shamsher M, Skibinski DOF. 1990. Restriction site mutation analysis, a proposed methodology for the detection and study of DNA base changes following mutagen exposure. *Mutagenesis* 5:209-212.

- Peinado MA, Malkhosyan S, Velazquez A, Perucho M.** 1992. Isolation and characterization of allelic losses and gains in colorectal tumors by arbitrarily primed polymerase chain reaction. *Proceedings of The National Academy of Sciences of The United States of America* 89:10065-10069.
- Perera FP, Hemminki K, Gryzbowska E, Motykiewicz G, Michalska J, Santella RM, Young TL, Dickey C, Brandtrauf P, Devivo I, et al.** 1992. Molecular and genetic damage in humans from environmental pollution in Poland. *Nature* 360:256-258.
- Pesch GG, Pesch CE, Malcolm AR.** 1981. *Neanthes arenaceodentata*, a cytogenetic model for marine genetic toxicology. *Aquatic Toxicology* 1:301-311.
- Pesch GG.** 1990. Sister chromatid exchange and genotoxicity measurements using polychaete worms. *Reviews in Aquatic Sciences* 2:19-25.
- Peters EC, Yevich PP, Harshbarger JC, Zaroogian GE.** 1994. Comparative histopathology of gonadal neoplasms in marine bivalve mollusks. *Diseases of Aquatic Organisms* 20:59-76.
- Peters LD, Morse HR, Waters R, Livingstone DR.** 1997. Responses of hepatic cytochrome P4501a and formation of DNA adducts in juveniles of turbot (*Scophthalmus maximus*) exposed to water borne benzo[a]pyrene. *Aquatic Toxicology* 38:67-82.
- Pfeifer GP, Drouin R, Holmquist GP.** 1993. Detection of DNA adducts at the DNA sequence level by ligation mediated PCR. *Mutation Research* 288:39-46.
- Pfeifer GP, Drouin R, Riggs AD, Holmquist GP.** 1992. Binding of transcription factors creates hot spots for UV photoproducts *in vivo*. *Molecular and Cellular Biology* 12:1798-1804.
- Phillips B, Harrison P.** 1999. Overview of the endocrine disrupters issue. In *Endocrine disrupting chemicals*, Hester RE, Harrison RM (eds), Issues in environmental Science and Technology, Royal Society of Chemistry, Cambridge, UK.
- Phillips DH.** 1983. Fifty years of benzo(a)pyrene. *Nature* 303:468-472.
- Pillaire MJ, Margot A, Villani G, Sarasin A, Defais M, Gentil A.** 1994. Mutagenesis in monkey cells of a vector containing a single d(GpG) *cis*-diaminedichloroplatinum(II) adduct placed on codon 13 of the human *H-ras* protooncogene. *Nucleic Acids Research* 22:2519-2524.
- Pirow R, Wollinger F, Paul RJ.** 1999. The sites of respiratory gas exchange in the planktonic crustacean *Daphnia magna*: an *in vivo* study employing blood haemoglobin as an internal oxygen probe. *Journal of Experimental Biology* 202:3089-3099.
- Ploskonosova II, Baranov VI, Gaziev AI.** 1999. PCR assay of DNA damage and repair at the gene level in brain and spleen of γ irradiated young and old rats. *Mutation Research* 434:109-117.
- Poulsen HE, Loft S, Vistisen K.** 1996. Extreme exercise and oxidative DNA modification. *Journal of Sports Sciences* 14:343-346.

- Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE.** 1990. Increased cell division as a cause of human cancer. *Cancer Research* 50:7415-7421.
- Pritsos CA, Sartorelli AC.** 1986. Generation of reactive oxygen radicals through bioactivation of mitomycin antibiotics. *Cancer Research* 46:3528-3532.
- Prutz WA, Butler J, Land EJ.** 1990. Interaction of copper(I) with nucleic acids. *International Journal of Radiation Biology* 58:215-234.
- Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP.** 1994. Estrogenic effects of effluents from sewage treatment works. *Chemistry and Ecology* 8:275-285.
- Quillardet P, Huisman O, Dari R, Hofnung M.** 1982. SOS chromotest, a direct assay of induction of an SOS function in *Escherichia coli* K12 to measure genotoxicity. *Proceedings of The National Academy of Sciences of The United States of America* 79:5971-5975.
- Rabbitts TH.** 1994. Chromosomal translocations in human cancer. *Nature* 372:143-149.
- Rabkin SD, Moore PD, Strauss BS.** 1983. *In vitro* bypass of UV induced lesions by *Escherichia coli* DNA polymerase I: specificity of nucleotide incorporation. *Proceedings of The National Academy of Sciences of The United States of America* 80:1541-1545.
- Randerath E, Agrawal HP, Weaver JA, Bordelon CB, Randerath K.** 1985a. ³²P-postlabeling analysis of DNA adducts persisting for up to 42 weeks in the skin, epidermis and dermis of mice treated topically with 7,12-dimethylbenz[a]anthracene. *Carcinogenesis* 6:1117-1126.
- Randerath K, Agrawal HP, Randerath E.** 1985b. 12-O-tetradecanoylphorbol-13-acetate-induced rapid loss of persistent 7,12-dimethylbenz[a] anthracene DNA adducts in mouse epidermis and dermis. *Cancer Letters* 27:35-43.
- Randerath K, Agrawal HP, Reddy MV.** 1983. Highly persistent polycyclic aromatic hydrocarbon DNA adducts in mouse skin: detection by ³²P-postlabeling analysis. *Cancer Letters* 20:109-114.
- Randerath K, Randerath E, Agrawal HP, Gupta RC, Schurdak ME, Reddy MV.** 1985c. Postlabeling methods for carcinogen DNA adduct analysis. *Environmental Health Perspectives* 62:57-65.
- Randerath K, Randerath E.** 1994. ³²P-postlabeling methods for DNA adduct detection: overview and critical evaluation. *Drug Metabolism Reviews* 26:67-85.
- Randerath K, Reddy MV, Gupta RC.** 1981. ³²P-labeling test for DNA damage. *Proceedings of The National Academy of Sciences of The United States of America* 78:6126-6129.
- Rank J, Nielsen MH.** 1993. A modified *Allium* test as a tool in the screening of the genotoxicity of complex mixtures. *Hereditas* 118:49-53.

- Rank J, Nielsen MH.** 1994. Evaluation of the *Allium* anaphase telophase test in relation to genotoxicity screening of industrial waste water. *Mutation Research* 312:17-24.
- Rank J, Nielsen MH.** 1997. *Allium cepa* anaphase telophase root tip chromosome aberration assay on *N*-methyl-*N*-nitrosourea, maleic hydrazide, sodium azide, and ethyl methanesulfonate. *Mutation Research* 390:121-127.
- Reddy M, Gowrishankar J.** 1997. A genetic strategy to demonstrate the occurrence of spontaneous mutations in nondividing cells within colonies of *Escherichia coli*. *Genetics* 147:991-1001.
- Reddy MV, Randerath K.** 1986. Nuclease P1 mediated enhancement of sensitivity of ³²P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis* 7:1543-1551.
- Rew DA.** 1994. Significance of aneuploidy. *British Journal of Surgery* 81:1416-1422.
- Richold M, Ashby J, Bootman J, Chandley A, Gatehouse DG, Henderson L.** 1990. *In vivo* cytogenetics assays. In *Basic mutagenicity tests: UKEMS recommended procedures*, Kirland DJ (ed), The Bath Press, Avon, UK.
- Riedy MF, Hamilton WJ, Aquadro CF.** 1992. Excess of non-parental bands in offspring from known primate pedigrees assayed using RAPD PCR. *Nucleic Acids Research* 20:918.
- Rieseberg LH.** 1996. Homology among RAPD fragments in interspecific comparisons. *Molecular Ecology* 5:99-105.
- Rittschof D, Clare AS, Gerhart DJ, Avelin S, Bonaventura J.** 1992. Barmnacle *in vitro* assays for biologically active substances: toxicity and settlement assays using mass cultured *Balanus amphitrite amphitrite*, Darwin. *Biofouling* 6:115-122.
- Rodriguez H, Drouin R, Holmquist GP, Oconnor TR, Boiteux S, Laval J, Doroshov JH, Akman SA.** 1995. Mapping of copper hydrogen peroxide induced DNA damage at nucleotide resolution in human genomic DNA by ligation mediated polymerase chain reaction. *Journal of Biological Chemistry* 270:17633-17640.
- Rodriguez H, Loechler EL.** 1993. Mutagenesis by the (+)-anti-diol epoxide of benzo[a]pyrene: what controls mutagenic specificity? *Biochemistry* 32:1759-1769.
- Roesijadi G.** 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology* 22:81-114.
- Roest PAM, Roberts RG, Sugino S, Van Ommen GJB, Dendunnen JT.** 1993. Protein truncation test (PTT) for rapid detection of translation-terminating mutations. *Human Molecular Genetics* 2:1719-1721.
- Rosenberg SM.** 1997. Mutation for survival. *Current Opinion in Genetics and Development* 7:829-834.
- Ross HL, Lee HM, Harvey RG, Dipple A.** 1994. Comparison of the sites of reaction of three polycyclic aromatic hydrocarbons in the *supF* gene. *Mutation Research* 306:91-101.

- Ross JA, Nesnow S.** 1999. Polycyclic aromatic hydrocarbons: correlations between DNA adducts and *ras* oncogene mutations. *Mutation Research* 424:155-166.
- Rossiter BJF, Caskey CT.** 1990. Molecular scanning methods of mutation detection. *Journal of Biological Chemistry* 265:12753-12756.
- Rothuizen J, Van Wolferen M.** 1994. Randomly amplified DNA polymorphisms in dogs are reproducible and display Mendelian transmission. *Animal Genetics* 25:13-18.
- Roy D, Liehr JG.** 1999. Estrogen, DNA damage and mutations. *Mutation Research* 424:107-115.
- Ruano G, Brash DE, Kidd KK.** 1991. PCR: the first few cycles. *Amplifications* :1-4.
- Ruano G, Fenton W, Kidd KK.** 1989. Biphasic amplification of very dilute DNA samples via 'booster' PCR. *Nucleic Acids Research* 17:5407.
- Rychlik W, Spencer WJ, Rhoads RE.** 1990. Optimization of the annealing temperature for DNA amplification *in vitro*. *Nucleic Acids Research* 18:6409-6412.
- Rydberg B.** 1975. The rate of strand separation in alkali of DNA of irradiated mammalian cells. *Radiation Research* 61:274-287.
- Rydberg B.** 1984. Repair of DNA double strand breaks in colcemid-arrested mitotic Chinese hamster cells. *International Journal of Radiation Biology* 46:299-304.
- Sadinski WJ, Levay G, Wilson MC, Hoffman JR, Bodell WJ, Anderson SL.** 1995. Relationships among DNA adducts, micronuclei, and fitness parameters in *Xenopus laevis* exposed to benzo[a]pyrene. *Aquatic Toxicology* 32:333-352.
- Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N.** 1985. Enzymatic amplification of β globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350-1354.
- Saitou N, Nei M.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4:406-425.
- Sambrook J, Fritsch EF, Maniatis T.** 1989. *Molecular cloning: a laboratory manual*, second edition, Cold Spring Harbor Laboratory Press, New York, USA.
- Samuni A, Chevion M, Czapski G.** 1981. Unusual copper induced sensitization of the biological damage due to superoxide radicals. *Journal of Biological Chemistry* 256:2632-2635.
- Sancar A, Sancar GB.** 1988. DNA repair enzymes. *Annual Review of Biochemistry* 57:29-67.
- Sancar A.** 1996. DNA excision repair. *Annual Review of Biochemistry* 65:43-81.
- Sandy MS, Chiocca SM, Cerutti PA.** 1992. Genotypic analysis of mutations in *Taq* I restriction recognition sites by restriction fragment length polymorphism polymerase chain

- reaction. *Proceedings of The National Academy of Sciences of The United States of America* 89:890-894.
- Sarkar G, Sommer SS.** 1990. Shedding light on PCR contamination. *Nature* 343:27.
- Savva D, Castellani S, Mattei N, Renzoni A, Walker CH.** 1994. The use of PCR and DNA fingerprints to detect the genotoxic effects of environmental chemicals. In *Environmental contamination*, Varnavas SP (ed), CEP Consultants, Edinburgh, UK.
- Savva D.** 1996. DNA Fingerprinting as a biomarker assay in ecotoxicology. *Toxicology and Ecotoxicology News* 3:110-114.
- Savva D.** 1998. Use of DNA fingerprinting to detect genotoxic effects. *Ecotoxicology and Environmental Safety* 41:103-106.
- Scates DK, Spigelman AD, Venitt S.** 1995. Appearance of artifacts when using ³²P-postlabeling to investigate DNA adduct formation by bile acids *in vitro*: lack of evidence for covalent binding. *Carcinogenesis* 16:1489-1491.
- Schaaper RM, Dunn RL.** 1987. Spectra of spontaneous mutations in *Escherichia coli* strains defective in mismatch correction: the nature of *in vivo* DNA replication errors. *Proceedings of The National Academy of Sciences of The United States of America* 84:6220-6224.
- Schierwater B, Ender A.** 1993. Different thermostable DNA polymerases may amplify different RAPD products. *Nucleic Acids Research* 21:4647-4648.
- Schoket B.** 1999. DNA damage in humans exposed to environmental and dietary polycyclic aromatic hydrocarbons. *Mutation Research* 424:143-153.
- Schubert I, Rieger R.** 1994. Sister chromatid exchanges in *Picea abies*: a test for genotoxicity in forest trees. *Mutation Research* 323:137-142.
- Scott D, Danford ND, Dean BJ, Kirland DJ.** 1990. Metaphase chromosome aberration assays *in vitro*. In *Basic mutagenicity tests: UKEMS recommended procedures*, Kirland DJ (ed), The Bath Press, Avon, UK.
- Scott MP, Haymes KM, Williams SM.** 1992. Parentage analysis using RAPD PCR. *Nucleic Acids Research* 20:5493.
- Seiler JP.** 1982. The ecological impact of environmental mutagens. *Mutation Research* 97:220-221.
- Seraj MJ, Umemoto A, Tanaka M, Kajikawa A, Hamada K, Monden Y.** 1996. DNA adduct formation by hormonal steroids *in vitro*. *Mutation Research* 370:49-59.
- Shimada A, Shima A.** 1998. Combination of genomic DNA fingerprinting into the medaka specific locus test system for studying environmental germ line mutagenesis. *Mutation Research* 399:149-165.

- Shimomura M, Higashi S, Mizumoto R.** 1992. ^{32}P -postlabeling analysis of DNA adducts in rats during estrogen induced hepatocarcinogenesis and effect of tamoxifen on DNA adduct level. *Japanese Journal of Cancer Research* 83:438-444.
- Shinoura Y, Kato T, Glickman BW.** 1983. A rapid and simple method for the determination of base substitution and frameshift specificity of mutagens. *Mutation Research* 111:43-49.
- Shou M, Harvey RG, Penning TM.** 1993. Reactivity of benzo[a]pyrene-7,8-dione with DNA: evidence for the formation of deoxyguanosine adducts. *Carcinogenesis* 14:475-482.
- Shugart L, McCarthy J, Jimenez B, Daniels J.** 1987. Analysis of adduct formation in the bluegill sunfish (*Lepomis macrochirus*) between benzo[a]pyrene and DNA of the liver and hemoglobin of the erythrocyte. *Aquatic Toxicology* 9:319-325.
- Shugart L.** 1986. Quantifying adductive modification of hemoglobin from mice exposed to benzo[a]pyrene. *Analytical Biochemistry* 152:365-369.
- Sibly RM, Calow P.** 1989. A life cycle theory of responses to stress. *Biological Journal of The Linnean Society* 37:101-116.
- Siegele DA, Kolter R.** 1992. Life after log. *Journal of Bacteriology* 174:345-348.
- Silva AJ, White R.** 1988. Inheritance of allelic blueprints for methylation patterns. *Cell* 54:145-152.
- Sims P, Grover PL, Swaisland A, Pal K, Hewer A.** 1974. Metabolic activation of benzo(a)pyrene proceeds by a diol epoxide. *Nature* 252:326-328.
- Singh N, Aggarwal S.** 1995. The effect of active oxygen generated by xanthine/xanthine oxidase on genes and signal transduction in mouse epidermal JB6 cells. *International Journal of Cancer* 62:107-114.
- Singh NP, Danner DB, Tice RR, McCoy MT, Collins GD, Schneider EL.** 1989. Abundant alkali sensitive sites in DNA of human and mouse sperm. *Experimental Cell Research* 184:461-470.
- Singh NP, Lai H, Khan A.** 1995. Ethanol induced single strand DNA breaks in rat brain cells. *Mutation Research* 345:191-196.
- Singh NP, McCoy MT, Tice RR, Schneider EL.** 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research* 175:184-191.
- Singh NP, Stephens RE, Schneider EL.** 1994. Modifications of alkaline microgel electrophoresis for sensitive detection of DNA damage. *International Journal of Radiation Biology* 66:23-28.
- Singh NP, Tice RR, Stephens RE, Schneider EL.** 1991. A microgel electrophoresis technique for the direct quantitation of DNA damage and repair in individual fibroblasts cultured on microscope slides. *Mutation Research* 252:289-296.

- Smit VTHBM, Cornelisse CJ, Dejong D, Dijkshoorn NJ, Peters AAW, Fleuren GJ.** 1988. Analysis of tumor heterogeneity in a patient with synchronously occurring female genital tract malignancies by DNA flow cytometry, DNA fingerprinting, and immunohistochemistry. *Cancer* 62:1146-1152.
- Smith CA, Taylor JS.** 1993. Preparation and characterization of a set of deoxyoligonucleotide 49-mers containing site-specific *cis-syn*, *trans-syn-I*, (6-4), and dewar photoproducts of thymidylyl(3' → 5')-thymidine. *Journal of Biological Chemistry* 268:11143-11151.
- Smith FDM, May RM, Pellew R, Johnson TH, Walter KS.** 1993. Estimating extinction rates. *Nature* 364:494-496.
- Soares AMVM, Baird DJ, Calow P.** 1992. Interclonal variation in the performance of *Daphnia magna* Straus in chronic bioassays. *Environmental Toxicology and Chemistry* 11:1477-1483.
- Sole M, Porte C, Albaiges J.** 1995. Seasonal variation in the mixed function oxygenase system and antioxidant enzymes of the mussel *Mytilus galloprovincialis*. *Environmental Toxicology and Chemistry* 14:157-164.
- Solomon E, Borrow J, Goddard AD.** 1991. Chromosome aberrations and cancer. *Science* 254:1153-1160.
- Sommer R, Tautz D.** 1989. Minimal homology requirements for PCR primers. *Nucleic Acids Research* 17:6749.
- Sommer SS.** 1995. Recent human germ line mutation: inferences from patients with hemophilia B. *Trends in Genetics* 11:141-147.
- Sorger PK.** 1991. Heat shock factor and the heat-shock response. *Cell* 65:363-366.
- Sparagano O, Sage T, Fossi MC, Lari L, Child P, Savva D, Depledge N, Bamber S, Walker C.** 1999. Induction of a putative monooxygenase of crabs (*Carcinus* spp.) by polycyclic aromatic hydrocarbons. *Biomarkers* 4:203-213.
- Speit G, Trenz K, Schutz P, Rothfuss A, Merk O.** 1999. The influence of temperature during alkaline treatment and electrophoresis on results obtained with the comet assay. *Toxicology Letters* 110:73-78.
- Stansbury KH, Flesher JW, Gupta RC.** 1994. Mechanism of aralkyl DNA adduct formation from benzo[a]pyrene *in vivo*. *Chemical Research in Toxicology* 7:254-259.
- Stapleton AE, Thornber CS, Walbot V.** 1997. UV-B component of sunlight causes measurable damage in field grown maize (*Zea mays*): developmental and cellular heterogeneity of damage and repair. *Plant Cell and Environment* 20:279-290.
- Stapleton AE.** 1992. Ultraviolet radiation and plants: burning questions. *Plant Cell* 4:1353-1358.
- Stebbing ARD.** 1985. *The effects of Stress and pollution on marine animals*, Praeger Scientific, New York, USA.

- Steffens GCM, Biewald R, Buse G.** 1987. Cytochrome C oxidase is a three copper, two heme A protein. *European Journal of Biochemistry* 164:295-300.
- Stegeman JJ, Brouwer M, Di Giulio RT, Forlin L, Fowler B, Sanders BM, Van Veld PA.** 1992. Molecular responses to environmental contamination: enzyme and protein synthesis as indicators of chemical exposure and effect. In *Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress*, Huggett RJ, Kimerle RA, Mehrle Jr PM, Bergman HL (eds), Lewis Publishers, Boca Raton, Florida, USA.
- Stegeman JJ, Lech JJ.** 1991. Cytochrome P450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. *Environmental Health Perspectives* 90:101-109.
- Stein JE, Collier TK, Reichert WL, Casillas E, Hom T, Varanasi U.** 1992. Bioindicators of contaminant exposure and sublethal effects: studies with benthic fish in puget sound, Washington. *Environmental Toxicology and Chemistry* 11:701-714.
- Steingrimsdottir H, Beare D, Carr AM, Cole J, Lehmann AR.** 1995. UV hypermutability of *xeroderma pigmentosum* cells demonstrated with a DNA based mutation system. *Oncogene* 10:2057-2066.
- Steingrimsdottir H, Beare D, Cole J, Leal JFM, Kostic T, LopezBarea J, Dorado G, Lehmann AR.** 1996. Development of new molecular procedures for the detection of genetic alterations in man. *Mutation Research* 353:109-121.
- Stewart HL.** 1976. Some observations on comparative vertebrate pathology: a summary discussion of the workshop. *Marine Fisheries Review* 38:46-48.
- Strauss BS.** 1985. Translesion DNA synthesis: polymerase response to altered nucleotides. *Cancer Surveys* 4:493-516.
- Stumm W, Morgan JJ.** 1981. *Aquatic chemistry. An introduction emphasizing chemical equilibria in natural waters*, Wiley and Sons, New York, USA.
- Sundelin B, Eriksson AK.** 1998. Malformations in embryos of the deposit feeding amphipod *Monoporeia affinis* in the Baltic sea. *Marine Ecology Progress Series* 171:165-180.
- Sutton HC, Winterbourn CC.** 1989. On the participation of higher oxidation states of iron and copper in Fenton reactions. *Free Radical Biology and Medicine* 6:53-60.
- Szymkowski DE, Lawrence CW, Wood RD.** 1993. Repair by human cell extracts of single (6-4) and cyclobutane thymine thymine photoproducts in DNA. *Proceedings of The National Academy of Sciences of The United States of America* 90:9823-9827.
- Tachibana A, Ohbayashi T, Takebe H, Tatsumi K.** 1990. Molecular changes in UV induced and γ ray induced mutations in human lymphoblastoid cells. *Mutation Research* 230:159-166.
- Taddei F, Vulic M, Radman M, Matic I.** 1997. Genetic variability and adaptation to stress. *Environmental Stress, Adaptation and Evolution* 83:271-290.

- Tang MS, Bohr VA, Zhang XS, Pierce J, Hanawalt PC.** 1989. Quantification of aminofluorene adduct formation and repair in defined DNA sequences in mammalian cells using the uvrABC nuclease. *Journal of Biological Chemistry* 264:14455-14462.
- Tatara CP, Mulvey M, Newman MC.** 1999. Genetic and demographic responses of mosquitofish (*Gambusia holbrooki*) populations exposed to mercury for multiple generations. *Environmental Toxicology and Chemistry* 18:2840-2845.
- Tates AD, Grummt T, Tornqvist M, Farmer PB, Van Dam FJ, Van Mossel H, Schoemaker HM, Ostermangolkar S, Uebel C, Tang YS, et al.** 1991. Biological and chemical monitoring of occupational exposure to ethylene oxide. *Mutation Research* 250:483-497.
- Taylor JS, Oday CL.** 1990. *Cis-syn* thymine dimers are not absolute blocks to replication by DNA polymerase I of *Escherichia coli* *in vitro*. *Biochemistry* 29:1624-1632.
- Taylor RM, Tobin AK, Bray CM.** 1997. DNA damage and repair in plants. In *Plants and UV-B responses to environmental change*, Lumsden P (ed), Cambridge University Press, Cambridge, UK.
- Thein SL, Jeffreys AJ, Gooi HC, Cotter F, Flint J, Oconnor NTJ, Weatherall DJ, Wainscoat JS.** 1987. Detection of somatic changes in human cancer DNA by DNA fingerprint analysis. *British Journal of Cancer* 55:353-356.
- Theodorakis CW, Bickham JW, Elbl T, Shugart LR, Chesser RK.** 1998. Genetics of radionuclide contaminated mosquitofish populations and homology between *Gambusia affinis* and *G. holbrooki*. *Environmental Toxicology and Chemistry* 17:1992-1998.
- Theodorakis CW, Blaylock BG, Shugart LR.** 1997. Genetic ecotoxicology I: DNA integrity and reproduction in mosquitofish exposed *in situ* to radionuclides. *Ecotoxicology* 6:205-218.
- Theodorakis CW, Dsurney SJ, Shugart LR.** 1994. Detection of genotoxic insult as DNA strand breaks in fish blood cells by agarose gel electrophoresis. *Environmental Toxicology and Chemistry* 13:1023-1031.
- Theodorakis CW, Elbl T, Shugart LR.** 1999. Genetic ecotoxicology IV: survival and DNA strand breakage is dependent on genotype in radionuclide exposed mosquitofish. *Aquatic Toxicology* 45:279-291.
- Therman E, Kuhn EM.** 1976. Cytological demonstration of mitotic crossing over in man. *Cytogenetics and Cell Genetics* 17:254-267.
- Thomas P.** 1990. Molecular and biochemical responses of fish to stressors and their potential use in environmental monitoring. In *Biological Indicators in Fish*, Adams SM (ed), American Fishery Society Symposium, Bethesda, Maryland, USA.
- Tice RR, Andrews PW, Singh NP.** 1990. The single cell gel assay: a sensitive technique for evaluating intercellular differences in DNA damage and repair. In *DNA damage and repair in Human tissues*, Sutherland BM, Woodhead AD (eds), Plenum Press, New York, USA.

- Timbrell JA.** 1991. *Principles of biochemical toxicology*, second edition. Burgess Science Press, Basingstoke, Hants, UK.
- Tinker NA, Fortin MG, Mather DE.** 1993. Random amplified polymorphic DNA and pedigree relationships in spring barley. *Theoretical and Applied Genetics* 85:976-984.
- Todo T, Takemori H, Ryo H, Ihara M, Matsunaga T, Nikaido O, Sato K, Nomura T.** 1993. A new photoreactivating enzyme that specifically repairs ultraviolet light induced (6-4) photoproducts. *Nature* 361:371-374.
- Todo T.** 1999. Functional diversity of the DNA photolyase blue light receptor family. *Mutation Research* 434:89-97.
- Tomatis L, Aitio A, Wilbourn J, Shuker L.** 1989. Human carcinogens so far identified. *Japanese Journal of Cancer Research* 80:795-807.
- Torkelson J, Harris RS, Lombardo MJ, Nagendran J, Thulin C, Rosenberg SM.** 1997. Genome wide hypermutation in a subpopulation of stationary phase cells underlies recombination dependent adaptive mutation. *Embo Journal* 16:3303-3311.
- Tornaletti S, Pfeifer GP.** 1994. Slow repair of pyrimidine dimers at p53 mutation hotspots in skin cancer. *Science* 263:1436-1438.
- Toyokuni S, Sagripanti JL.** 1996. Association between 8-hydroxy-2'-deoxyguanosine formation and DNA strand breaks mediated by copper and iron. *Free Radical Biology and Medicine* 20:859-864.
- Trentini M.** 1979. Chromosome numbers of nine species of Daphniidae (Crustacea, Cladocera). *Genetica* 54:221-223.
- Troll W, Wiesner R.** 1985. The role of oxygen radicals as a possible mechanism of tumor promotion. *Annual Review of Pharmacology and Toxicology* 25:509-528.
- Trucco RG, Engelhardt FR, Stacey B.** 1983. Toxicity, accumulation and clearance of aromatic hydrocarbons in *Daphnia pulex*. *Environmental Pollution* 31:191-202.
- Truhaut R.** 1977. Ecotoxicology: objectives, principles and perspectives. *Ecotoxicology and Environmental Safety* 1:151-173.
- Tucker JD, Preston RJ.** 1996. Chromosome aberrations, micronuclei, aneuploidy, sister chromatid exchanges, and cancer risk assessment. *Mutation Research* 365:147-159.
- Tylerwalters H, Hawkins AR.** 1995. The application of RAPD markers to the study of the bivalve mollusk *Lasaea rubra*. *Journal of The Marine Biological Association of The United Kingdom* 75:563-569.
- Van Beerendonk GJM, Van Gog FB, Vrieling H, Pearson PG, Nelson SD, Meerman JHN.** 1994. Blocking of *in vitro* DNA replication by deoxycytidine adducts of the mutagen and clastogen 2-bromoacrolein. *Cancer Research* 54:679-685.

- Van Beneden RJ, Gardner GR, Blake NJ, Blair DG.** 1993. Implications for the presence of transforming genes in gonadal tumors in two bivalve mollusk species. *Cancer Research* 53:2976-2979.
- Van Beneden RJ, Henderson KW, Blair DG, Papas TS, Gardner HS.** 1990. Oncogenes in hematopoietic and hepatic fish neoplasms. *Cancer Research* 50:S5671-S5674.
- Van Beneden RJ, Rhodes LD, Gardner GR.** 1999. Potential alterations in gene expression associated with carcinogen exposure in *Mya arenaria*. *Biomarkers* 4:485-491.
- Van Beneden RJ.** 1994. Molecular analysis of bivalve tumors: models for environmental genetic interactions. *Environmental Health Perspectives* 102:81-83.
- Van Hummelen P, Zoll C, Paulussen J, Kirschvolders M, Jaylet A.** 1989. The micronucleus test in *Xenopus*: a new and simple *in vivo* technique for detection of mutagens in fresh water. *Mutagenesis* 4:12-16.
- Varanasi U, Stein JE, Hom T.** 1981. Covalent binding of benzo[a]pyrene to DNA in fish liver. *Biochemical and Biophysical Research Communications* 103:780-787.
- Varga-Weisz PD, Becker PB.** 1998. Chromatin remodeling factors: machines that regulate? *Current Opinion in Cell Biology* 10:346-353.
- Varghese AJ, Wang SY.** 1968. Thymine-thymine adduct as a photoproduct of thymine. *Science* 160:186-187.
- Venier P, Canova S.** 1996. Formation of DNA adducts in the gill tissue of *Mytilus galloprovincialis* treated with benzo[a]pyrene. *Aquatic Toxicology* 34:119-133.
- Venier P, Maron S, Canova S.** 1997. Detection of micronuclei in gill cells and haemocytes of mussels exposed to benzo[a]pyrene. *Mutation Research* 390:33-44.
- Verlaan De Vries M, Bogaard ME, Van Denelst H, Van Boom JH, Van Dereg AJ, Bos JL.** 1986. A dot-blot screening procedure for mutated *ras* oncogenes using synthetic oligodeoxynucleotides. *Gene* 50:313-320.
- Vogel EW.** 1992. Tests for recombinagens in somatic cells of *Drosophila*. *Mutation Research* 284:159-175.
- Vogelstein B, Kinzler KW.** 1992. Cancer: carcinogens leave fingerprints. *Nature* 355:209-210.
- Vonarx EJ, Mitchell HL, Karthikeyan R, Chatterjee I, Kunz BA.** 1998. DNA repair in higher plants. *Mutation Research* 400:187-200.
- Wachsman JT.** 1997. DNA methylation and the association between genetic and epigenetic changes: relation to carcinogenesis. *Mutation Research* 375:1-8.
- Wagner M, Lehner A, Brandl E.** 1998. Comparison of three techniques for molecular differentiation of foodborne and clinical listeria strains: single strand conformation polymorphisms, random amplified polymorphic DNA polymorphisms and pulsed field gel electrophoresis. *Archiv fur Lebensmittelhygiene* 49:57-61.

- Walbot V, Cullis CA.** 1985. Rapid genomic change in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 36:367-396.
- Walker CH, Hopkin SP, Sibly RM, Peakall DB.** 1996. *Principles of ecotoxicology*, Taylor and Francis, UK.
- Walker CH, Livingstone DR.** 1992. *Persistent Pollutants in Marine Ecosystems*, Pergamon Press, Oxford.
- Walker CH, Newton I, Hallam SD, Ronis MJJ.** 1987. Activities and toxicological significance of hepatic microsomal enzymes of the kestrel (*Falco tinnunculus*) and sparrowhawk (*Accipiter nisus*). *Comparative Biochemistry and Physiology* 86:379-382.
- Walker CH.** 1995. Biochemical biomarkers in ecotoxicology: some recent developments. *Science of The Total Environment* 171:189-195.
- Walker CH.** 1998a. Biochemical biomarkers and potentiation of toxicity. *Biotherapy* 11:113-117.
- Walker CH.** 1998b. The use of biomarkers to measure the interactive effects of chemicals. *Ecotoxicology and Environmental Safety* 40:65-70.
- Walton DG, Acton AB, Stich HF.** 1984. DNA repair synthesis following exposure to chemical mutagens in primary liver, stomach, and intestinal cells isolated from rainbow trout. *Cancer Research* 44:1120-1121.
- Wang CI, Taylor JS.** 1991. Site specific effect of thymine dimer formation on dAn.dTn tract bending and its biological implications. *Proceedings of The National Academy of Sciences of The United States of America* 88:9072-9076.
- Wang CI, Taylor JS.** 1992. *In vitro* evidence that UV induced frameshift and substitution mutations at T-tracts are the result of misalignment mediated replication past a specific thymine dimer. *Biochemistry* 31:3671-3681.
- Wang CI, Taylor JS.** 1993. The *trans-syn-I* thymine dimer bends DNA by $\approx 22^\circ$ and unwinds DNA by $\approx 15^\circ$. *Chemical Research in Toxicology* 6:519-523.
- Wang GQ, Van Dam AP, Spanjaard L, Dankert J.** 1998. Molecular typing of *Borrelia burgdorferi* sensu lato by randomly amplified polymorphic DNA fingerprinting analysis. *Journal of Clinical Microbiology* 36:768-776.
- Wang MY, Liehr JG.** 1995. Induction by estrogens of lipid peroxidation and lipid peroxide-derived malonaldehyde-DNA adducts in male Syrian hamsters: role of lipid peroxidation-mediated carcinogenesis. *Carcinogenesis* 16:1941-1945.
- Wardlaw AC.** 1985. *Practical statistics for experimental biologists*, John Wiley and Sons, UK.
- Wei D, Maher VM, McCormick JJ.** 1995. Site-specific rates of excision-repair of benzo[a]pyrene diol epoxide adducts in the hypoxanthine phosphoribosyltransferase gene

of human fibroblasts: correlation with mutation spectra. *Proceedings of The National Academy of Sciences of The United States of America* 92:2204-2208.

Wei Y, Mizzen CA, Cook RG, Gorovsky MA, Allis CD. 1998. Phosphorylation of histone H3 at serine 10 is correlated with chromosome condensation during mitosis and meiosis in *Tetrahymena*. *Proceedings of The National Academy of Sciences of The United States of America* 95:7480-7484.

Weitzman SA, Turk PW, Milkowski DH, Kozłowski K. 1994. Free radical adducts induce alterations in DNA cytosine methylation. *Proceedings of The National Academy of Sciences of The United States of America* 91:1261-1264.

Welch WJ. 1993. How cells respond to stress. *Scientific American* 268:56

Welsh J, McClelland M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* 18:7213-7218.

Welsh J, Petersen C, McClelland M. 1991. Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. *Nucleic Acids Research* 19:303-306.

Welsh J, Pretzman C, Postic D, Saintgiron I, Baranton G, McClelland M. 1992. Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves *Borrelia burgdorferi* into three distinct phyletic groups. *International Journal of Systematic Bacteriology* 42:370-377.

Welsh J, Ralph D, McClelland M. 1995a. DNA and RNA fingerprinting using arbitrary primed PCR. In *PCR Strategies*, Innis MA, Gelfand DH, Sninsky JJ (eds), Academic Press, San Diego, USA.

Welsh J, Rampino N, McClelland M, Perucho M. 1995b. Nucleic acid fingerprinting by PCR based methods: applications to problems in aging and mutagenesis. *Mutation Research* 338:215-229.

Wester PW, Canton JH. 1986. Histopathological study of *Oryzias latipes* (medaka) after long term β hexachlorocyclohexane exposure. *Aquatic Toxicology* 9:21-45.

Westerhoff HV, Van Workum M. 1990. Control of DNA structure and gene-expression. *Biomedica Biochimica Acta* 49:839-853.

White JJ, Neuwirth H, Miller CD, Schneider EL. 1990. DNA alterations in prostatic adenocarcinoma and benign prostatic hyperplasia: detection by DNA fingerprint analyses. *Mutation Research* 237:37-43.

White PA, Rasmussen JB. 1996. SOS chromotest results in a broader context: empirical relationships between genotoxic potency, mutagenic potency, and carcinogenic potency. *Environmental and Molecular Mutagenesis* 27:270-305.

White PA, Robitaille S, Rasmussen JB. 1999. Heritable reproductive effects of benzo[a]pyrene on the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry* 18:1843-1847.

- White R, Jobling S, Hoare SA, Sumpter JP, Parker MG.** 1994. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* 135:175-182.
- WHO.** 1994. *Environmental Health Criteria 160, Ultraviolet Radiation*, World Health Organisation, Geneva.
- Widdows J, Johnson D.** 1988. Physiological energetics of *Mytilus edulis*: scope for growth. *Marine Ecology Progress Series* 46:113-121.
- Wight DC, Wagner TE.** 1994. Transgenic mice: a decade of progress in technology and research. *Mutation Research* 307:429-440.
- Wilkie SE, Isaac PG, Slater RJ.** 1993. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. *Theoretical and Applied Genetics* 86:497-504.
- Williams GM, Watanabe K.** 1978. Quantitative kinetics of development of *N*-2-fluorenylacetamide induced, altered (hyperplastic) hepatocellular foci resistant to iron accumulation and of their reversion or persistence following removal of carcinogen. *Journal of The National Cancer Institute* 61:113-121.
- Williams GM.** 1992. DNA reactive and epigenetic carcinogens. *Experimental and Toxicologic Pathology* 44:457-464.
- Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18:6531-6535.
- Williams JGK, Reiter RS, Young RM, Scolnik PA.** 1993. Genetic mapping of mutations using phenotypic pools and mapped RAPD markers. *Nucleic Acids Research* 21:2697-2702.
- Wilson AC, Carlson SS, White TJ.** 1977. Biochemical evolution. *Annual Review of Biochemistry* 46:573-639.
- Wilson EO.** 1988. *Biodiversity*, National Academy Press, Washington, USA.
- Wilson VL, Jones PA.** 1984. Chemical carcinogen-mediated decreases in DNA 5-methylcytosine content of BALB/3T3 cells. *Carcinogenesis* 5:1027-1031.
- Winner RW, Farrell MP.** 1976. Acute and chronic toxicity of copper to four species of *Daphnia*. *Journal Fisheries Research Board of Canada* 33:1685-1684.
- Witkin EM.** 1976. Ultraviolet mutagenesis and inducible DNA Repair in *Escherichia coli*. *Bacteriological Reviews* 40:869-907.
- Wolff K, Schoen ED, Petersvanrijn J.** 1993. Optimizing the generation of random amplified polymorphic DNAs in *Chrysanthemum*. *Theoretical and Applied Genetics* 86:1033-1037.
- Wolff SP, Dean RT.** 1986. Fragmentation of proteins by free radicals and its effect on their susceptibility to enzymatic hydrolysis. *Biochemical Journal* 234:399-403.

- Wolffe AP, Hayes JJ.** 1999. Chromatin disruption and modification. *Nucleic Acids Research* 27:711-720.
- Wood RD.** 1996. DNA repair in eukaryotes. *Annual Review of Biochemistry* 65:135-167.
- Woodward LA, Mulvey M, Newman MC.** 1996. Mercury contamination and population level responses in chironomids: can allozyme polymorphism indicate exposure? *Environmental Toxicology and Chemistry* 15:1309-1316.
- Wurgler FE, Kramers PGN.** 1992. Environmental effects of genotoxins (ecogenotoxicology). *Mutagenesis* 7:321-327.
- Wurgler FE.** 1992. Recombination and gene conversion. *Mutation Research* 284:3-14.
- Yamamoto K, Kawanishi S.** 1989. Hydroxyl free-radical is not the main active species in site-specific DNA damage induced by copper (II) ion and hydrogen-peroxide. *Journal of Biological Chemistry* 264:15435-15440.
- Yang LL, Maher VM, McCormick JJ.** 1980. Error free excision of the cytotoxic, mutagenic N2-deoxyguanosine DNA adduct formed in human fibroblasts by (\pm)-7 β ,8 α -dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. *Proceedings of The National Academy of Sciences of The United States of America* 77:5933-5937.
- Yauk C.** 1998. Monitoring for induced heritable mutations in natural populations: application of minisatellite DNA screening. *Mutation Research* 411:1-10.
- Yauk CL, Quinn JS.** 1996. Multilocus DNA fingerprinting reveals high rate of heritable genetic mutation in herring gulls nesting in an industrialized urban site. *Proceedings of The National Academy of Sciences of The United States of America* 93:12137-12141.
- Youil R, Kemper BW, Cotton RGH.** 1995. Screening for mutations by enzyme mismatch cleavage with T4 endonuclease 7. *Proceedings of The National Academy of Sciences of The United States of America* 92:87-91.
- Zacharias W, Jaworski A, Wells RD.** 1990. Cytosine methylation enhances Z DNA formation *in vivo*. *Journal of Bacteriology* 172:3278-3283.
- Zahn RK, Zahn G, Muller WEG, Kurelec B, Rijavec M, Batel R, Given R.** 1981. Assessing consequences of marine pollution by hydrocarbons using sponges as model organisms. *Science of The Total Environment* 20:147-169.
- Zahn RK, Zahndaimler G, Muller WEG, Michaelis ML, Kurelec B, Rijavec M, Batel R, Bihari N.** 1983. DNA damage by PAH and repair in a marine sponge. *Science of The Total Environment* 26:137-156.
- Zarbl H, Sukumar S, Arthur AV, Martinzanca D, Barbacid M.** 1985. Direct mutagenesis of Ha-ras-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. *Nature* 315:382-385.
- Zhivotovsky LA.** 1997. Environmental stress and evolution: a theoretical study. *Environmental Stress, Adaptation and Evolution* 83:241-254.

Zhou JL, Fileman TW, Evans S, Donkin P, Mantoura RFC, Rowland SJ. 1996. Seasonal distribution of dissolved pesticides and polynuclear aromatic hydrocarbons in the humber estuary and humber coastal zone. *Marine Pollution Bulletin* 32:599-608.

Zimmermann FK. 1992. Tests for recombinagens in fungi. *Mutation Research* 284:147-158.

Zingg JM, Jones PA. 1997. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis* 18:869-882.