THE DETECTION OF GENETIC TOXICITY IN MARINE ORGANISMS

by

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Abstract

Studies were undertaken to measure genetic toxicity at the molecular and cytogenetic levels of biological organisation in marine invertebrates and flatfish. The responses in the early life stages and adult life stages of marine invertebrates (*Mytilus edulis* and *Platynereis dumerilit*) were measured following exposures to the physical agent ultraviolet radiation (UVR) and representative compounds of the chemical group, polycyclic aromatic hydrocarbons (PAHs) in laboratory conditions. Also, the potential changes of toxicity of PAH contaminants caused by interactive effects with UVR were evaluated. In a collaborative study with researchers at the Centre for Environment, Fisheries and Aquaculture Science (CEFAS), genotoxic measurements were carried out in marine flounder (*Platichthys flesus*) which had been chronically exposed *in vivo* to PAHs administered *via* their food in controlled laboratory conditions. Significantly elevated levels of DNA damage were only observed in fish exposed to the highest PAH concentrations.

Dose-dependent cytogenetic responses were observed in the early life stages of both of the invertebrate species investigated following exposure to UVR. However, statistically significant increases in chromosomal aberration induction and sister chromatid exchange (SCE) induction, and decreases in proliferation rate index (PRI) and normal development were only observed at UVR levels exceeding that of equivalent environmentally realistic conditions, when related to historical and predicted ozone levels in the south west of England.

The interactive effects of UVR and PAHs indicated that the genotoxic potential of PAHs is increased when photoactivation takes place, measured by increases in genotoxic responses in both the early and adult life stages. Following a series of laboratory studies, a field study was conducted whereby indigenous populations of marine invertebrates (*Mytilus edulis* and *Cerastoderma edule*) were used as target organisms. Haemolymph samples were collected from the animals and tested for genotoxic and cytotoxic effects, employing assays validated in the earlier laboratory studies. Correlations between samples showing high levels of DNA damage measured with the comet assay and sites with high levels of heavy metals in the sediments and soft tissue of the animals were established.

Finally, an attempt was made to evaluate the DNA repair capabilities of haemocytes collected from *M. edulis*. A series of *in vitro* exposures were carried out using hydrogen peroxide (H$_2$O$_2$) an oxidising agent, and methyl methane sulfonate (MMS) an alkylating agent to induce DNA damage. These were incubated with cytosine β-D-arabinofuranoside (AraC), to establish whether these cells had the capacity to repair DNA damage induced by the model chemicals. The results showed that DNA damage induced by H$_2$O$_2$ required DNA polymerases for repair, whereas MMS-induced damage and repair did not appear to be affected by the presence of AraC.

In conclusion, the data collated from the research undertaken for this project showed that UVR and PAHs are able to induce genotoxic lesions measurable at the cytogenetic level and whole organism levels in early life stages; and at the molecular and cellular levels in the adult stages. The assays employed appeared to be useful for identifying potential “hotspots” for genotoxic agents in the environment as indicated by the results of the field study, however, the endpoints measured are not contaminant-specific and provide little information about the type of contaminant or agent which might be present. Nonetheless, it has been shown here that comet assay is applicable to different marine species (including *Cerastoderma edule* and *Platichthys flesus*) and it is a potentially useful method for detecting DNA damage as well as DNA repair.
CONTENTS

Copyright statement ........................................... i
Title page ....................................................... ii
Abstract ......................................................... iii
List of contents ................................................ iv
List of figures, table & plates ................................ xi
Acknowledgements .............................................. xvi
Author’s declaration .......................................... xvii
Abbreviations ................................................... xix

Chapter 1: Introduction

1.1 Thesis outline .............................................. 1
1.2 Aims & objectives ......................................... 4
1.3 Background ................................................ 4
1.4 Genotoxins .................................................. 6
  1.4.1 Genetic toxicology in invertebrates vs. vertebrates .... 7
  1.4.2 Ecotoxicology ........................................... 9
    1.4.2.1 Biomarkers ......................................... 10
    1.4.2.2 The use of biomarkers in the environmental monitoring 13
    1.4.2.3 Biomarkers of genotoxicity ......................... 14
1.5 Genotoxic agents .......................................... 17
  1.5.1 Ozone depletion and ultraviolet radiation (UVR) .... 18
    1.5.1.1 UVR in the aquatic environment .................... 21
    1.5.1.2 UV-B photobiology ................................ 22
    1.5.1.3 The repair of UV-induced DNA damage ............... 26
  1.5.2 Organic chemical contaminants ........................ 28
  1.5.3 Synergistic effects ................................... 32
1.6 Target species ............................................. 33
1.7 Selected endpoints ....................................... 34
  1.7.1 Molecular endpoints ................................... 34
    1.7.1.1 The ‘Comet’ assay ................................ 34
  1.7.2 Cytogenetic endpoints ................................ 37
    1.7.2.1 Chromosomal aberrations (CAbs) .................. 38
    1.7.2.2 Sister chromatid exchanges (SCEs) ................. 39
    1.7.2.3 Micronucleus (MN) induction ....................... 41
  1.7.3 Cytological endpoints ................................ 42
    1.7.3.1 Proliferation rate index (PRI) .................... 42
    1.7.3.2 Neutral red retention (NRR) assay ................ 43
  1.7.4 Individual responses .................................. 44
    1.7.4.1 Embryo-larval development assay ................. 44
1.8 Summary .................................................. 45

Chapter 2: Materials & Methods

2.1 Test organisms ............................................. 46
  2.1.1 Mytilus edulis (Mollusca: Bivalvia) .................. 46
    2.1.1.1 Collection and maintenance of Mytilus edulis .... 47
  2.1.2 Cerastoderma edule (Mollusca: Bivalvia) ............... 49
    2.1.2.1 Collection of Cerastoderma edule ................ 49
  2.1.3 Platynereis dumerili (Polychaete: Annelida) .......... 51
    2.1.3.1 Culture and maintenance of Platynereis dumerili 51
2.1.4 Platichthys flesus

2.1.4.1 Culture and maintenance of Platichthys flesus

2.2 Spawning and collection of embryo-larvae

2.2.1 Mytilus edulis

2.2.2 Platynereis dumerilii

2.3 Collection of haemolymph from adult animals

2.3.1 Mytilus edulis

2.3.2 Cerastoderma edule

2.3.3 Platichthys flesus

2.4 UV exposures

2.4.1 Sources of UVR

2.4.2 UV measurements and dosage calculations

2.4.3 Biological weighting and calculation of ozone depletion

2.4.4 Exposure of biological material

2.4.4.1 Early life stages

2.4.4.2 Adult life stages

2.5 Statistical analyses

Chapter 3: Validation studies

3.1 Introduction

3.2 Mytilus edulis: Calculation of Average Generation Time (AGT)

3.2.1 Aims & objectives

3.2.2 Materials & methods

3.2.2.1 Exposure of embryo-larvae to BrdU

3.2.2.2 Fixing of embryo-larvae and preparation of metaphases

3.2.2.3 Fluorescence plus Giemsa (FPG) differential staining of the chromosomes

3.2.2.4 Analysis of metaphase spreads

3.2.3 Results

3.3 Mytilus edulis: Validation of cytogenetic and developmental endpoints by exposure to Methyl Methane Sulfonate

3.3.1 Aims & objectives

3.3.2 Materials & Methods

3.3.2.1 Exposure of embryo-larvae to methyl methane Sulfonate

3.3.2.2 Staining of metaphase spreads

3.3.2.3 Analysis of metaphase spreads

3.3.3 Results

3.4 Mytilus edulis: Validation of the classification method for the Comet assay - comparison of this method vs. the Image analysis automated method (Kinetic Imaging)

3.4.1 Aims & objectives

3.4.2 Materials & methods

3.4.2.1 Sampling of haemolymph

3.4.2.2 In vitro exposure of haemolymph samples

3.4.2.3 Eosin Y cell viability assay

3.4.2.4 Comet assay

3.4.3 Results

3.5 Cerastoderma edule: Evaluation of the sensitivity of haemocytes for the Comet assay

3.5.1 Aims & objectives

3.5.2 Materials & methods

3.5.2.1 Sampling of haemolymph
Chapter 4: Genotoxic effects of ultraviolet radiation in the early life stages of marine invertebrates

4.1 Introduction
4.2 Aims & objectives
4.3 Materials & methods
4.3.1 UV exposure of embryo-larvae
4.3.1.1 Metaphase preparation and chromosome aberrations analysis
4.3.1.2 Staining and scoring of chromosomes for chromosomal aberration analysis
4.3.1.3 Differential staining and scoring for proliferation rate index (PRI) and sister chromatid exchanges (SCEs)
4.3.2 Statistical analyses
4.4 Observations
4.5 Results
4.5.1 Effects of UVR on the induction of chromosomal aberrations
4.5.1.1 Mytilus edulis
4.5.1.2 Platynereis dumerilii
4.5.2 Effects of UVR on the induction of sister chromatid exchanges
4.5.2.1 Mytilus edulis
4.5.2.2 Platynereis dumerilii
4.5.3 Effects of UVR on the proliferative rate index (PRI)
4.5.3.1 Mytilus edulis
4.5.3.2 Platynereis dumerilii
4.5.4 Effects of UVR on development
4.5.4.1 Mytilus edulis
4.5.4.2 Platynereis dumerilii
4.5.5 Effects of UVR on growth
4.5.5.1 Mytilus edulis
4.5.5.2 Platynereis dumerilii
4.6 Discussion
4.6.1 Chromosomal aberrations
4.6.2 Sister chromatid exchanges
4.6.3 Proliferation rate index
4.6.4 Developmental responses
4.6.5 Conclusions

Chapter 5: PAHs & UVR: An investigation of the interactive toxic effects in the early life stages of marine invertebrates

5.1 Introduction
5.2 Aims & objectives
5.3 Materials & methods
5.3.1 Collection of embryo-larvae
5.3.2 Preparation of PAH solutions
5.3.3 Exposure of embryo-larvae
5.3.3.1 Metaphase preparation and chromosome aberration (CAb) analysis
5.3.3.2 Staining and scoring of chromosomes for CAb analysis
5.3.3.3 Differential staining and scoring for proliferation rate index (PRI) and sister chromatid exchange (SCEs)
5.3.4 Development of embryo-larvae
5.3.5 Statistical analyses
5.4 Results
5.4.1 Effects of PAHs on the induction of chromosomal aberrations
5.4.1.1 Mytilus edulis exposed to PAHs (photoactivated and non-photoactivated)
5.4.1.2 Platynereis dumerilii exposed to PAHs (photoactivated and non-photoactivated)
5.4.2 Effects of PAHs on the induction of sister chromatid exchanges
5.4.2.1 Mytilus edulis exposed to PAHs (photoactivated and non-photoactivated)
5.4.2.2 Platynereis dumerilii exposed to PAH (photoactivated and non-photoactivated)
5.4.3 Effects of PAHs on the proliferative rate index (PRI)
5.4.3.1 Mytilus edulis exposed to PAH (photoactivated and non-photoactivated)
5.4.3.2 Platynereis dumerilii exposed to PAH (photoactivated and non-photoactivated)
5.4.4 Effects of PAHs on the development of embryo-larvae
5.4.4.1 Mytilus edulis exposed to PAH (photoactivated and non-photoactivated)
5.4.4.2 Platynereis dumerilii exposed to PAH (photoactivated and non-photoactivated)
5.5 Discussion
5.5.1 Chromosomal aberrations (CAbs)
5.5.2 Sister chromatid exchanges (SCEs)
5.5.3 Proliferation rate index (PRI)
5.5.4 Photoactivation of PAH contaminants
5.6 Conclusions

Chapter 6: PAHs & UVR: An investigation of the interactive toxic effects in the adult life stages of marine organisms

6.1 Introduction
6.2 Aims & objectives
6.3 Materials & methods
6.3.1 Exposure of adult Mytilus edulis to B(a)P
6.3.2 Exposure of adult Mytilus edulis to B(a)P and simultaneous UVR
6.3.3 Measurements of UV-B penetration through the shells of M. edulis
6.3.4 Chemical analyses
6.3.5 Exposure of Platichthys flesus to PAHs
6.3.6 Sampling of haemolymph
6.3.7 Micronucleus assay
6.3.7.1 Slide preparation
6.3.7.2 Staining and scoring of micronuclei (MN)
6.3.8 Eosin Y assay
6.3.9 Trypan blue assay
6.3.10 Comet assay
   6.3.10.1 Slide preparation
   6.3.10.2 Cell lysis
   6.3.10.3 Alkaline DNA unwinding and electrophoresis
   6.3.10.4 Neutralisation and staining of slides
   6.3.10.5 Scoring of comets
6.3.11 Neutral red retention (NRR) assay
   6.3.11.1 Preparation of slides
   6.3.11.2 Neutral red probe treatment
   6.3.11.3 Analysis of haemocytes
6.3.12 Statistical analyses

6.4 Results
   6.4.1 Measurements of UV-B penetration through the shells of adult *M. edulis*
   6.4.2 Chemical analysis of water samples containing benzo(a)pyrene exposed to ultraviolet radiation
   6.4.3 *Mytilus edulis* exposed to benzo(a)pyrene
      6.4.3.1 Micronucleus (MN) assay
      6.4.3.2 Comet assay
      6.4.3.3 Neutral red assay
   6.4.4 *Platichthys flesus* exposed to PAHs
      6.4.4.1 Micronucleus (MN) assay
      6.4.4.2 Comet assay
      6.4.4.3 Other endpoints
6.5 Discussion
   6.5.1 Comet assay
      6.5.1.1 Bivalves
      6.5.1.2 Fish
   6.5.2 Micronucleus assay
      6.5.2.1 Bivalves
      6.5.2.2 Fish
   6.5.3 Neutral red retention assay
   6.5.4 Whole organism responses
   6.5.5 Other sublethal effects
6.6 Conclusions

Chapter 7: The detection of genotoxic effects in marine bivalves: A field assessment

7.1 Introduction
7.2 Aims & objectives
7.3 Materials & methods
   7.3.1 Sampling sites
   7.3.2 Biological sampling
      7.3.2.1 *Mytilus edulis*
      7.3.2.2 *Cerastoderma edule*
   7.3.3 Water sampling
   7.3.4 Sediment sampling
   7.3.5 Chemical analyses for heavy metals
      7.3.5.1 Sediments
      7.3.5.2 Bivalve soft tissues
         7.3.5.2.1 Analysis of samples
         7.3.5.2.2 Data output
Chapter 8: DNA repair processes occur in the haemocytes of *Mytilus edulis*

**8.1 Introduction** ....... 258
**8.2 Aims & objectives** ....... 266
**8.3 Materials & methods** ....... 266
  **8.3.1** Sampling of haemolymph ....... 266
  **8.3.2** Exposure of haemocytes to $H_2O_2$ and MMS ....... 267
  **8.3.3** Eosin Y cell viability assay ....... 267
  **8.3.4** Comet assay ....... 268
    **8.3.4.1** Slide preparation ....... 268
    **8.3.4.2** Cell lysis ....... 268
    **8.3.4.3** Alkaline DNA unwinding and electrophoresis ....... 270
    **8.3.4.4** Neutralisation and staining of comets ....... 270
    **8.3.4.5** Scoring of comets ....... 270
  **8.4 Results** ....... 271
    **8.4.1** $H_2O_2$ with or without AraC ....... 271
    **8.4.2** MMS with or without AraC ....... 271
Chapter 9: General discussion

9.1 Genetic toxicology ........................................... 280
9.2 Genotoxic effects of ultraviolet radiation ................ 281
9.3 Genotoxic effects of PAH exposure ......................... 285
9.4 Interactions between UVR & PAHs ......................... 287
9.5 Application of biomarkers to field studies ............... 288
9.6 DNA repair mechanisms in M.edulis haemocytes ....... 290
9.7 Implications of genotoxic effects ......................... 292
9.8 Future directions ........................................... 293
9.9 Conclusions .................................................. 293

Appendix I: Formulations of reagents and solutions ........ 296
Appendix II: Extra data ........................................... 300
Appendix III: Scientific contribution .......................... 302
References ........................................................ 349
LIST OF FIGURES, TABLES & PLATES

Chapter 1: Introduction

Figure 1.1 Outline of thesis ........................................... 3
Figure 1.2 The relationship of ecotoxicology to other scientific disciplines and to environmental risk assessment and management .................. 11
Figure 1.3 The ozone layer and its influence on UVR penetration ............... 19
Figure 1.4 Photoprodusts of ultraviolet radiation ................................ 24
Figure 1.5 The repair of photodamaged DNA ................................ 27
Figure 1.6 Structures of some commonly occurring aromatic compounds ....... 29
Figure 1.7 Formation of differentially labelled sister chromatids ................. 40
Figure 1.8 The formation of micronuclei in mitotically dividing cells .......... 40

Table 1.1 Examples of biomarkers at different levels of biological organisation ........................................... 15
Table 1.2 Anthropogenic sources of PAHs ................................... 29

Plate 1.1 *Mytilus edulis* blood cells exhibiting increasing levels of DNA damage detected with the 'Comet' assay ........................................... 35

Chapter 2: Materials & Methods

Figure 2.1 The location of the sampling point at Whitsand Bay, from which stock *Mytilus edulis* were collected ........................................... 48
Figure 2.2 The life cycle of *Platynereis dumerilii* ................................ 52
Figure 2.3a Correlation between UV-A broad-band cosine sensor and MACAM double monochromator spectroradiometer ...................... 65
Figure 2.3b Correlation between UV-B broad-band cosine sensor and MACAM double monochromator spectroradiometer ...................... 65
Figure 2.4a Spectrum of irradiance emitted by Q-Panel 313, 340 and cool lamps fitted in the exposure cabinet ........................................... 66
Figure 2.4b Spectrum of irradiance emitted by sunlight ........................................... 66

Plate 2.1 Adult *Mytilus edulis* collected from Sharrow Point, Whitsand, Cornwall ........................................... 48
Plate 2.2 Adult *Cerastoderma edule* collected from field sites along the River Tamar, Plymouth ........................................... 50
Plate 2.3 Sexually mature adult *Platynereis dumerilii*: (a) Mature female; (b) mature male ........................................... 52
Plate 2.4 Cultures of sexually immature (juvenile) *Platynereis dumerilii* .... 53
Plate 2.5 Adult *Platichthys flesus* (taken from Bristow, 1987) ................ 53
Plate 2.6 (a) Unfertilised *Mytilus edulis* eggs; (b) Fertilised *Mytilus edulis* eggs with polar bodies ........................................... 57
Plate 2.7. Haemolymph extraction from the posterior adductor muscle of *Mytilus edulis* ........................................... 59
Plate 2.8. The internal view of an adult *Mytilus edulis*, showing the position of the posterior adductor muscle ........................................... 59
Plate 2.9. The internal view of an adult *Cerastoderma edule* ........................................... 60
Plate 2.10 The Macam spectroradiometer ........................................... 62
Chapter 3: Validation studies

Figure 3.1. Outline of the timings of each stage in the exposure of embryo-larval material to BrdU
Figure 3.2. The effect of temperature upon the proliferation rate index of Mytilus edulis embryo-larval cells.
Figure 3.3. The effect of temperature upon the generation time of Mytilus edulis embryo-larval cells.
Figure 3.4. The effect of MMS on the induction of chromosomal aberrations in Mytilus edulis embryo-larvae.
Figure 3.5. The effect of MMS on the induction of sister chromatid exchanges and proliferation rate index in Mytilus edulis embryo-larvae.
Figure 3.6. The effect of MMS upon the normal development of early life stages of M. edulis based upon external morphology.
Figure 3.7. The relationship between visual classification of DNA damage and automated image analysis measurements.
Figure 3.8. DNA damage effects following in vitro exposure of Cerastoderma edule haemocytes to H2O2.
Table 3.1 The results of the PRI, GT and AGT of Mytilus edulis embryo-larval cells.
Table 3.2 Range of SCE frequency in M2 metaphase cells in Mytilus edulis embryo-larval cells exposed of MMS.
Plate 3.1 Embryo-larval metaphase spreads from Mytilus edulis in M1, M2, and M3.
Plate 3.2 (a) Viable and (b) Non-viable Mytilus edulis haemocytes stained with Eosin Y solution.

Chapter 4: Genotoxic effects of ultraviolet radiation in the early life stages of marine invertebrates

Figure 4.1 Schematic representation of the inside of the Fisons UV irradiation cabinet used for exposing samples to UVR.
Figure 4.2 Flow chart representing the protocol for the exposure of marine invertebrate larvae to UVR.
Figure 4.3 Flow diagram for the amended protocol for the differential staining of sister chromatids.
Figure 4.4 The effect of UV exposure on the induction of chromosomal aberrations in Mytilus edulis embryo-larvae.
Figure 4.5 The effect of UV exposure on the induction of chromosomal aberrations in P. dumerilii embryo-larvae.
Figure 4.6. The effect of UV exposure on the induction of SCEs in M. edulis embryo-larvae.
Figure 4.7 The effect of UV exposure on the induction of SCEs in P. dumerilii embryo-larvae.
Figure 4.8 The effect of UVR on PRI in Mytilus edulis embryo-larvae.
Figure 4.9 The effect of UVR on PRI in Platynereis dumerilii embryo-larvae.
Figure 4.10 The effect of UVR exposure upon the normal development of Mytilus edulis larvae aged 48 hours.
Figure 4.11 The effect of UVR exposure upon the normal development of Platynereis dumerilii larvae aged 48 hours (metatrochophores).
Figure 4.12 Mean *Mytilus edulis* D-shell larvae length aged 48h following UV exposure . . . . . . 126
Figure 4.13 *Platynereis dumerilii* metatrochophore larvae length aged 48h following UV exposure . . . . . . 126
Table 4.1 Measurements of unweighted and Setlow weighted UV-B levels 104

Plate 4.1 A partial metaphase spread showing typical chromosomes observed from *Mytilus edulis* embryo-larvae following UVR exposure, in the presence of 1.0x10^-6 BrdU solution . . . . . . 111
Plate 4.2 *Mytilus edulis* (a) normal metaphase chromosome spread and (b) metaphase spread with chromosomal aberrations . 113
Plate 4.3. *Platynereis dumerilii* (a) normal metaphase chromosome spread and (b) metaphase spread with chromosome type break . . . 113
Plate 4.4 (a) Normal and (b) Abnormal trochophore *Mytilus edulis* larvae following UVR exposure . . . . . . 123
Plate 4.5 (a) Normal and (b) Abnormal prodissoconch ‘D-shell’ *Mytilus edulis* larvae following UVR exposure . . . . . . 123
Plate 4.6 (a) Normal and (b) Abnormal 48 hour old metatrochophore *Platynereis .dumerilii* larvae following UVR exposure . . . . . . 125

Chapter 5: PAHs & UVR: An investigation of the interactive toxic effects in the early life stages of marine invertebrates

Figure 5.1 Flow chart representing the protocol for the exposure of marine invertebrate larvae to PAHs . . . . . . 140
Figure 5.2 The effect of photoactivated and non-photoactivated PAH exposure on the induction of CAbs in *Mytilus edulis* embryo-larvae . . . . . . 149
Figure 5.3 The effect of photoactivated and non-photoactivated PAH exposure on the induction of CAbs in *Platynereis dumerilii* embryo-larvae . . . . . . 150
Figure 5.4 The effect of photoactivated and non-photoactivated PAH exposure on the induction of SCEs in *Mytilus edulis* . . . . . . 152
Figure 5.5 The effect of photoactivated and non-photoactivated PAH exposure on the induction of SCEs in *Platynereis dumerilii* . . . . . . 152
Figure 5.6 The effect of PAH exposure on PRI in *Mytilus edulis* embryo-larvae . . . . . . . . . . . . 156
Figure 5.7 The effect of PAH exposure on PRI in *Platynereis dumerilii* 156
Figure 5.8 The effect of PAH exposure on the normal development of *Mytilus edulis* larvae . . . . . . . . . . . . 157
Table 5.1 Quantities of individual PAHs (µg/ml) in the mixed stock solution used in the experiments (dissolved in dimethyl sulfoxide) . . . . 142
Plate 5.1 An abnormal prodissoconch *Mytilus edulis* larva with a ‘pinched’ hinge, observed in samples exposed to PAHs . . . . . . . . . . . . 157
Chapter 6: PAHs & UVR: An investigation of the interactive toxic effects in the adult life stages of marine organisms

Figure 6.1 Possible pathways for the activation of B(a)P ................................................................. 170
Figure 6.2 The exposure of adult marine mussels (Mytilus edulis) to B(a)P or B(a)P with simultaneous UVR ................................................................. 178
Figure 6.3a Standard curve measuring fluorescence emission peaks (430nm) for B(a)P at an excitation wavelength of 340 nm ....................................................... 196
Figure 6.3b The effect of UVR on the stability of B(a)P measured by fluorescent spectrophotometry ................................................................. 196
Figure 6.4 The effect of prolonged exposure of adult Mytilus edulis to B(a)P on the induction of micronucleated haemocytes ................................................................. 198
Figure 6.5 The effect of simultaneous UVR on the genotoxic potential of B(a)P measured with the MN ................................................................. 200
Figure 6.6 The effect of simultaneous UVR on the DNA damaging potential of B(a)P measured with the comet assay ................................................................. 201
Figure 6.7 The effect of UVR on the cytotoxic potential of B(a)P measured with the NRR assay ................................................................. 204
Figure 6.8 The effect of PAH exposure on MN induction in Platichthys flesus haemocytes ................................................................. 206
Figure 6.9 The effect of PAH exposure on the level of DNA damage in Platichthys flesus blood cells ................................................................. 208
Figure 6.10 The effect of PAH exposure on the induction of DNA adducts in the liver of Platichthys flesus ................................................................. 208
Figure 6.11 The effect of PAH exposure on the EROD activity detected in the liver of Platichthys flesus ................................................................. 208

Table 6.1 Measurements of the penetration of UV-B through the shells of adult Mytilus edulis ................................................................. 194

Plate 6.1 Three-tier flow-through tank system for Platichthys flesus ................................................................. 182
Plate 6.2 (a) Normal, and (b) and (c) micronucleated Mytilus edulis haemocytes ................................................................. 198
Plate 6.3 (a) Healthy, and (b) stressed Mytilus edulis haemocytes, the lysosomes of which are stained with the neutral red probe ................................................................. 203
Plate 6.4 Normal and micronucleated blood cells from Platichthys flesus ................................................................. 206

Chapter 7: The detection of genotoxic effects in marine bivalves: A field assessment

Figure 7.1 Location of the field sampling stations ................................................................. 224
Figure 7.2 Summary of the endpoints measured ................................................................. 230
Figure 7.3 DNA damage measured with the comet assay in haemocytes from Mytilus edulis ................................................................. 241
Figure 7.4 DNA damage measured with the comet assay in haemocytes from Cerastoderma edule ................................................................. 243
Figure 7.5 Neutral red retention time of the lysosomal compartment of Mytilus edulis haemocytes ................................................................. 245
Figure 7.6 Neutral red retention time of the lysosomal compartment of Cerastoderma edule haemocytes ................................................................. 245

Table 7.1 Metals analysed in sediments and biological material from the selected field sites ................................................................. 229
Table 7.2 Results of the hydrological measurements 234
Table 7.3 Concentrations of total heavy metals in sediments 236
Table 7.4 Concentrations of total heavy metals in the soft tissue of adult Mytilus edulis 238
Table 7.5 Concentrations of total heavy metals in the soft tissue of adult Cerastoderma edule 239
Table 7.6 Summary of mean data collated from metallothionein, micronucleus and heart rate assays in Mytilus edulis and Cerastoderma edule 248

Chapter 8: DNA repair mechanisms in the haemocytes of Mytilus edulis

Figure 8.1 Summary of the mechanisms involved during phagocytosis and the potential for oxidative damage 261
Figure 8.2 The direct reversal of damage to a DNA guanine residue by an alkyltransferase 265
Figure 8.3 DNA damage measured with the comet assay in Mytilus edulis haemocytes exposed in vitro to hydrogen peroxide +/- AraC 272
Figure 8.4 DNA damage measured with the comet assay in Mytilus edulis haemocytes exposed in vitro to methyl methane sulfonate 273

Table 8.1 Classification of DNA repair 265
Table 8.2 Summary of the concentrations of the test chemicals 269
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**Author's Declaration**

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Relevant scientific seminars and conferences were regularly attended at which work was always presented, the details of which follow.

**Publications & Reports (please refer to Appendix III for peer-reviewed manuscripts):**


National and International Conference Presentations (please refer to Appendix III):

- April 1997: 9th International symposium on Pollutant Responses in Marine Organisms (PRIMO 9), Bergen, Norway. Poster presentation entitled ‘Molluscan lysosomal responses as a diagnostic tool for the detection of a pollution gradient in Tolo Harbour, Hong Kong.’


- April 1998: 8th Annual meeting of the Society of Environmental Toxicology and Chemistry (SETAC-Europe), Bordeaux, France. Poster presentation entitled ‘Genotoxic and developmental effects of arsenic-containing samples on marine invertebrates.’


Signed

Date
Abbreviations

%  percent
<  less than
>  greater than
°C  degrees centigrade
(6-4)PP  6-4 photoproducts
µg  microgram
µl  microlitre
µm  micrometer
µM  micromolar
AGT  average generation time
ANOVA  analysis of variance
AraC  cytosine β-D-arabinofuranoside
ASTM  American Society for Testing Materials
ATP  adenosine-tri-phosphate
B(a)P  benzo (alpha) pyrene
BrdU  5-bromodeoxyuridine
CAbs  chromosomal aberrations
cc  cell cleavage
CEFAS  The Centre for Environment, Fisheries & Aquaculture Science
CFC  chlorofluorocarbon
CHO  Chinese hamster ovary
cm  centimetre
CPD  cyclobutane pyrimidine dimers
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
dsb  double strand break
EROD  7-ethoxyresorufin O-deethylase
FPG  fluorescence plus giemsa
g  gram
GT  generation time
h  hour
H₂O₂  hydrogen peroxide
J  joules
kg  kilogram
kJ  kilojoules
L  litre
LSD  least significant difference
m  metre
M  molar
ma  milliamp
MFO  mixed function oxygenase
mg  milligram
min  minute
ml  millilitre
mm  millimetre
mM  millimolar
MMS  methyl methane sulfonate
MN  micronucleus / micronuclei
MT  metallothionein
nm  nanometer
NRC  National Research Council
NRR  neutral red retention
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Oz.</td>
<td>ozone</td>
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<tr>
<td>P450</td>
<td>cytochrome P450</td>
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<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
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<tr>
<td>pb</td>
<td>polar body</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PRE</td>
<td>photoreactivation enzyme</td>
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<tr>
<td>PRI</td>
<td>proliferation rate index</td>
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<tr>
<td>RAPD</td>
<td>random amplified polymorphic DNA</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>s</td>
<td>second</td>
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<tr>
<td>SCD</td>
<td>sister chromatid differential</td>
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<tr>
<td>SCE</td>
<td>sister chromatid exchange</td>
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<tr>
<td>SCG</td>
<td>single cell gel</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>ssb</td>
<td>single strand break</td>
</tr>
<tr>
<td>SW</td>
<td>seawater</td>
</tr>
<tr>
<td>UNEP</td>
<td>United Nations Environment Programme</td>
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<tr>
<td>UV-A</td>
<td>ultraviolet-A</td>
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<td>ultraviolet-B</td>
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<td>UV-C</td>
<td>ultraviolet-C</td>
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<tr>
<td>UVR</td>
<td>ultraviolet radiation</td>
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<tr>
<td>V</td>
<td>volts</td>
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<td>v/v</td>
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<tr>
<td>W</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WMO</td>
<td>World Meteorological Organisation</td>
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<tr>
<td>w/v</td>
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Chapter 1

Introduction
1.1 Thesis Outline

An outline of this thesis is summarised in figure 1.1. The studies are based upon a number of methods for detecting genotoxic and cytologic effects, following the exposure of marine organisms to physical and chemical agents. The physical agent selected is ultraviolet radiation (UVR), with particular reference to biologically active radiation with wavelengths ranging between 280 and 315 nm. This is known as ultraviolet-B (UV-B) radiation. UV-B radiation is a known DNA-damaging agent and has been selected due to its occurrence both in the aquatic and terrestrial environments. While elevated levels of UV-B radiation has been attributed to higher incidences of skin cancer in humans, its ecogenotoxicological significance in the marine environment has not been adequately investigated. In addition, recent public interest following the discovery of ozone holes within the stratosphere and subsequent increased levels of UVR has generated a need for further understanding of the potential ecological effects.

The group of chemical agents which have been investigated are polycyclic aromatic hydrocarbons (PAHs). These are also ubiquitously found in the marine environment and are usually associated with the occurrence of oil spills and leaks. The current chapter (chapter 1) provides some background information regarding some of the other sources of PAHs and their biological effects. Furthermore, the potential for alterations in toxicity of PAH contaminants has been investigated when occurring in synchrony with UVR.

Within Chapter 2 descriptions of the general methods, including the collection and maintenance of test organisms, the protocols for the extraction of biological samples from the test organisms, the methods for UVR exposure of the biological material and the statistical methods applied are provided. The protocols for specific assays are described in further detail in the chapters within which they have been applied.

Chapter 3 presents the findings of some of the validation studies which were completed to test the sensitivity and robustness of the methods and species used. A
number of pilot studies were also carried out prior to the commencement of this PhD thesis which have not been included here, which however proved the reproducibility, sensitivity and reliability of the methods employed.

In Chapters 4 and 5 the biological impacts of UVR, PAH contaminants and photo-altered PAH contaminants are investigated, focusing on the early life stages of marine invertebrates. The use of two different target organisms has allowed inter-species comparison for the different endpoints and identification of any differences of sensitivity.

In Chapter 6 the hypothesis that PAH contaminants induce genotoxic and cytotoxic responses which can be detected using molecular, cytogenetic and cytological methods is tested. The studies included in this chapter have been undertaken in the adult stages of invertebrates and vertebrates. Furthermore, the potential for UVR to alter the toxicity of PAH contaminants is investigated. For these studies, adult invertebrates were exposed to both agents simultaneously. In addition, the level of photo-degradation of benzo(a)pyrene, a representative compound of PAHs was assessed using chemical analytical methods.

In Chapter 7 the results of a field study where an attempt was made to identify a pollution gradient are described. A suite of biomarker techniques were applied to samples collected from indigenous populations of two marine bivalve species (Mytilus edulis and Cerastoderma edule) from a series of sampling stations along the Tamar estuary, Devon, UK. The second test species Cerastoderma edule had not previously been used in genotoxicity assays and it’s suitability as an alternative test species was assessed.

In Chapter 8 the DNA repair capacity of haemocytes collected from adult M.edulis is investigated. In vitro studies were carried out using the DNA repair inhibitor cytosine β-d-Arabinofuranoside (AraC). M.edulis is the main sentinel species that was used throughout this thesis.

Finally, Chapter 9 provides a summary of findings from this research. It includes the discussion of the overall results and how these compare with previous work. Recommendations for future work are also presented.
Figure 1.1. Outline of the thesis.
1.2 Aims and Objectives

The aims and objectives of the studies undertaken within the duration of this thesis, were to ascertain whether exposure of the early life and/or adult stages of marine organisms to environmentally realistic levels of UV-B radiation and polycyclic aromatic hydrocarbon (PAH) contaminants could cause detectable genetic damage. Also, to determine whether any interactive reactions between simultaneous UV-B radiation and PAH exposure occurred, resulting in alterations in toxicity of the contaminants to early and adult life stages of marine organisms. A number of investigations were conducted to examine the applicability of various biomarkers as tools for the detection of genotoxic and cytotoxic damage and, where possible, comparisons made between the responses at different life stages and between different species. In addition, a field study was conducted in an attempt to detect a pollution gradient and the use of an alternative bivalve species was assessed for environmental monitoring.

1.3 Background

It is without doubt that anthropogenic activity has had an impact upon Earth’s natural environment and biota. The aquatic environment constitutes approximately 70% of this planet and the contamination of water, especially in the coastal zone, has been due to an increase in urbanisation and industrialisation along the shoreline. It has been estimated that approximately 70% of the human population resides within 60 km of the coastal ocean, with a significant proportion of the world’s largest cities being connected either directly or indirectly, to the marine environment (Forbes & Forbes, 1994). The production, consumption, and disposal of new chemicals from industry, agriculture and waste continues to increase, and estimates of the number of organic compounds synthesised by man total over 1.8 million, with at least 250,000 new formulations per year being created (Walker & Livingstone, 1992; Zhou et al., 1996; Walker et al., 2001).
Although a link between human population increase and environmental change has long been identified, only relatively recently have there been attempts to assess the impacts of contaminants in the aquatic environment, by examining the physical, chemical and biological consequences (Peakall, 1992). Studies investigating the environmental impacts of anthropogenic activity have been driven by concerns about environmental devastation by the green lobby and forced by legislation (Shaw & Chadwick, 1999). Semi-enclosed areas of the shoreline are increasingly showing signs of contamination, and the biological impact of human activities can be observed as changes to inshore ecosystems (Peakall, 1992). Considerable effort is therefore required to monitor the pathways, bioavailability, and fate of pollutants in the marine environment, as it has been emphasised that different pollutants of various origin, from terrestrial to aerosols, often eventually reach this environment (Depledge, 1992).

In addition to the potential problems caused by chemical contaminants, anthropogenic activity has resulted in the indirect degradation of environmental quality. An example of this has been the use of chlorine-containing chemicals which have destroyed stratospheric ozone, and consequently led to an increase in the amount of biologically active wavelengths of solar radiation reaching the Earth's surface.

When considering the impacts of pollution, investigations into the way environmental contaminants interact with genetic material have been of particular interest. Deoxyribonucleic acid (DNA) is the basis of all the biological processes and structures of life. This macromolecule has a structure that accounts for two of the key properties of life, replication and the generation of form (Griffiths et al., 1993). In humans genetic damage in the form of mutations have been linked to serious effects on health, including hereditary diseases, cancer, congenital anomalies and reduced life expectancy. Furthermore, the induction of damage to the germ line and to the mechanisms that control cellular division have potential detrimental impacts on all living organisms (UNEP, 1992). Any alterations in the information carried in the DNA due to pollutants therefore may affect the function
and survival of individual organisms, and could potentially have catastrophic consequences at higher levels of biological organisation at both intra- and interspecies levels. However, extrapolations of the relationships between effects at lower levels of biological organisation, with those of the population, community and ecosystem levels, have proven to be a challenge (Würgler & Kramers, 1992; Anderson & Wild, 1994; Jha, 1998).

1.4 Genotoxins

Genotoxic agents, or genotoxins when considered as genetic damage-causing agents in terms of ecotoxicological impacts can be defined as:

".... chemical and physical agents capable of inducing mutations and related genetic changes in living cells of living organisms." (Würgler & Kramers, 1992).

Mutations are a natural phenomenon in the evolution of plant and animal species. They result in the genetic heterogeneity that is required for the survival of species in the face of environmental change, and occur spontaneously during replication of hereditary molecules during cell division. The theory of evolution proposed by Charles Darwin is partly based upon the existence of environmentally-induced genetic variability (UNEP, 1992). While spontaneously occurring, high levels of induced mutations could have detrimental effects on individuals and populations. Furthermore, heritable changes in the DNA form have been identified in a number of studies.

Mutations caused by genotoxic agents can be categorised into three main classes depending upon the type of effects produced, and the level at which the effects are detected. Mutagenesis is the loss, addition or alteration of a small number of base pairs and is therefore observed at the gene level (point-mutations). Clastogenesis is the loss, addition or rearrangement of sections or parts of a chromosome and is therefore observed at the chromosomal level (modification of the chromosomal structure). Aneuploidism is the acquisition or loss of a whole chromosome and is therefore observed at the genome
level (modification of the number of chromosomes) (Susanne, 1984; Timbrell, 1991; UNEP, 1992).

The term carcinogen is given to genotoxins which have the potential to induce cancer, and those which have the potential to induce malformations in embryos are termed teratogens (Thain & Hickman, 1995). The mutations described above occur in somatic cells and can for example, potentially lead to cancer or alterations in germinal cells which, in turn could give rise to congenital diseases. As well as damaging DNA and interfering with cell division, mutagenic mechanisms can also affect the replication, transcription and repair of DNA. For example, mutagenic conditions include modifications of nucleotides resulting in mispairing and base pair substitutions, local misalignments in base pairing (frameshift mutations), modifications of bases leading to aborted DNA synthesis, and a number of different factors which influence DNA metabolism (such as deprivation of some bases, analogues of amino acids, alkylation of enzymes of DNA metabolism) (Susanne, 1984). Mutations which are genetically significant, are those which result in a hereditary modification of the sequences of DNA which can lead to a decrease or increase of genetic diversity (Kirsh-Volders et al., 1984).

1.4.1 Genetic toxicology in invertebrates vs. vertebrates.

Research into genetic toxicology has been widely investigated in mammalian systems, with particular focus upon the search for a greater understanding of the mechanisms and causes of carcinomas, as well as degenerative diseases, autoimmune defects, diabetes and ageing processes (Würgler & Kramers, 1992). Kurelec (1993) instigated that invertebrates rarely developed tumours and in a number of pollution studies where the presence of tumours in marine invertebrates were reported, it was suggested that the tumours may have resulted from viral aetiology (Dixon, 2000). However, it has been documented that many invertebrate species do in fact develop tumour-like lesions and the lack of information previously accumulated may simply have been due to the fact that
little research in this field had been carried out, and the incidence of tumours had not been recorded. In addition, it was previously considered to be of little importance to study induced somatic mutations since these disappear with the death of an individual, whereas germ-line mutations were thought to be of greater significance in terms of population impacts (UNEP, 1992). There have been a number of reports of neoplasms in bivalve species such as clams, mussels, oysters and arthropods (Harshbarger, 1969, 1974, 1977, 1997; Harshbarger et al., 1979; Couch & Harshbarger, 1985; Hesselman et al., 1988; Gardner et al., 1991; van Beneden et al., 1993, 1998; Peter et al., 1994; van Beneden, 1994). Furthermore, uncontrolled proliferation of haemocytes has been reported in arthropods and molluscs (Harshbarger & Dawe, 1973; Couch & Harshbarger, 1985). Reports of the genotoxic effects in aquatic vertebrates in contrast to invertebrates, have been well documented and tumours have been found in a number of fish species, and marine mammals (Malins et al., 1988; Mix, 1986; Myers et al., 1990; van Beneden et al., 1990). These responses serve as practical biological monitors of pollution.

Studies with aquatic invertebrate organisms are of importance since 70% of the Earth’s surface consists of water, and invertebrates constitute 95% of all living organisms (Barnes, 1968). They are major components of all ecosystems, are found to occupy all trophic levels and are therefore of high significance within the aquatic food webs. Any detrimental effect on the population dynamics of this taxa, would therefore have a significant impact on organisms higher up the food chain (Maciorowski & Clarke, 1980). Invertebrates usually meet the criteria for the selection of test organisms (NRC, 1985), and their populations are often numerous, so samples can be readily collected for analysis without significantly impacting upon the population dynamics. They occur over an extended geographic range, and have a wide distribution. Furthermore, they come from or represent marine habitats likely to be impacted by the test agents of interest. In addition, work with invertebrates is considered to be ethical, and legal considerations favour their use in studies. With an increasing amount of research being carried out on invertebrates,
knowledge of their biochemistry and physiology allows reasonable interpretation of biomarker responses, and understanding of the well-being of individual organisms (Depledge & Fossi, 1994; Fossi et al., 2000).

Kurelec et al. (1988) suggested that in comparison with vertebrate systems, the reduced ability of marine invertebrates to biotransform and excrete xenobiotics that can form DNA adducts (e.g. PAHs) and cause DNA strand breaks, may lead to relatively higher levels of bioaccumulation. This was also supported by James (1989) who reported that PAH metabolites are eliminated inefficiently by aquatic invertebrates leading to relatively high steady state body burdens.

1.4.2 Ecotoxicology

The term 'ecotoxicology' was introduced by Truhant in 1969, deriving from the words ecology and toxicology (Walker et al., 2001). It has been defined as being: 

"....concerned with the toxic effects of chemical and physical agents on living organisms, especially on populations and communities within defined ecosystems; it includes the transfer pathways of those agents and their interactions with the environment." (Butler, 1978).

Ecotoxicology is a relatively new science, and integrates the ecological and toxicological effects of chemical pollutants on populations, communities, and ecosystems with the fate of such pollutants in the environment (Forbes & Forbes, 1994). How ecotoxicology is related to other scientific disciplines and environmental management, is depicted in figure 1.2. Toxicology and ecotoxicology tend to deal with the study of damaged systems, whereas ecology and physiology generally study 'normal' systems. Ecotoxicological research provides the basis for strategies of risk assessment and for improved environmental decision making (Forbes & Forbes, 1994).

The effects of a pollutant or xenobiotic agent on an organism can be observed at the various biological levels of organisation, from the biochemical and subcellular, to the population and ecosystems levels. In principle, ecotoxicological tests can be carried out at any biological level. However, moving down from the ecosystem to the molecular level
tests become easier to control and reaction times decrease, thus reproducibility, reliability, robustness and repeatability all increase (Luoma & Ho, 1993).

The effects of genotoxicity are of interest to ecotoxicological studies, where attempts to link responses of organisms at the lower levels of biological organisation, to those at the community and ecological levels of organisation are made. Alterations in reproductive output and success, normal development and survival of early life stages, the development of neoplasia, and the presence of heritable mutations, are all inherently of concern, with potential adverse effects upon the population dynamics of a species and in extreme cases extinction (Smith et al., 1993; Depledge, 1998). Where mutations transpire, but do not manifest as neoplasia, teratogenic effects or genetic disease, alterations to phenotype and Darwinian fitness (including survival, reproductive output and time to maturity between breeding) parameters may occur. Such responses may also have implications upon populations and communities (Forbes & Forbes, 1994; Depledge, 1994b).

1.4.2.1 Biomarkers

Indicators of adverse biological effect at the biochemical, cellular, physiological, histological, morphological and behavioural levels of biological organisation are termed "biomarkers". They provide a measure of exposure, and at times, also of the toxic effect of a chemical or physical pollutant (or more realistically for in situ studies, a number of pollutants) on organisms (Depledge & Fossi, 1994). Biological responses detected at higher levels of biological organisation, such as the population, community and ecosystem level are termed "bioindicators" since these changes are too general to be linked specifically to causal factors (Walker et al., 2001). The ability to measure characteristic pathological responses of organisms to an agent in the environment can provide the evidence required to establish a link between exposure and effect.
Figure 1.2. The relationship of ecotoxicology to other scientific disciplines and to environmental risk assessment and management. (Adapted from Forbes & Forbes, 1994).
Furthermore, it may be possible to relate responses at the biochemical level to consequent effects at the population level (Peakall, 1992). This could potentially be of use for the prediction of the effects of environmental agents at higher biological levels, from the biomarker responses observed at the cellular level.

Trump and Arstila (1975) divided pathological changes resulting from cell injury, into two phases: a ‘reversible phase’, which is associated with sublethal cell injury, and may proceed onto the second, ‘irreversible phase’. The irreversible phase is reached when the injurious stimulus is removed, and the cell is unable to recover, even though it has been returned to a normal environment; this is the point of cell death (Moore, 1982). Biomarkers are usually associated with the reversible stage of cell injury. Such changes in cell function and pathology, caused by pollutant exposure, can be used as early warning signals of possible injury at higher levels of biological organisation.

A further classification of ecotoxicological biomarkers was proposed by Depledge (1994a). These consist of four types:

(i) Biomarkers of exposure;
(ii) Biomarkers of effect;
(iii) Biomarkers of exposure-effect;
(iv) Latent effect biomarkers.

Biomarkers of exposure indicate that an organism, population or a community have been exposed to pollutants, but do not provide information of the degree of adverse effect this exposure causes. Endpoints such as metallothionein induction following metal exposure, and DNA adducts and sister chromatid exchanges following genotoxin exposure are considered to be biomarkers of exposure and they can be repaired (Depledge, 1994a; Albertini, 1996; Walker et al., 2001).

Biomarkers of effect indicate that an organism, population or a community have been adversely affected by one or a combination of pollutants. This type of biomarker
does not necessarily give information regarding the nature of the pollutant stress, i.e. the response is non-specific to a pollutant. Examples of this type of biomarker include, altered gill Na, K-ATPase activity, or the induction of chromosomal aberrations. Furthermore, these responses tend to be irreversible (Depledge, 1994a; Albertini et al., 1996; Walker et al., 2001).

Biomarkers of exposure/effect specifically link the exposure to an effect, as well as indicate that an organism, population or community has been exposed to one or a combination of pollutants. Example of this are acetylcholinesterase inhibition occurring in organisms which have been exposed to insecticides, or the formation of cyclobutane pyrimidine dimers following ultraviolet irradiation (Depledge, 1994a; Klug & Cummings, 1991).

The fourth class, latent effect biomarkers, occur when an organism appears to have had no detrimental effect following exposure to a pollutant or pollutants. However, in other circumstances the unseen effects may limit the ability of an organism to adapt to environmental changes, therefore affecting health or survival. Scope for growth is such a possible effect (Depledge, 1994a).

1.4.2.2 The use of biomarkers in environmental monitoring

Biomarker responses represent an integrated effect of a combination of pollutants or biological agents present in the environment. Many biomarker studies have been carried out in laboratory conditions, and have often investigated the effects of single agents. It is first necessary to investigate the effects of individual agents in preliminary studies of biomarker responses, to determine the mechanisms of the biomarker response or responses observed, before attempting to identify responses in environmental exposure scenarios (Bucheli & Fent, 1995).

Some biomarkers are classed as ‘non-destructive’, where measurements of responses are carried out in tissue samples or the whole organism without causing
mortality to the target organism. This is particularly useful where the loss of individuals from a population would cause an imbalance in an already critical species. Another advantage of these types of biomarkers is that repeated temporal sampling in the same individual is tenable, and therefore long-term studies of the responses can be carried out (Depledge, 1994a).

As mentioned in section 1.3.2.1 biomarker responses can be observed at a number of different levels of biological organisation. Should the impact of a toxicant at the lower levels of biological organisation, such as the biochemical, cytological or physiological level, exceed the compensatory responses, then potentially the effects may pass to successively higher levels of biological organisation (Stebbing, 1985; Newman, 1998). Biological effects at the behavioural level, may lead to reduced growth or affect reproductive ability or output, which in the long-term may have impacts at the population or community levels where changes in species diversity, density and genotypic frequencies may occur (McCarthy & Shugart, 1990). The application of biological effects observed at the lower levels of biological organisation to predict possible effects at higher levels of organisation is a key feature underlying the rationale of the biomarker approach. Table 1.1 gives some examples biomarkers detected at different organisational levels.

1.4.2.2 Biomarkers of genotoxicity

More than 150 assay systems have been developed to detect changes in DNA, using the full spectrum of organisms, from bacteria to human cells as well in vivo systems using experimental animals (UNEP, 1992). Biomarkers of genotoxicity have been identified at various levels of biological organisation, depending upon the type of lesions which manifest. The number of methods available for the detection of genotoxic endpoints has increased in recent years along with developments in genetic techniques (Kotelevstev et al., 1994; Pfau, 1997; Livingstone et al., 2000).
Table 1.1. Examples of biomarkers observed at different levels of biological organisation.

<table>
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<tr>
<th>Level of Biological Organisation</th>
<th>Biomarker</th>
<th>Reference</th>
</tr>
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</table>
| **Molecular / Biochemical**      | - Induction of Cytochrome P450 monooxygenases following organic contaminant exposure.  
- Induction of metallothionein following heavy metal exposure.  
- Formation of DNA adducts following exposure to PAHs. | - Stegeman, 1979;               
| **Cytogenetical**                | - Induction of sister Chromatid exchanges following genotoxic exposure.  
- Induction of chromosomal aberrations or micronuclei.  
- Aneuploidy.             | - Brunetti et al., 1986;       
Dixon, 1983.  
- Scott et al., 1990.  
- Tucker & Preston, 1996. |
| **Cellular / Histopathological** | - Increased permeability of lysosomal membranes detected with the neutral red assay. | - Moore, 1982, 1985, 1988, 1993;  
| **Physiological**                | - Changes in osmoregulation.  
- Bamber & Depledge, 1997a, 1997b. |
| **Behavioural**                  | - Changes in avoidance, motility, feeding, predation behaviours. | - Rand, 1984;               
In studies of human cancer, the tools of molecular epidemiology can be divided into a number of categories. These include biomarkers of exposure or dose; biomarkers of susceptibility; and biomarkers of effect (Albertini et al., 1996). These biomarkers may be detected at different levels of biological organisation, for example, biomarkers of genotoxicity at the molecular level include the formation of DNA adducts. These are formed when chemical compounds (such as benzo(a)pyrene) covalently bind to the DNA biomolecule. If these adducts are not repaired, they can induce gene mutation and have been associated with the initiation of carcinogenesis. The formation of adducts at particular loci in DNA is known to lead to the activation of oncogenes (Zarbl et al., 1985) and has also been associated with the inactivation of anti-oncogenes (Bressac et al., 1991; Hsu et al., 1991). As well as being recognised as potential indicators of carcinogenic processes, adducts can also lead to the disruption of physiological functions such as defence mechanisms and hematopoiesis (Rose et al., 2001). DNA adduct formation can be detected with $^{32}$P post labelling techniques (Kurelec, 1990; Dunn, 1991; Venier & Canova, 1996; Harvey et al., 1997; Lyons et al., 1997; Malmström et al., 2000). Limitations of this method include the potential inclusion of artefacts (Scates et al., 1995) and the relatively large quantities of DNA required for analysis. In comparison, other methods for detecting genotoxicity such as polymerase chain reaction, require relatively less DNA material.

Strand breaks within the DNA macromolecule have been detected using the alkaline/neutral elution technique, first proposed by Rydberg (1975). This was used to detect DNA breakage in irradiated mammalian cells. The single cell gel electrophoresis (or Comet assay) was then developed by Ostling & Johanson (1984) for the direct visualisation of DNA in individual cells. Since then, a number of modifications have been made to the method, allowing the detection of DNA strand breaks in a wide range of cell types from different species, due to exposure to a number of agents which cause strand breakage either directly or during the process of DNA excision repair (Singh et al., 1989;
Cytogenetic anomalies are known to be of many types and to be induced by a variety of agents by several different mechanisms (Tucker & Preston, 1996). The detection of chromosome changes using the light microscope is the basis of cytogenetic tests. Such changes include structural chromosomal aberrations (CAbs), sister chromatid exchanges (SCEs) and numerical changes (Scott et al., 1990). Some of these methods have been used to detect genotoxicity during the present studies, and have been described in more detail in section 1.4.

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 the replication of non-repaired modified bases such as 8-hydroxyguanine, thymine dimers, or bulky adducts. Previous authors have found that the DNA damage either blocked the replication process (Brown & Romano, 1991; Broschard et al., 1995) or that the enzymes involved in the replication (i.e. DNA polymerases) bypassed the DNA lesion and incorporated the wrong base opposite the lesion (Taylor & Oday, 1990; Chary & Lloyd, 1995; Nelson et al., 1996).

1.5 Genotoxic agents

The marine environment receives a wide range of natural and anthropogenic contaminants, many of which have the capacity to damage genetic material. Bioassays have been used to assess the genotoxic potential of a wide range of industrial effluents and wastes. Industries such as petroleum refineries, organic compound industries, resin manufacturers, dye manufacturers, metal refining operations, and pulp and paper mills have been identified to produce genotoxic effluents (McGeorge et al., 1985; Houk, 1992; White et al., 1996; Jha et al., 1997). In some cases the treatment and remediation of
wastes, such as pulp and paper mill effluents have been shown to reduce or negate the genotoxic potential. In other cases, the opposite has been reported, whereby the genotoxic potential of the wastes are enhanced following treatment, such as the fungal treatment of oils (Claxton et al., 1998).

Physical as well as chemical agents are known to cause genotoxicity (Pruski & Dixon, 2002). Ionising and non-ionising radiations are well-known for their DNA-damaging potential in mammalian systems, and more recently have been investigated in aquatic species. In the presented studies, non-ionising radiation namely ultraviolet radiation (UVR) and organic compounds, polycyclic aromatic hydrocarbons (PAHs), have been selected for study. Their effects upon the target organisms at the genetic, cellular, and whole organism level have been investigated.

1.5.1 Ozone depletion and ultraviolet radiation (UV-R)

The Earth’s atmosphere is divided into layers that are defined approximately by the distance above the Earth’s surface (figure 1.3). Ozone is present in both the stratosphere and the troposphere, with the stratospheric ozone being generated by the action of ultraviolet light on molecules of oxygen, and tropospheric ozone by the action of ultraviolet light on molecules of nitrogen dioxide:

Atmospheric ozone = \( \text{O}_2 \rightarrow \text{O} + \text{O} \)
\( \text{O} + \text{O}_2 \rightarrow \text{O}_3 \)

Tropospheric ozone = \( \text{NO}_2 \rightarrow \text{NO} + \text{O} \)
\( \text{O} + \text{O}_2 \rightarrow \text{O}_3 \)

(Jones & Wigley, 1989).

Since the early 1970’s the possibility of global decreases in stratospheric ozone, due to anthropogenic activities have been of great interest (Watson, 1988; Tevini, 1993; Young et al., 1993; Madronich, 1994). Of particular concern has been the release of man-made organic halogen compounds, the chlorofluorocarbons (CFCs). These compounds were widely used as propellants for aerosols, in refrigeration units, as cleaning agents, and in

- 18 -
Figure 1.3. The earth's atmosphere is comprised of a series of layers: The troposphere is the layer nearest the earth; the stratosphere ranges from 13-35 miles in altitude; and the mesosphere above this. The ozone layer is found in the stratosphere. Ozone does not affect UV-A penetration at all; UV-C is totally absorbed by the ozone layer; and UV-B is filtered by the ozone layer.
some industrial foams (Madronich, 1994). As well as CFCs, other compounds such as methyl bromide, a pesticide used on crops, also have ozone depleting properties (http://www.essential.org/ orgs/Ozone_Action, 1999). When these chemicals diffuse into the atmosphere, they can be photo-dissociated by sunlight to release chlorine atoms (Cl).

These chlorine atoms then attack ozone as follows:

\[
\begin{align*}
\text{Cl} + \text{O}_3 &= \text{ClO} + \text{O}_2 \\
\text{ClO} + \text{O} &= \text{Cl} + \text{O}_2 \\
\text{Result: } \text{O}_2 + \text{O}_2 &= 2\text{O}_2
\end{align*}
\]

In the first reaction, the chlorine atoms react catalytically with ozone in a process that generates chlorine monoxide (ClO) and oxygen (O\(_2\)). In the second reaction, the Cl is regenerated and is free to commence the cycle again, therefore each chlorine atom can result in the destruction of tens of thousands of ozone molecules (Roan, 1989; http://www.inform, 1999).

A proportion of the electromagnetic spectrum emitted by the sun is ultraviolet radiation (UV-R). UV-R can be divided into three categories according to the wavelength of emission; UV-A (315-400nm), UV-B (280-315nm) and UV-C (200-280nm). Whilst both UV-A and UV-B can penetrate to the Earth’s surface, UV-C is effectively absorbed by the atmosphere and never penetrates the Earth’s atmosphere, and is therefore not biologically significant (Karentz et al., 1994; Madronich, 1993) (figure 1.3). Of the UV-R that reaches the Earth’s surface, approximately 5% is UV-B radiation (Fraikin et al., 2000). The ozone hole over Antarctica has now become a predictable event during the spring, and at times has been reduced by as much as 90% (Farman et al., 1985; Soloman & Schoeberl, 1988; Pyle, 1997). In addition to the reduction of ozone in the Antarctic area, ozone models have shown that temperate regions may also be affected (Kerr & McElroy, 1993; Pyle, 1997; Madronich et al., 1998). Measurements which have routinely been carried out have supported these predictions, with northern mid-latitudes suffering reductions, and models indicate that significant thinning of the ozone layer has occurred.
This reduction of the Earth’s ozone layer is of concern since it is this trace gas that shields the earth from biologically damaging solar ultraviolet radiation in the 280-320nm wavelength range (UV-B) (see figure 1.4). Although it is estimated that the total energy contained in the UV-B part of the solar spectrum is less than 1%, the photons which comprise UV-B are the most energetic which results in its high potential to initiate biological and chemical reactions (Department of the Environment, 1996). In contrast, the level of UV-A, which is involved with photoreactivation processes as well as being damaging to the biosphere, remains constant, since it is able to pass through the ozone layer regardless of its thickness. It is estimated that a 1% decrease in total ozone content can result in a 2-3% increase in the UV-B radiation doses weighted by the DNA action spectrum (UNEP, 1989).

The majority of the anthropogenic ozone-depleting compounds are now strictly controlled under the Montreal Protocol (1987) and its amendments, and at the time it was thought that the ozone should be able to fully recover by the year 2050 (WMO, 1994; Henderson, 2000). However, a more recent report, has indicated that the ozone layer is not repairing as quickly as was originally predicted. It has been found that this has been due to the continued use of chemicals previously considered to be not significantly detrimental to the ozone layer, and therefore not banned by the Montreal Protocol or its amendments (Pearce, 2001).

1.5.1.1 UV-R in the aquatic environment.

There is a great concern that aquatic ecosystems may be at risk from the depletion of ozone concentrations, since UV-B radiation can penetrate to ecologically significant depths in water (Smith & Baker, 1989; Karentz & Lutze, 1990; Soloman, 1990). In aquatic environments the amount of radiation that reaches any given depth is dependent upon the total amount reaching the surface; this is largely a function of latitude, season, time of day and cloudiness. Other factors that influence the levels of radiation at specific depths
include the degree of water-surface roughness (which determines the amount that is reflected back into space), and the amount of scattering and absorption within the water column (Hardy & Gucinski, 1989). Within the water column, water itself contributes little to the absorption of UV-B. However, it has been noted that high concentrations of suspended particulate matter in the water can lead to an almost complete absorption of UV radiation (Kirk, 1994; Nagl & Hofer, 1997). Decreases in the ozone have been noted in both the northern and southern hemispheres, with the greatest changes in ozone having been observed in the austral spring in Antarctica, where depletions have reached 50% (Hardy & Gucinski, 1989).

With regard to biomass production, aquatic ecosystems balance terrestrial ecosystems, fixing large quantities of atmospheric carbon into organic material. It is, therefore, important to discern what effect increased ultraviolet (UV) radiation will have on aquatic productivity, as well as the potential impact such changes may have on ecosystems and global climate (Häder et al., 1995; Williamson et al., 1994).

The effects of increases of UV-B radiation due to ozone depletion in aquatic animals have been studied at the various levels of biological organisation from the biochemical level up to the cellular, individual, population and community levels. Laboratory and field studies have shown that microalgae as well as larval stages of fishes and invertebrates are extremely sensitive to UV-B radiation (Smith & Baker, 1989). Phyto- and zooplankton play a vital role in the food chain and are the sole food source for a number of pelagic fishes and invertebrate predators, hence any negative impact of UV-B radiation on these organisms as well as the organisms of higher trophic levels is of great significance (Karanas et al., 1979).

1.5.1.2 UV-B photobiology.

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 (Environmental Health Criteria, 1994). Studies in mammalian systems have shown that UVR has the potential to induce photochemical changes in the superficial tissues of animals which in turn can lead to a number of acute or chronic adverse health effects (Cridland & Saunders, 1994). UVR readily activates biologically important molecules such as amino acids and the nucleic acids, RNA and DNA, therefore mutagenic damage such as changes in chromosomal structure, mutations and morphological transformations and cell death have been observed following exposure of prokaryotic and eukaryotic cells (Cridland & Saunders, 1994; Environmental Health Criteria, 1994).

Ultimately DNA is the most critical target for damage by UV radiation (Environmental Health Criteria, 1994). The three main classes of damage caused by UV radiation are (i) cyclobutane pyrimidine dimers (CPD); (ii) pyrimidine (6-4) pyrimidone photoproducts (often referred to as (6-4)photoproducts or (6-4)PP); and (iii) DNA-protein crosslinks. The absolute and relative amounts of each type of damage vary according to the wavelength of radiation (Siebeck et al., 1994; Kielbassa et al., 1997). Figure 1.4 represents the first two of these types of photoproducts that may be formed. UV-B tends to damage DNA directly, whereas UV-A causes genotoxicity indirectly, through sensitizer radicals or reactive oxygen species (ROS) (Griffiths et al., 1998). UV-B photolysis may also generate biologically harmful photochemicals such as the interaction of free radicals with cell membranes and the generation of highly reactive oxygen species within and outside the organism which can oxidise and cross-link membrane lipids and other cell compounds (Siebeck et al., 1994). The formation of CPDs and (6-4)PPs interfere with normal base-pairing, inhibit replication, have been correlated with mutagenesis, and hence pose detrimental effects upon the viability and functional integrity of cells (Klug & Cummings, 1991; Griffiths et al., 1993; Friedburg et al., 1995; Carroll 1998). The rate of induction of CPDs and (6-4)PPs depends on the base sequence of the DNA, its physical confirmation and its level of metabolic activity. Cleaver et al. (1990) found that (6-4)PPs
Figure 1.4. Ultraviolet Radiation photoproducts from undamaged DNA: (a) Cyclobutane-pyrimidine photodimer, in this example thymidine residues are joined by UV irradiation to produce a thymine dimer; (b) 6-4 Photoprodut, in this example the C-6 of a thymine molecule is linked by UV to the C-4 of an adjacent thymine (Based upon Griffiths et al., 1993).
occur with much greater frequency in actively transcribing genes than in non-expressed genes, although such damage may be more accessible to excision repair enzymes (Mitchell & Karentz, 1993; Mitchell et al., 1993).

DNA extracted from human fibroblasts and used as a solar dosimeter in the ocean was studied by Regan et al. (1992). In their experiments, UV-B-induced photoproducts (CPDs) were quantified in the isolated DNA molecules exposed to UV radiation at the ocean surface and at various depths. In addition, bacteriophage DNA inactivation by solar UV-B was assayed by plaque formation in spheroplasts of Escherichia coli. The results of these experiments showed that pyrimidine dimer induction was linear with time. In Antarctic zooplankton, the extent of DNA damage in pelagic icefish eggs correlated with daily incident UV-B irradiance (Malloy et al., 1997). Similar DNA damage (quantified by the induction of pyrimidine dimers) was measured in amphibians (Licht & Grant, 1997), and Xiphophorus hybrid fish (Ahmed, 1993; Mitchell et al., 1993). A dose-dependent response was observed in anaphase aberration frequencies, in the embryos of Sterechinus neumayero (the Atlantic sea urchin) exposed to UV-B radiation at levels ranging from 0-1000 J/m², both in the laboratory and in field studies (Anderson et al., 1993). UV-B-induced endonuclease-sensitive sites (ESS) were not detected in the DNA of the tail fin of the Medaka after increasing the doses of UV-B up to 500 J/m². However, when the cells were disassociated with trypsin, ESS were detected in the epithelial cells near the tissue surface but not in the inner cells (Funayama et al., 1993). Kidambi et al. (1996), studied the effects of UV-A and UV-B irradiation upon the level of RecA protein in Pseudomonas aeruginosa. The protein concentration increased two-fold following 2 hours of irradiation. The authors also showed that the induction of damage-inducible genes was RecA dependent.

Adenosine-tri-phosphate (ATP) is continuously formed in catabolism and used in all energy-consuming reactions of living organisms. Therefore any impact upon the synthesis of ATP will be reflected in all other reactions of an organism. Vosjan et al.
(1990) assessed the effect of UV-B radiation on the ATP content of micro-organisms from the upper layer of the Weddell Sea, an important group of organisms in both the foodweb and the carbon cycle of marine ecosystems. They found that after 2 hours of irradiation with UV-B of 1.35Wm$^{-2}$, the ATP content of the micro-organisms decreased by 75% and showed a strong reduction after a longer period of exposure. Ultimately this could have severe deleterious effects on the functioning of the organisms concerned.

1.5.1.3 The repair of UV-induced DNA damage

All cells have the capacity to repair damage to nuclear DNA. There are two main mechanisms of repair for UV-induced DNA damage. Firstly, photoreactivation is a temperature-dependent phenomenon and occurs when following irradiation, the cells are exposed to light in the blue range of the visible spectrum. The process is initiated by photoreactivation enzymes (PRE) such as photolyase. The reaction involves the cleavage of the bonds between thymine dimers, and although the process can occur in the dark, firstly the enzyme requires absorption of a photon of light (Klug & Cummings, 1991). Secondly, another method of DNA repair is excision repair. This is a temperature-dependent enzymatic process which removes (6-4)PPs more rapidly than CPDs, and consists of the processes of damage recognition by a nuclease, dual excision that cleaves the phosphodiester bonds, release of an oligonucleotide fragment which carries the damage, resynthesis of the gap by DNA polymerase, and finally ligation which seals the 'nick' and closes the gap (Klug & Cummings, 1991; Siede, 1998). It has been inferred from this preferential activity that the (6-4)PP is intrinsically more dangerous. Many species use the light-activated repair systems (photoreactivation) which deal specifically with CPDs; in these species at least, it might be expected that the excision repair system would target those types of damage which the photorepair system cannot deal with. Nevertheless, the (6-4)PP and its photoisomer, the Dewer pyrimidone, have been shown to be potentially important agents of the lethal and mutagenic effects of solar UV (Mitchell

-26-
Excision of dimer

Blue light
PRE enzyme

Dimer repaired, normal pairing restored

(b) Photoreactivation

DNA polymerase fills gap
DNA ligase seals gap

Excision of dimer

(b) Excision Repair

Figure 1.5. The repair of photodamaged DNA: (a) Photoreactivation: PRE cleaves the bonds between thymine dimers, and reverses the damage induced by UV-R; (b) Excision repair: involves the excision and removal of a DNA segment carrying the lesion (represented by a blue square), followed by repair synthesis (dotted line), and ligation. (Adapted from Klug & Cummings, 1991; Livneh et al., 1993).
The repair of DNA damage should be error-free, however, when confronted with excessive doses of mutagenic factors they may saturate resulting in the occurrence of mutations (Susanne, 1984; Zakrzewski, 1997). Figure 1.5 illustrates the mechanisms of each type of DNA repair. In photoreactivation repair a photoreactivation enzyme (PRE) cleaves bonds between thymine dimers and the UV-R-induced damage is reversed. The second DNA repair mechanism involves excision repair, where the damage is excised, and the DNA is resynthesised and then ligation occurs.

Reports of PRE activity in aquatic animals have been documented. A study by Blaustein et al. (1994) found that in different species of amphibians, the levels of photolyase were different and certain species (such as Hyla regilla) exhibited higher levels of the enzyme. It is of interest that the populations of this particular species are not known to be in decline and in field studies their eggs had a higher hatching success compared to the other species studied which had lower levels of photolyase. Worrest & Kimeldorf (1975, 1976) found that the timing of exposure of toad tadpoles to UV-B, relative to the timing of exposure to photoreactivating light, had a very large influence on the occurrence of damage and this was taken as evidence that CPDs played an important part in the harmful effects they observed. Kim et al. (1996) have subsequently described a light-activated system for repairing (6-4)PPs in amphibians. The relative importance of CPDs and (6-4)PPs therefore may need to be reassessed.

1.5.2 Organic chemical contaminants

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants of aquatic ecosystems. They occur as a result of natural processes such as forest fires, volcanic activity, erosion of sedimentary rocks and oil seeps, and are synthesised by some bacteria, plants and fungi. However, the predominant sources of PAHs in the environment are linked to anthropogenic activity such as incidences of oil leaks and spills in addition to the burning and combustion of fossil fuels (Grimmer, 1979; Neff, 1979; National Research...
Table 1.2 Anthropogenic sources of PAHs.

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<tr>
<th>STATIONARY SOURCES</th>
<th>MOBILE SOURCES</th>
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<tr>
<td>Residential Heating:</td>
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<tr>
<td>- Furnaces, fireplaces, and stoves (wood and coal)</td>
<td>- Gasoline-engine vehicles</td>
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<td>- Gas burners</td>
<td>- Diesel-engine vehicles</td>
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<td>Industry:</td>
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<tr>
<td>- Coke production</td>
<td>- Rubber tyre wear</td>
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<td>- Carbon black production</td>
<td>- Aeroplanes</td>
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<td>- Petroleum catalytic cracking</td>
<td>- Sea traffic</td>
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<td>- Asphalt production</td>
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<td>- Aluminium smelting</td>
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<td>- Iron and steel sintering</td>
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<td>- Ferroalloy industry</td>
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<td>Power &amp; Heat generation:</td>
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<td>- Coal- and oil-fired power plants</td>
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<td>- Wood- and peat-fired power plants</td>
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<td>- Industrial and commercial boilers</td>
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<td>Incineration &amp; open fires:</td>
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<td>- Municipal &amp; industrial incinerators</td>
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<td>- Refuse burning</td>
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<td>- Structural fires</td>
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<td>- Agricultural burning</td>
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Figure 1.6. Structures of some commonly occurring aromatic compounds. This figure includes benzene. (Adapted from Jacob & Grimmer, 1979; McElroy et al., 1987; Harrison, 1990).
It has been estimated that approximately 5 million tonnes of petroleum hydrocarbons are released into the marine environment each year, of which PAHs are derivatives (Fossi et al., 2000). The highest concentrations of PAHs are generally found nearby urban centres (Meador et al., 1995).

Table 1.2 lists the main sources of PAHs from anthropogenic activity. They are divided into two categories: stationary and mobile sources. The stationary sources include industrial sources, the generation of heat and power, incineration and open fires. Mobile sources include gasoline-engine vehicles, diesel-engine vehicles, aeroplanes, and sea traffic (Bjørseth & Ramdahl, 1985). It has been suggested that PAHs from pyrogenic sources are less bioavailable than fuel based PAHs (Kaag et al., 1998). The group of contaminants classed as PAHs generally refer to hydrocarbons which have two or more fused benzene rings (McElroy et al., 1987; Harrison, 1990). Figure 1.6 shows the structures of some commonly found PAHs.

Of particular concern are PAHs which range in molecular weight from 128 to 300, since PAHs within this group tend to be more acutely toxic to aquatic organisms (Arfsten et al., 1996; Law et al., 1997). PAHs represent a very important group of chemical carcinogens and/or mutagens, and some have been extensively studied in biological experiments with the carcinogenic potency of tetra- and pentacyclic compounds being repeatedly proven (Law, 1986; Gert-Jan de Maagd et al., 1998). It has been estimated that approximately 60 to 90% of human cancers are caused by exposure to environmental chemicals, including PAHs (Miller et al., 1988). The US Environmental Protection Agency has recognised 16 PAHs as priority contaminants of which eight are included as possible carcinogens (Okay et al., 2000). Benzo(a)pyrene is included as one of these carcinogens and has been used in some of the presented studies as a representative of PAHs since it is always present whenever PAHs have been detected. However, it is
usually less than 5% of the total amount of PAHs (Egan, 1979; Bjørseth & Ramdahl, 1985).

Much toxicological concern has focused upon PAHs since they can interact with cellular components in two ways to cause toxic responses. Firstly, they may bind reversibly to lipophilic sites in the cell and cause interference with the cellular processes. Secondly, metabolites of the PAH, being more hydrophilic, reactive and electrophilic may bind covalently to many cellular structures including DNA causing long-term damage and potentially forming DNA adducts (Neff, 1979; Kurelec et al., 1990; Malmström et al., 2000). Often the metabolic activation of these compounds to daughter products results in the formation of compounds that are far more toxic than their parent compounds (Mekenyan et al., 1994). The binding of PAHs to cellular DNA is linked to monooxygenase activity, is dependent on NADPH, can be stimulated by inducers of monooxygenases (e.g. benzo[a]pyrene, 3-methylcholanthrene) and inhibited by the inhibitors of this enzyme (7,8-benzoflavone). This demonstrates how PAHs require conversion into their epoxides prior to their reaction with DNA (Jacob & Grimmer, 1983).

In aquatic organisms it has been found that petroleum hydrocarbons bind to the membrane of neural tissues. More specifically, paraffins penetrate into and are associated with the hydrophobic interior of membranes. In contrast, aromatic hydrocarbons are bound to various nonpolar and electron-interactive sites on the membrane surface (Neff, 1979). Many physiological processes are dependent directly on the unique properties of biological membranes, hence the ability of aromatic hydrocarbons to alter membrane surfaces indicates that they may be toxic to living organisms.

Benthic communities are deleteriously affected by oil pollution due mainly to the sedimentation of oil and their near or sub-sediment lifestyle. One mechanism for the sedimentation of oil offshore is by adsorption onto sediment particles suspended in the water (Meyers & Quinn, 1973). An alternative mechanism, is uptake by phytoplankton and subsequent sedimentation of the phytoplankton (Lee et al., 1978). A further
mechanism is sedimentation of oil in zooplankton faecal pellets (Conover, 1971). These organisms have been reported to feed directly upon particles of suspended oil following oil spills (Conover, 1971; Johansson, 1977).

1.5.3 Synergistic effects of UVR and PAH

In the case of petroleum and other oil products which have been introduced to the marine environment, a number of processes may transform the chemical or physical state of the contaminants, including photo-oxidation and photolysis. The photo-transformation of the PAHs has been reported to alter the toxic potential of the samples. In some cases, certain promutagens or even non-mutagenic compounds can be photoactivated to derivatives exhibiting a direct mutagenicity in prokaryotic or eukaryotic target cells (De Flora et al., 1989).

Synergistic effects of UV-B along with other contaminants or environmental stressors have been investigated, as well as the effects of UV-B in isolation. A common variable often related to UV radiation is an increase of temperature. Studies into the effects of UV radiation in conjunction with alternative stressors include the synergistic effects of pH, pathogens and PAHs (Long et al., 1995; Kiesecker et al., 1995; Ankley et al., 1994, 1995). Of particular interest has been the action of UV-B on the toxicity of PAHs. A number of authors have demonstrated that phototoxicity is a function of both PAH dose and light intensity and the mortality rates of test organisms such as amphipods and oligochaetes have increased significantly compared to tests performed under otherwise analogous conditions with fluorescent light (Ankley et al., 1994, 1995; Arfsten et al., 1996). The interaction of light and PAHs to cause phototoxic effects in mammalian systems, protozoa and bacteria has been documented by Santamaria & Prino (1964). They found that laboratory animals exhibited responses such as acute skin reactions and UV-enhanced carcinogenesis.
Phototoxicity occurs when UVR is absorbed by the conjugated bonds of PAH molecules exciting them to a triplet state. The toxicity is elevated due to the transfer of the absorbed energy from the excited PAHs to ground-state dissolved oxygen forming singlet-oxygen intermediaries. The singlet oxygen and other oxygen free radicals which are formed are of high oxidising potential and are able to induce oxidative stress and subsequently destroy biomolecules in tissues. Cell membranes in particular are susceptible to disruption caused via lipid peroxidation (Arfsten et al., 1996). In seawater, the half life of singlet oxygen is very limited (less than 2 μs), however, it is much greater in lipophylic tissues where hydrophobic PAHs accumulate, resulting in the greater potential for tissue destruction (Vaca et al., 1988; Kagan et al., 1990; Ankley et al., 1995; Pelletier et al., 1997). Kagan et al. (1990) concluded there are at least three targets of PAH phototoxicity; firstly the cell membrane components, secondly DNA, and thirdly essential cytoplasmic components (such as superoxide dismutase). It has also been found that UVR enhances the covalent binding of PAHs to DNA (Sinha & Chignell, 1983).

The effects of enhanced UV-B on fungal infections was investigated by Keisecker & Blaustein (1995) who found that ambient levels of UV-B exacerbated mortality of amphibian species due to infection by the pathogenic fungus Saprolegnia. Long et al. (1995) studied the synergistic effects of low pH with UV-B. There was no increase in frog embryo mortality where only one variable (either low pH or UV-B) was present. However, UV-B and low pH acting together led to a significant decrease in embryo survival. Each of these studies indicated that the effect of UV radiation was to activate and increase the negative effect of a single variable and resulted in an increase in the rates of mortality.

1.6 Target species

There are currently a number of freshwater and marine invertebrate and vertebrate species which are recommended for performing acute toxicity tests (ASTM, 1997). These
species are selected on the basis of availability, commercial and ecological importance, past successful use and ease of manipulation in the laboratory.

There is a lack of adequately validated in vivo test methods which could be used effectively to evaluate genotoxicity in ecologically relevant organisms under environmentally realistic 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 urchins Strongylocentrotus purpuratus (Hose & Puffer, 1983; Hose et al., 1983; Hose, 1985; Anderson et al., 1994) and Sphaerechinus granularis (Pagano et al., 1982, 1996), the polychaete worms Platynereis dumerilii (Jha et al., 1996; 1997; Hutchinson et al., 1998) and Neathes arenaceodentata (Pesch et al., 1981; Harrison & Anderson, 1989), the marine mussels Mytilus edulis (Dixon, 1982, 1985; Dixon & Clarke, 1982; Dixon & Prosser, 1986; Al-Sabti, 1989; Jha et al., 2000) and Mytilus galloprovincialis (Al-Sabti & Kurelec, 1985; Brunetti et al., 1986; Akcha, 2000), and the freshwater mussel Dreissena polymorpha (de Lafontaine et al., 2000).

The target species used in the present work include the marine bivalves Mytilus edulis and Cerastoderma edule, the marine polychaete Platynereis dumerilii, and the marine flatfish Platichthys flesus. These have been described in more detail in the second chapter of this thesis.

1.7 Selected endpoints

The present work focuses on molecular, cytogenetic and whole organism responses to genotoxic agents.

1.7.1 Molecular endpoints

1.7.1.1 The ‘Comet’ Assay

The single cell gel electrophoresis or ‘Comet’ assay was first introduced by Östling and Johanson (1984), and subsequently modified by Singh et al. (1988) and Olive et al.
Plate 1.1 *Mytilus edulis* blood cells exhibiting increasing levels of DNA damage detected with the 'Comet' assay. Class O indicates negligible amounts of damage, increasing up to class III. Class IV cells are considered to be apoptotic. Classes of damage are based upon that of Collins *et al.* (1997).
(1990) as a method for DNA damage in single cells. DNA strand breakage is not contaminant-specific, however, it is an easy and sensitive method for detecting cellular exposure responses to genotoxic agents (Nacci, 1996). The advantages of this assay include the relatively few numbers of cells required (compared to other techniques), the non-destructive element (and therefore potential for temporally repeated sampling) and prospective application to any nucleated cell type in a single cell suspension. Furthermore this assay allows the measurement of responses in individual cells and is relatively inexpensive and quick thus making this a simple and useful method of detecting DNA damage (Nacci, 1996). In addition this technique is independent of the chromosome number or karyotype of the species. Furthermore, for species which have small-sized and high numbers of chromosomes and hence poor suitability for cytogenetic studies, the comet assay provides an alternative endpoint for measuring DNA damage.

Following exposure to the test agents, individual cells are lysed to remove the nuclear membrane surrounding the DNA. They are then exposed to alkaline conditions which allows the unwinding of nuclear DNA, following which they are electrophoresed and then stained with a fluorescent stain to allow subsequent detection of strand breaks in individual cells (Wilson et al., 1998). Damaged DNA migrates towards the anode during electrophoresis and the level of migration is proportional to the amount of damage as described by Fairbairn et al. (1995). Plate 1.1 illustrates examples of cells exhibiting different levels of DNA damage as defined by visual classification.

The interpretation of the results is based on the hypothesis that the alkaline comet assay is able to detect nuclear DNA damages caused by non-crosslinking genotoxic agents to produce low molecular weight DNA strands, these being formed either directly through DNA single and double strand breaks, or indirectly by excision-repair of damaged DNA, or by alkali-labile sites (Hartmann & Speit, 1997; Choucroun et al., 2001). Single strand breaks (SSB) are easily repaired and are not considered to be significant lethal or mutagenic lesions. However, many genotoxic agents do not directly induce strand breaks,
but induce alkali-labile sites. These are likely to be converted into breaks during the comet assay when the DNA is in the electrophoresis solution at high pH conditions. These can then be detected by the Comet assay (Collins et al., 1997a).

The comet assay has the potential to provide information about other mechanisms such as DNA repair by employing lesion specific enzymes and incorporating their application into the protocol (Collins et al., 1996, 1997a, 2001; Pruski & Dixon, 2002). This method had been recognised as a useful technique for human biomonitoring studies (Kassie et al., 2000). Several reviews have appeared in relation to Comet assay methodology and application areas (McKelvey-Martin et al., 1993; Fairbairn et al., 1995; Mitchelmore & Chipman, 1998b; Cotelle & Férard, 1999; Rojas et al., 1999).

More recently, the comet assay has been used to assess the genotoxicity of contaminants using cell lines from tunicates and fish (Nehls & Segner, 2001; Kamer & Rinkevich, 2002). Also organic extracts from marine sediments were tested for genotoxicity using leukocytes isolated from carp (Kammann et al., 2000). The erythrocytes from gilthead bream, Sparus aurata, have also been identified as useful target cells for the evaluation of genetic toxicity following organotin exposure (Gabbianelli et al., 2002). In addition to fish cells, in vivo cells from aquatic organisms including freshwater and marine mussels (Mitchelmore et al., 1998; Rank, 1999; Steinert et al., 1998; Wilson, 1998; Steinert, 1999; Pavlica et al., 2001), oysters (Nacci et al., 1996), grass shrimp larvae (Lee et al., 2000), Daphnia magna (Den Besten & Tuk, 2000), tadpoles from the green frog Rana clamitans (Ralph & Petras, 1997, 1998, 1998a), and cetaceans (Taddei et al., 2001) have been reported to be useful bioindicators for genotoxicity.

1.7.2 Cytogenetic endpoints

Cytogenetic endpoints are usually observed with light microscopy. Of the aquatic species, the number of cytogenetic studies of fish have been relatively scarce, with fewer
than 2% of the 13000 existing marine species having been studied (Galetti et al., 2000). Fish species have often been found to be unsuitable for cytogenetic research due to their large number of small chromosomes, in contrast some invertebrate species have been found to produce easily examined anaphase and/or metaphase chromosome spreads and have therefore been put forward as useful potential indicator species for cytogenetic studies (Hooftman & Vink, 1981).

1.7.2.1 Chromosomal Aberrations (CAbs)

Among various molecular, biochemical and cytogenetic parameters for the evaluation of induced genetic damage the analysis of CAbs have been considered to be the most important because of their known role for the induction of malignancies, congenital abnormalities and foetal wastage (Natarajan et al., 1994; Tucker & Preston, 1996). The induction of CAbs have been found to be associated with the initiation and promotion of malignancies. CAb assays aim to detect the induction of clastogenesis (chromosome breakage). CAbs are commonly scored in metaphase and anaphase cells, with the former usually providing more information on the types of induced aberration (Gómez-Arroyo & Villalobos-Pietrini, 1995). Tumour cells have been examined in detail and in most cases have been found to contain structural and/or numerical alterations of chromosomes.

Although physical and chemical agents readily induce structural CAbs, the types of aberrations induced and the stage of the cell cycle which is sensitive to the genotoxic agent may vary for different types of mutagens. Although the mechanisms may be different the end-point is a discontinuity in the chromosomal DNA. This can be rejoined or repaired to restore the original structure, left unrejoined, or rejoined inaccurately to produce a rearrangement of the chromosome (Wolff, 1982; Scott et al., 1990). DNA breaks of a single strand (ssb) or double strand (dsb) type can lead to mutations. Dsb are very mutagenic and lethal and if unrepaired or repaired with errors result in the loss and/or rearrangement of genetic material (Dugle et al., 1976). Preparation of metaphase
chromosomes and their staining will be described in more detail in the sections where they were assayed. The classification and recording of CAbs was based upon the criteria and definitions detailed by Scott et al. (1990).

1.7.2.2 Sister Chromatid Exchanges (SCEs)

SCEs affect both chromatids because they are formed during the replication part of the cell cycle. They are more analogous to isocentric chromatid aberrations in which the sister chromatids are translocated (Wolff, 1982). Although SCEs are not mutations in the conventional sense, since they involve the reciprocal exchange of DNA between sister chromatids, they have been found to be a sensitive and quantitative assay of genetic damage (Takehisa, 1982; Brunetti et al., 1986). SCEs were first demonstrated by Taylor (1958), in autoradiographic studies of tritium labelled chromosomes that contained one labelled and one unlabelled chromatid. More recently, other techniques have been developed without using radiography. These involve growing the cells for two cell cycles, in the presence of a thymidine analogue, the halogenated nucleoside 5-bromodeoxyuridine (BrdU), leading to one unifilarly substituted and one bifilarly substituted chromatid. Sister chromatids substituted in this way stain differentially with Giemsa with the fluorescent compound Hoechst 33258 (as well as other fluorescent dyes such as acridine orange and quinacrine). Figure 1.7 illustrates how BrdU is incorporated into DNA and leads to differential staining of the sister chromatids. Perry & Wolff (1974) described the fluorescence plus Giemsa (FPG) technique where Hoechst 33258 and short-wave light (blacklight) were used to treat the chromosomal preparations, which was then followed by staining with Giemsa solution. These produce non-fading, permanently stained chromosomes in which SCEs can be easily visualised and scored (Perry & Evans, 1975; Stetka & Wolff, 1976; Gómez-Arroyo & Villalobos-Pietrini, 1995). It has been shown that the frequency of SCEs increases following exposure to a number of physical and chemical agents including tritium, x-rays and ultraviolet light as well as alkylating agents. In some
Figure 1.7 The formation of differentially labelled sister chromatids during two cell cycles in BrdU. $G_1$ represents the pre-DNA synthesis stage; $S$ phase represents the DNA synthesis stage; $G_2$ represents the post-DNA synthesis stage; $M$ is metaphase.

Figure 1.8 The formation of micronuclei in mitotically dividing cells.
cases, it was found that the induction of SCEs was more sensitive to chemicals than was the induction of CAbs (Stetka & Wolff, 1976). It has been reported that an increase in SCE frequency of as much as a third (compared with controls) can be induced by potential carcinogens and mutagens (Redei, 1998).

This endpoint has been applied to study chromosome damage both *in vitro* and *in vivo* conditions, and has been found to be a rapid, sensitive and quantitative assay of genetic damage (Stetka & Wolff, 1976a; Brunetti *et al.*, 1986; Cooke *et al.*, 1989). The precise molecular mechanism or mechanisms involved in the formation of SCEs has not been made clear though there have been suggestions of a link between cell-cycle or DNA replication kinetics and SCE formation (Lamberti *et al.*, 1983; Das & Sharma, 1984). Some chemical agents used at the early stage of SCE research were ones that had previously been proven to be DNA-damaging agents. However, SCE-inducing agents are not necessarily agents which act with DNA directly (Takehisa, 1982). Furthermore, caution has been raised when applying this method, Tucker and Preston (1996) stated that positive SCE results did not necessarily imply that the test agent was mutagenic, for example, the authors found that sodium chloride (NaCl) induced increased frequencies of SCEs. Preparation of metaphase chromosomes and their differential staining for the detection of SCEs will be described in more detail in the sections where they were assayed.

### 1.7.2.3 Micronucleus (MN) induction

The micronuclei (MN) test is a method for detecting chromosomal breaks in mitotically dividing cells. It was originally developed for testing mammalian systems in samples collected from bone marrow (Schmid, 1975). It has since been developed for use in aquatic and amphibious organisms. MN can be formed from the mitotic loss of acentric chromosomal fragments; a variety of mechanical consequences of chromosomal breakage and exchange such as lagging chromosomes during anaphase, inactive kinetochore, strong
chromosomal bridges and tangled chromosomes; and mitotic loss of whole chromosomes (Heddle et al., 1991). MN appear as separate, smaller nuclei within the cytoplasm of the cell, but separate to the main nucleus (Bauchinger & Braselmann, 1990). Since MN are induced by clastogens and agents which have an effect upon the spindle apparatus the MN assay is widely used (Evans, 1976; Schmid, 1975; Heddle et al., 1983; Hogstedt, 1984; MacGregor et al., 1987; Ciaravino et al., 1993). Figure 1.8 illustrates the formation of micronuclei in mitotically dividing cells.

1.7.3 Cytological Endpoints

1.7.3.1 Proliferation Rate Index (PRI)

The rate of cell division has been used as a biomarker of cellular immune competence in lymphocytes in previous work (Gonsebatt et al., 1995). PRI is an arbitrary measurement of cell duplication, calculated by measuring the proportion of cells which have passed through 1, 2 or 3 cell cycles following incorporation of BrdU (see section 1.6.2.2). The presence of BrdU allows the differential staining of sister chromatids and visual observation of the staining of the chromosomes within a metaphase cell and allows the determination of the number of mitotic cell cycles which have been passed through. This method has been developed based upon the observations that responses to chemical and physical trauma often result in altered rates of cell proliferation (Moore & Morss, 1996). The universal process of cell division is well documented in eukaryotic cells, however, specifics such as cycle time and cell size differ for each species as well as for tissue types within species. Toxins can affect the rate and effectiveness of the replication cycle by slowing it down, resulting in a lower PRI value. PRI is essentially dependent on the toxic agent(s) involved, the organism's response and factors influencing the normal cell replication rate. As such, indexes and their values are relative only to themselves, although generally and under expected conditions where toxins reduce the number of cells in M3, low PRI is interpreted as an increased toxicity. Preparation of metaphase...
chromosomes, their staining and analysis for the determination of PRI is described in more detail in the sections where they were assayed.

1.7.3.2 Neutral Red Retention (NRR) assay

The NRR assay is based upon the identification of early changes at the subcellular level, specifically in the lysosomal compartment as a biomarker of the process of cellular injury. It is concerned with the examination of responses in living cells, as changes in vesicular events can only be directly evaluated through the use of live cells (Moore & Simpson, 1992). Lysosomes are organelles found within the cell and are defined as membrane-bound vesicles involved in intracellular digestion, and they contain a variety of hydrolytic enzymes (Alberts et al., 1992). As such they form part of the cells' defence mechanism towards xenobiotic insult and have known responses to contaminants. The main lysosomal stress responses are changes in size, lysosomal content, rate of fusion events, lysosomal permeability and alterations in uptake and detoxification ability (Hawkins, 1980).

The lysosomes found in invertebrate haemocytes have been successfully used in lysosomal stress studies both in laboratory experiments and from field samples (Lowe et al., 1995a, 1995b; Cheung et al., 1996; Grundy et al., 1996a, 1996b; Moore et al., 1996; Femley et al., 2000). Haemocytes make suitable cell types because they are rich in lysosomes, the cells are easily handled without significant change to the sensitivity of the lysosomal response, and haemolymph can be extracted from live organisms without the requirement to sacrifice the animal. Alterations in the stability of the lysosomal membrane and increases in the volume of the lysosomes have been used as biomarkers of environmental stress in a number of situations (Lowe, 1981; Moore, 1985; Moore 1992). By the microinjection of the supravital dye, neutral red, into the lysosomal compartment, the injury to these organelles can be examined. The neutral red dye is taken up specifically in the lysosomes of live cells. Due to its lipophylic nature, neutral red is
able to freely permeate the cell membrane in the non-ionised form, but diffusion is slow or negligible once ionisation has taken place (De Duve et al., 1974). The extent to which this molecular probe is retained is dependent on the pH of the lysosome, and the efficiency of its membrane associated proton pump. Any deleterious effect on the proton pump system will result in a reduced ability for the lysosomes to retain the dye. As mentioned above, a number of authors have reported that a number of contaminants may induce destabilisation of the lysosomal membrane, thus permeability of the membrane and hence retention of the neutral red dye within the lysosome will be affected (Moore, 1982).

1.7.4 Individual responses

1.7.4.1 Embryo-larval development assay

The development of the early life stages of bivalve molluscs such as oysters, is an established bioassay technique (ASTM, 1997b). The oyster (Cerastoderma gigas) embryo bioassay is one of the earliest modern biological measures of water quality applied to marine waters around the UK (Matthiessen & Law, 2002). It has however, been noted that many benthic marine invertebrate species have high juvenile mortality rates, with mortality being particularly high early in early life stages (Gosselin & Qian, 1997). Due to this factor, the normal morphological (anatomical) development of embryo-larval stages of the target species was used as an endpoint to identify whole organism responses to exposure to agents, as opposed to survival rates. The deformation of individuals is the visible part of a complex process of toxicity stress in a sample collected from a population (De Bistoven, 1999).

The principle responses of development effects were characterised by the three main stages of growth of the early life stages. The first being the fertilisation rate of the eggs, this was recognised by the presence of a polar body or signs of cellular cleavage. The second was that of the normal development to the free-swimming trochophore stage. The third was the normal development to the prodissococonch stage (otherwise known as the
D-shell stage) in *Mytilus edulis*, and nectochaete larval stage in *Platynereis dumerilii*. Previous workers have identified inhibition of fecundation, cell cleavage in the eggs, and larval development in oyster (*Crassostrea gigas*) larvae, following exposure to organotin compounds (Alzieu & Heral, 1984). Also, a number of sea urchin species have been identified to be useful test organisms with distinct development stages (Pagano *et al.*, 1982, 1988, 1993; Anderson *et al.*, 1994; Trieff *et al.*, 1995; Quiniou *et al.*, 1999).

### 1.8 Summary

In summary, this chapter has stated the objectives of the thesis, indicated the target species used and the methodologies selected for detecting DNA-damaging effects of UVR and PAH contaminants. The background information provided has indicated the knowledge which has been collated so far by previous authors regarding the effects of the genotoxic agents of interest, and has also highlighted the gaps in the research undertaken where certain genotoxic biomarkers have not yet been used. In the thesis that here follows, attempts have been made to evaluate the usefulness of using different life stages of different marine species, and assessing the applicability of a number of biomarker techniques for the identification of genotoxic, cytotoxic and phenotypic impacts of UV and/or PAH contaminant exposure in the marine environment.
Chapter 2

Materials & Methods
2.1 Test organisms

The target organisms used in these investigations were selected for their ease of collection from the field and or maintenance in the laboratory, as well as their environmental and commercial relevance. Invertebrate species were of particular interest for reasons discussed in section 1.1.1 and the species were selected since their genotoxic responses to ultraviolet radiation, and ultraviolet radiation with organic contaminants had not been previously studied in detail.

2.1.1 *Mytilus edulis* (Mollusca: Bivalvia)

*Mytilus* typifies the mussels or Mytilidae which are one of the most successful and prolific of the shore bivalves. *Mytilus edulis*, the common blue mussel, is a well researched species and has been widely used as a test organism in ecotoxicological and ecological studies. It is often used as a sentinel organism since it is cosmopolitan with a wide geographical distribution, can be found in high abundance and is easily collected from the shore without significant disturbance to the population. It is a filter-feeder and as much as 45-65 litres of water may be pumped through and filtered by an adult of 7.5cm in 24 hours (Dare, 1980). Due to the feeding mechanisms of this bivalve, food particles (namely plankton and organic detritus) are filtered from a current of water drawn through an inhalant siphon created by the gills and into the shell cavity. Mussels collected for commercial use from wild and cultured beds are carefully purified before marketing, this is necessary to remove any pathogenic bacteria or toxins filtered by them from the water (Beedham, 1972).

The sexually mature adults release eggs and sperm into the surrounding water for fertilisation. Once fertilised, the early larval stages are planktonic and drift in the surface layers of the water for 1-2 months, before settling to the sea bottom and metamorphosing into spat. The spat secrete byssus threads and attach themselves to suitable substrates at or below the low water mark. When the spat have grown to 1-1.5mm in size, they detach
their byssus and are carried by currents until they reach an existing mussel bed or other suitable grounds. From this stage, the mussels are sedentary and therefore provide an indication of the contaminants available in the water column where they are found. Marine mussels reproduce externally, therefore allowing the controlled collection and fertilisation of gametes (Dare, 1980; Mackie, 1984). *Mytilus* has a stable karyotype of 28 chromosomes (2n), and its metaphase chromosomes can be examined easily (Thiriot-Quievreux & Ayraud, 1982). Plate 2.1 shows the external morphology of typical adults collected from the field and used in the experiments described in this thesis.

### 2.1.1.1 Collection and maintenance of *Mytilus edulis*.

Adult *Mytilus edulis* within a size range of 3.0-3.5 cm were collected from their natural beds on rocky outcrops, within the lower intertidal zone at Sharrow Point, Whitsand Bay, Southeast Cornwall, UK (SX 396 520), illustrated in figure 1.1. This site met with the guideline coliform standards (Environment Agency, 1996a) as specified in the EEC bathing waters directive (76/160/EEC), which are recognised as high standards indicating a low sewage input (Kay et al., 1990). Since there are no nearby river outflows these mussels are considered to be from a relatively clean environment. The mussels were removed from their rock substrates by carefully cutting the byssus threads and then transported to the laboratory in a cool box. On return to the laboratory, they were cleaned of epibionts and maintained in 20 litre glass static aquaria containing clean, aerated 10μm carbon filtered seawater (salinity of 35-37), and held at 15 ± 1°C. They were fed twice weekly with ‘Marine Aquiphy’ (Interpet, Dorking). 80% water changes were made twice weekly to remove faeces, and any animals which did not close their valves on exposure to air were considered to be of poor health and removed to avoid contamination of the remaining animals.
Plate 2.1 Adult *Mytilus edulis* collected from Sharrow Point, Whitsand, Cornwall.

Figure 2.1. The location of the sampling point at Whitsand Bay, from which stock *Mytilus edulis* were collected.
2.1.2 *Cerastoderma edule* (Mollusca: Bivalvia)

*Cerastoderma edule*, the edible common cockle, is the best known member of the family Cardiidae. It can be found in many shore areas, particularly in fine sediments around the coast and can occur in enormous numbers in dense beds beneath the surface of the sand, reaching densities of up to 10,000 individuals per square metre. In some areas, the cockles may occupy 40% of the surface area of the beach in their preferred zones (Hayward, 1994). Due to its high prevalence, *C. edule* are raked out of the sediments and sold for food on a commercial scale. This species can tolerate salinity reductions as low as 10‰ and can be commonly found in estuaries (Gibson *et al.*, 2001). *C. edule* does not attach to the substrate with byssus threads like *Mytilus edulis*, but burrows into soft sediment with the aid of its large, powerful and angled foot. It is also known to use this appendage to 'leap' along the sediment surface to escape its predators, in particular the starfish (Beedham, 1972). The development of the larvae of *C. edule* is planktotrophic. The pelagic stages feed on phytoplankton and usually take 1-4 weeks to grow and develop into young adults. The external morphology of typical adults collected from the field is presented in plate 2.2.

Relatively few studies have been reported with *C. edule* as the test species, particularly when comparing the frequency of studies with the vast amount of literature available with *Mytilus* species. For this reason the cockle was used in some of the studies undertaken for this thesis, for the evaluation of it's suitability as a test organism.

2.1.2.1 Collection of *Cerastoderma edule*

Adult *Cerastoderma edule* within a size range of 3.5-4.0 cm were collected by hand from the lower tidal zone of a series of field sites. After they were removed from the fine-medium grained sediments they were transported to the laboratory in a cool box. On return to the laboratory they were cleaned externally of residual sediments with filtered seawater and used immediately for experiments.
Plate 2.2 Adult *Cerastoderma edule* collected from field sites along the River Tamar, Plymouth.
2.1.3 *Platynereis dumerilii* (Polychaete: Annelida)

*Platynereis dumerilii* is a polychaete worm, which can be found in many temperate regions. In nature it usually resides in parchment-like tubes for protection, which are constructed on detached kelp which form sublittoral drift weed accumulations. They feed mainly on the macroalgae with the aid of their rough, horny jaws. *P. dumerilii* has been established as a potential biomarker organism, and is useful for cytogenetic monitoring since it is amenable to culture and maintenance in the laboratory, and has a relatively short life cycle (approximately 3 months from trochophore to sexually mature adult), as shown in figure 2.2. This species has a stable karyotype of 28 chromosomes (2n) (Jha et al., 1995). It is a species of importance in the food chain, being primary consumers and prey to higher organisms such as fish (Bedford & Moore, 1985; Hutchinson et al., 1995; Jha et al., 1995, 1996, 1997).

2.1.3.1 Culture and maintenance of *Platynereis dumerilii*

Broodstocks of *Platynereis dumerilii* were cultured and maintained in the laboratory held at the University of Plymouth from stocks originally provided by Dr. Adriann Dorresteijn, University of Mainz, Germany. The animals were maintained in 10 litre static tanks composed of perspex and containing clean, aerated 10μm carbon filtered seawater (salinity 35-37), held at 20 ± 1°C. To induce sexual maturation of the polychaetes, the photoperiod was manipulated to give a cycle of 16 hours light followed by 8 hours darkness, with continuous low level illumination for 8 hours once every 4 weeks, to simulate ‘full-moon’ conditions (Hutchinson et al., 1995). Plate 2.3 shows the external appearance of sexually mature male and female *P. dumerilii*. The animals were fed with chopped spinach thrice weekly, and with fish flake food (Aquarian, Waltham, Elland, UK) on alternating days. 50% water changes were made once weekly to remove faeces and to help prevent the build-up of bacteria in the stock vessels. Plate 2.4 illustrates the appearance of the tubes within which the polychaetes reside until sexually mature.
6. Sexually mature adults spawning

I. Newly fertilised embryo (6 hours old)

2. Trochophore (24 hours old)

3. Metatrochophore (48 hours old)

4. Necrochaeta larvae (5 days old)

5. Tubed animal (3 months old)

Figure 2.2 The life cycle of *Platynereis dumerilii*. (Reproduced from Hutchinson et al., 1995).

Plate 2.3 Sexually mature adult *Platynereis dumerilii*: (a) female; (b) male.
Plate 2.4 Cultures of sexually immature (juvenile) *P. dumerilii* residing within their tubes (indicated by arrows) in laboratory conditions. Note the chopped spinach collected at the tube entrances.

Plate 2.5 Adult *Pleuronectes flesus* (taken from Bristow, 1992).
2.1.4 *Platichthys flesus* (Pleuronectidae)

*P. flesus* is the only species of flatfish which can be found in fresh water and are common in the brackish waters of estuaries, particularly during the summer season. They live from the intertidal zone to a depth of 60 m, and tend to live on sandy or clayey sea bed substrates, burying themselves in the sediment during the day, and being more active at night. This species can commonly be found along the coast of Europe, in the Mediterranean, the Black Sea and the Sea of Arov. They feed upon benthic animals including molluscs and polychaete worms, and on midge larvae when in brackish or fresh water. Flounder migrate with the tide, swimming shorewards with the incoming tide in search of food and back to deeper water as the tide recedes. They spawn from January to approximately June, producing pelagic eggs, larvae and fry. This species is considered to be economically an important species in some regions, such as the Baltic Sea (Fitter & Ray, 1984; Bristow, 1992).

2.1.4.1 Maintenance of *Platichthys flesus*

A stock of healthy 0-group flounder (*Platichthys flesus*) were obtained from Port Erin Marine Laboratory and grown to their second year before the start of the experiments. The fish were maintained in 200 litre glass-fibre tanks with a flow-through and pumped water return system. The fish were introduced into the exposure tanks 2 weeks prior to the commencement of the exposures at a density of 15 individuals per tank. They were fed a diet of nutra marine 4mm pellets, both during the acclimatisation of the animals in the tanks and during the experiment. They were fed daily at 0.9% body weight/day. This was calculated by weighing each fish at the beginning of the study and at one month intervals thereafter. The animals were exposed for a total of 11 months before they were sacrificed. Plate 2.5 illustrates the morphology of an adult *Platichthys flesus*. 
2.2 Spawning & collection of embryo-larvae

2.2.1 *Mytilus edulis*

*M. edulis* become sexually mature from approximately one year in age. The main spawning season in the British Isles is during the spring, with a subsidiary peak in the autumn (Dare, 1980). During the spawning seasons, *M. edulis* were collected as described in section 2.1.1.1 and allowed to acclimatise in 20 litre aquaria containing filtered seawater held at <15°C, for at least 24 hours before the induction of spawning was carried out. Individual animals were transferred into 250ml glass beakers containing clean 10μm filtered seawater, the temperature of which had been adjusted to 18°C (±2°C) to induce thermal shock of the animals as described by Harrison and Jones (1982). The water temperature was not allowed to exceed 20°C as directed in the ASTM guidelines (1997a). On occasion that following a period of an hour, no evidence of spawning was observed, the mussels were removed from the water, their valves carefully prised open with the point of a pair of scissors and 0.5ml of 0.5M potassium chloride solution (KCl dissolved in distilled water) injected into the posterior adductor muscle, using a 1ml syringe fitted with a 0.8mm x 40mm hypodermic needle. They were then replaced into the beakers which had the contents replenished with new seawater held at 19°C. The sexes of the individuals were easily distinguished by the form and colour of their gametes; females produced a mass of orange-pink coloured eggs and males produced a thin steady stream of white sperm. In general, the males tended to spawn more readily than females following thermal shock treatment.

After filtration through a sterile 100μm nylon sieve to remove tissue debris and faeces, the gametes were checked for quality (the eggs for roundness and normal morphology, the sperm for motility and viability) and quantity. Gametes which were of poor quality were discarded and only gametes showing good quality were used for experiments.
In order to achieve optimum fertilisation, the eggs were pooled and suspended in clean seawater at a density of approximately 20 eggs per ml seawater. To fertilise the eggs, the sperm suspension from one or more viable males was added to the egg suspension in the ratio of approximately 1:1000 (egg:sperm), as described by Sprung and Bayne (1984). The gametes were mixed with a perforated nylon plunger and incubated at 15°C (± 1°C) for approximately one hour, before they were checked for fertilisation success. This was verified by microscopic examination, with the eggs being examined for the presence of polar bodies or the first stages of cell cleavage (plate 2.6). Once the maximum rate of fertilisation was achieved, the suspension was sieved through a 30μm nylon sieve and excess sperm gently rinsed away with clean seawater to prevent polyspermy. Only samples where the fertilisation rate exceeded 90% were used for the experiments included in this thesis. The embryos were then re-suspended in clean seawater at a maximum concentration of 30 embryos per ml seawater, and incubated at 15°C (± 1°C) (ASTM, 1997).

Following an incubation period of 12 hours, the embryos were transferred to sterile exposure vessels, maintaining a maximum concentration of 30 embryos per ml seawater before the start of the exposure of the samples to test agents.

2.2.2 *Platynereis dumerilii*

Cultures of *Platynereis dumerilii* were maintained in laboratory conditions as described in section 2.1.3.1. Sexually mature adults were identified following emergence from their tubes and swimming near the surface of the water. Males could be distinguished from females by the colour of their anterior end, males being white in colour due to the white, milky appearance of the sperm. In contrast, the anterior end of females was of a yellow-green colouration, since this was the colour of their eggs. Plate 2.3 illustrates the difference between the sexually mature polychaetes.
Plate 2.6 (a) Unfertilised *Mytilus edulis* eggs; (b) Fertilised *Mytilus edulis* eggs with polar bodies (pb) or cell cleavage (cc) indicated with arrows.
Males and females were placed together in glass evaporating dishes containing approximately 150 ml of 10 μm carbon-filtered seawater held at room temperature (20±1°C), and were allowed to spawn. The females would release their gametes spontaneously on detection of the hormones released from the males, and would swim vigorously as the gametes were released to maximise the mixing of the gametes and ensure optimum fertilisation. Approximately one hour following gamete release, the gamete suspension was passed through a 100 μm nylon sieve to remove the adults and faeces, and carefully rinsed with filtered seawater. The eggs were then checked for fertilisation success by taking samples of known volume and examined for signs of cell cleavage. Only samples where the fertilisation rate was of at least 95% were used in experiments. The embryos were then re-suspended in clean seawater at a maximum concentration of 30 embryos per ml seawater, and incubated at 20°C.

Following an incubation period of 12 hours, the embryos were transferred to sterile exposure vessels, maintaining a maximum concentration of 30 embryos per ml, before the start of the exposure to test agents.

2.3 Collection of haemolymph from adult animals

2.3.1 *Mytilus edulis*

Haemolymph samples were collected from adult *M. edulis* by carefully prising open the valves using the point of a pair of scissors. The excess seawater held within the valves was allowed to drain out, and approximately 0.2 ml haemolymph was drawn from the posterior adductor muscle (plate 2.7) into a 1 ml syringe which contained 0.2 ml physiological saline (20 mM Hepes, 10 mM calcium chloride, 10 mM potassium chloride, 436 mM sodium chloride, 53 mM magnesium sulphate, adjusted to pH 7.36) from the recipe of Peek & Gabbot (1989). The cell density in the 400 μl haemolymph-physiological saline suspension ranged from approximately 15000-25000 cells. The samples were then transferred to siliconised 2.0 ml microcentrifuge tubes, and held on ice until ready for
Plate 2.7. Haemolymph extraction from the posterior adductor muscle of *Mytilus edulis*. The hypodermic syringe contains physiological saline.

Plate 2.8. The internal view of an adult *M. edulis*, showing the position of the posterior adductor muscle (pam), anterior adductor muscle (aam), muscular foot (mf), gill (g). The haemolymph samples were collected from the pam.
Plate 2.9. The internal view of an adult *C. edule*, showing the position of the posterior adductor muscle (pam), anterior adductor muscle (aam), and muscular foot (mf). The haemolymph samples were collected from the pam.
assaying. Plate 2.8 illustrates the position of the posterior adductor muscle and other organs.

2.3.2 *Cerastoderma edule*

Haemolymph samples were collected from *C. edule* in a similar manner to *Mytilus edulis* as described in section 2.2.3.1. Briefly, 0.2 ml of haemolymph was extracted into 0.2 ml physiological saline. The composition of the physiological saline was the same as that used for maintaining the haemocytes from *M. edulis*. The density of the haemocytes in 400 µl was lower than that of *M. edulis*, ranging from approximately 10000-15000 cells. Plate 2.9 shows the position of the posterior adductor muscle in this species.

2.3.3 *Platichthys flesus*

Haemolymph samples were collected from *P. flesus* by staff at CEFAS laboratories holding a Home Office licence entitling them to do so. Approximately 1ml of haemolymph was extracted from the caudal artery using 5 ml hepinarised syringes fitted with a hypodermic needle. The samples were then transferred into siliconised 2.0 ml microcentrifuge tubes, and held on ice until ready for assaying. For the micronucleus assay, the haemolymph samples were used undiluted for the preparation of blood smears. For the comet assay, the haemolymph samples were diluted 1:1000 (by volume) with buffered salt solution (23.5 g l⁻¹ sodium chloride, 0.7 g l⁻¹ potassium chloride, 0.1 g l⁻¹ potassium phosphate, 0.2 g l⁻¹ sodium carbonate, 1.1 g l⁻¹ calcium chloride, adjusted to pH 7.5) as per the recipe by Nacci *et al.* (1996) in 2.0 ml siliconised microcentrifuge tubes.

2.4 UV Exposures

2.4.1 Sources of UVR

The simulation of solar radiation was provided by a combination of 8 x Osram cool white light lamps, 2 x Q-panel 340 lamps, and 2 x Q-panel 313 lamps. Each lamp had been fitted in an incubation cabinet (Fisons), arranged parallel to one another and pre-
Plate 2.10. The Macam spectroradiometer measuring the emission of the lamps fitted in the Fisons incubation cabinet.
burned for at least 60 minutes before the commencement of any exposures. This ensured the output of the lamps had stabilised before the biological samples were exposed. Plate 2.10 illustrates the external view of the exposure cabinet and the spectroradiometer used to carry out UV measurements within the cabinet.

To ensure that a relatively even distribution of radiation was achieved, the lamps were installed and arranged in a regular spatial sequence. To identify areas of uneven radiation, measurements of UVR were made prior to the commencement of experiments within all areas of the cabinet at points approximately 15 cm apart. The exposure shelf was raised and lowered to ascertain the optimum shelf position, which would reduce small-scale localised hot-spots of elevated UVR due to the distribution of the lamps and yet provide an environmentally realistic level of irradiation. Any areas of uneven UV exposure (identified to be towards the extreme sides, back and front of the cabinet) were avoided during the experiments.

Measurements of the UV-A and UV-B levels within the exposure areas were routinely carried out, and to further ensure even exposure of the samples, the exposure vessels were moved around at regular intervals throughout the duration of experiments wherever possible. Within the closed exposure cabinet, a constant temperature was maintained throughout the experiments, and the cabinet was fitted with a sheet of Optiwhite float-glass (4mm thick, Pilkington special glass). This has 0% transmission below 280nm and therefore removed any wavelengths within the UV-C range.

2.4.2 UV measurements & dosage calculations

Measurements of UV were originally made with UV-A (MP-236) and UV-B (MP-229) broad-band cosine sensors (Micropulse Technology Ltd.). These had been calibrated against a double monochromator spectroradiometer (model SR 9910, Macam Photometrics Ltd., UK). Correlations between measurements made with the broad-band cosine sensors and the spectroradiometer are presented in figures 2.3a and 2.3b. The
relationship between the two methods of detection was not of a ratio of 1:1 indicating that
the broad-band sensors were detecting wavelengths of light outside of their range of
sensitivity. During later experiments, all measurements of UV-A and UV-B were carried
out with a Macam spectroradiometer (model SR9910-V7, Macam Photometrics Ltd., UK)
which was subsequently acquired by the University. This instrument provided a
significantly increased sensitivity for the measurement of irradiances, and wavelengths
within specific ranges could be measured more accurately. Plate 2.10 illustrates the set up
of the Macam spectroradiometer in relation to the exposure cabinet which was fitted with
Q-313, Q-340 and white lamps. The instrument was calibrated against a deuterium and
tungsten halogen lamp, which in turn had been calibrated by the National Physics
Laboratory. The calibration was performed by an expert operator from Macam
Photometries. The spectrum measured by the spectroradiometer, of the emission of the
lamps in the exposure cabinet are presented in figure 2.4a, with the emission of sunlight is
presented in figure 2.4b for comparison.

Measurements were routinely carried out at the position of each exposure vessel
and the total dose calculated by summation of the mean irradiance (Wm⁻²) and the period
of exposure (seconds). The total dose was then presented as a dose in kilojoules per metre
squared (kJm⁻²).

2.4.3 Biological weighting and calculation of ozone depletion

The action spectrum used in these studies was the Setlow DNA weighting function,
since this particular biological weighting function is related specifically to DNA damage
(Setlow, 1974; Regan AYoshida, 1995; Horneck, 1995).

The Setlow DNA-weighted values for UV-B irradiance present in the exposure
cabinet was measured at the start of each experiment with the Macam spectroradiometer
and was converted from total UVR as part of the Björg and Murphy computer software.
For the calculation of the relative ozone depletion according to the dose of the UV-B
Fig. 2.3a. Correlation between UV-A (316-400 nm, MP-236) broad-band cosine sensor and MACAM double monochromator spectroradiometer.

Fig. 2.3b. Correlation between UV-B (280-315 nm, MP-229) broad-band cosine sensor and MACAM double monochromator spectroradiometer.
Figure 2.4a Spectrum of irradiance emitted by Q-Panel 313, 340 and Cool lamps fitted in the exposure cabinet.

Figure 2.4b Spectrum of irradiance emitted by sunlight.
radiation, the long-term monthly mean ozone levels for Camborne, Cornwall, UK, (http://www.meto.gov.uk/sec5/OZONE/ozsttc.html, 1998) were used and incorporated into the Björg and Murphy UVB programme (Björg and Murphy, 1985).

2.4.4 Exposure of Biological Material

2.4.4.1 Early Life Stages

Embryo-larval stages were placed into 500 ml glass evaporating dishes filled with 100 ml filtered (10 μm carbon filter) seawater held at the appropriate temperature (15°C for *M.edulis*, 20°C for *P.dumerili*), at a concentration not exceeding 30 embryos per ml seawater. Each dish was covered with a circle of filter material: Mylar D, which excludes light of wavelengths within the UV-B range for control samples; and cellulose diacetate which allows UV-B light through but excludes light of wavelengths within the UV-C range for exposed samples. Each circle of filter film was held in place on the dishes with small pieces of Blu Tack® (Bostik, UK). The cellulose diacetate film was used as a means for preventing evaporation from the dishes at the same rate as those in the control samples, rather than a filter of UV-C since the Optiwhite glass fitted in the exposure cabinet had already adequately removed radiation from the UV-C range. Vessels containing samples which were treated as “dark controls” were covered with aluminium foil to totally exclude light. The dishes were then placed within the exposure cabinet at specific positions. Each dish was rotated to an adjacent position at regular intervals during the exposure period to ensure a comparable dose between samples. The animals were not fed for the duration of the experiments.

2.4.4.2 Adult life stages

Adult *M.edulis* were exposed in 2 litre glass beakers, filled with 1.5 litres of filtered (10μm carbon filter) seawater held at 15° ± 2°C), with a maximum of 3 animals per beaker. Air was supplied via plastic airlines, to the ends of which were attached glass
pasteur pipettes. Only the glass pipettes were submerged into the seawater to ensure that any chemical agents did not adsorb onto the surfaces of the plastic airlines, also to prevent the leaching of thallates from the plastic airlines into the dilution water. Each beaker was covered with filter material, which was held in place with sticky tape. Vessels containing animals which were to be treated in the absence of light, were covered with aluminium foil to exclude light. The animals were not fed during the experiments.

Adult *C. edule* were exposed *in situ* in the field, presumably from their early life stages when they settled in the areas from which they were collected. Following collection from their sites, they were sampled for haemolymph immediately on return to the laboratory.

Adult *P. flesus* were exposed in 200 litre glass-fibre tanks, filled with filtered seawater held at 12 ±2°C, with 15 fish per tank. Seawater was supplied to the tanks at 2.5 litres per minute, providing a 90% partial water replacement time of approximately 4 hours. The fish were administered PAHs via their food, which was a diet of nutra marine 4 mm pellets dosed with a mix of four PAHs.

### 2.5 Statistical analyses

Statistical analysis was carried out using the statistical package Statgraphics Plus, Version 4.0. Some graphical data was constructed using Microsoft Excel XP, box and whisker plots were constructed using Statgraphics Plus 4.0. Where sufficient data points for each treatment were available and the data was normally distributed, one-way analysis of variance (ANOVA) was carried out to compare the means between different populations. The method used to discriminate among the means was the Fisher’s least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0 (i.e. 95.0% confidence level). In such cases where the data were non-parametric and could not be normalised by transformation, the non-parametric Kruskall-Wallis test was used to
compare the medians between populations. P-values have been included where appropriate to provide an indication of the level of significance between treatments. For the statistical analysis of data produced from the analysis of chromosomal aberrations, the z-test was used to assess the level of significance from one treatment compared with the control.

Where dose-dependent relationships were observed, linear regression analyses were carried out using the least squares approach and $R^2$ values have been presented where significant correlations have been calculated.
Chapter 3

Validation Studies
3.1 Introduction

Before employing biomarkers as tools for evaluating the genotoxic potential of physical or chemical agents, it is necessary to evaluate the robustness and sensitivity of the assays being used. In the following studies, compounds known to be genotoxic agents were selected for exposures of the target organisms, acting as positive controls to validate the endpoints in the specific species used. All of the chemicals were supplied by Sigma-Aldrich, Poole, UK, unless otherwise stated. For cytogenetic studies, the direct-acting mutagen methyl methane sulfonate (MMS) was used for comparison of the response with other species examined by previous authors. For the comet assay studies, hydrogen peroxide (H$_2$O$_2$) was used as the positive control compound. This is thought to cause base modifications directly, particularly following the Fenton reaction to form the highly reactive hydroxyl radical (·OH) (Halliwell & Aruma, 1991).

For the cytogenetic studies involving sister chromatid differential (SCD) staining, it was first necessary to determine the rate at which cells proliferated, the average generation time (Ivett & Tice, 1982). This would allow the design of suitable protocols for the determination of cytogenetic endpoints such as the detection of chromosomal aberrations (CAbs) and sister chromatid exchanges (SCEs). The latter requires the incorporation of the thymidine analogue, 5-Bromo-2-deoxyuridine (BrdU) into the cells. It is necessary for the cells to pass through at least two cell cycles in the presence of this halogenated pyrimidine to allow preferential incorporation into the DNA in place of thymidine and therefore make provision for the differential staining of the sister chromatids. This method would then allow the determination of the frequency of the induction of SCEs and also of the proliferation rate index (PRI). Furthermore, the timing of one cell cycle is required for the determination of the frequency of CAbs, which are measured in cells which have passed through one cell cycle only. Work carried out by Jha et al. (1996) had already determined the cell cycle kinetics of the embryo-larval stages of the polychaete worm, *Platynereis dumerilii*, on the other hand the cell cycle kinetics of the
embryo-larval stages of *Mytilus edulis* had not been previously clearly documented and therefore needed to be established.

The ‘Comet’ assay was optimised for single cells from marine invertebrates and was used to detect DNA strand breaks in studies included in this thesis. During the early stages of the application of this assay, the level of DNA damage was determined by visual classification of the cellular damage, as described in plate 1.8 in section 1.4.1.1. Later, a computer software package was obtained, which allowed the automated measurement of the ‘comets’ by image analysis. It was therefore necessary to evaluate the correlation, if any, between the visual class system and the automated image analytical system. To this end, an *in vitro* study was carried out to validate and correlate the two methods.

The use of *Cerastoderma edule* in comet assay studies had not been documented before, therefore it was necessary to determine the sensitivity and robustness of haemocytes from this species, for use with the comet assay, before applying its use as a test organism in field studies. An *in vitro* study was undertaken to determine the usefulness of the blood cells extracted from *C.edule* whereby the cells were exposed to hydrogen peroxide, a known DNA damaging agent.

This chapter outlines some of the validation studies carried out during the period of research for this thesis. Wherever necessary, assays used have been verified, in advance of the studies carried out for the main thesis.

**3.2 *Mytilus edulis*: Calculation of Average Generation Time (AGT)**

**3.2.1 Aims & Objectives**

The average generation time (AGT) of the embryo-larval cells of *Mytilus edulis* was not known and it was necessary for this to be calculated before undertaking cytogenetical studies. Temperature is known to have an influence upon the rate of cell division and thus rates of embryonic development (Hoegh-Guldberg & Pearse, 1995). The aim of this study therefore was to determine the proliferation rate index (PRI) and the
average generation time (AGT) in the embryo-larval stages of *Mytilus edulis* at two different temperatures, 15 and 20°C.

### 3.2.2 Materials & Methods

#### 3.2.2.1 Exposure of embryo-larvae to BrdU

*Mytilus edulis* embryo-larvae were collected by the stimulated spawning of adult animals, under controlled laboratory conditions, as described in section 2.2.1. In this study, three females and one male spawned. The morphology of the oocytes and activity of the spermatocytes were examined and on finding each respective gamete type to be of normal morphology and high motility, the gametes were mixed. An hour following the mixing of the gametes, the suspension was condensed by filtering through a nylon mesh (mesh size of 30 μm), the excess sperm rinsed away by gently rinsing with filtered seawater before resuspension in 5 litres of clean filtered seawater. The fertilisation rate was then determined by microscopic examination, fertilised eggs being identified as those with evidence of polar bodies or cell cleavage as described in section 2.2.1. The fertilisation rate in this study was 97%, with a total of 38,800 embryos being produced.

5 x 1 litre beakers were filled with 500 ml filtered seawater (10 μm carbon-filtered) and incubated at 15°C. A further 5 x 1 litre beakers were filled with 500 ml filtered seawater and incubated at 20°C (Sanyo Gallenkamp incubators) during the period that the gametes were allowed to fertilise. Once the fertilisation rate had been calculated and found to be at least 95%, 500 ml of the embryo suspension was carefully poured into each of the 1 litre glass beakers to produce a density of approximately 10 embryos per ml of seawater, which is well below that of the maximum density recommended by the ASTM (1997b). The embryos were incubated in separate incubators (Sanyo Gallenkamp) at the respective temperatures for 12 hours before the first treatments were exposed to BrdU.
A stock solution of BrdU was made up with filtered seawater at a concentration of 1.0x10^-5 M and stored in a 500 ml amber glass bottle. This in turn was covered in aluminium foil to keep the solution in the dark since BrdU is photosensitive. The embryo-larvae were exposed to a working solution of 1.0 x 10^-5 M BrdU for periods of 4, 6, 8 and 12 hours at 15°C or 20°C before fixing. To synchronise the time at which the samples were fixed (when the embryo-larvae were 24 hours in age), the times of addition of BrdU were staggered. Figure 3.1 illustrates the outline of the protocol and the times at which the embryo-larvae were transferred to seawater containing 1.0x10^-5 M (working concentration) of BrdU.

3.2.2.2 Fixing of embryo-larvae and preparation of metaphases

The methods of slide preparation, chromosome staining and chromosome analysis were adopted from earlier studies by Harrison & Jones (1982) and subsequently modified by other workers such as Jha et al. (1995, 1996, 2000). When the embryo-larvae were 24 hours old and had received 1.0 x10-5 M BrdU exposure for 4, 6, 8 or 12 hours, they were treated with 0.025% colchicine solution (w/v) dissolved in seawater, for 30 minutes. Following this, the embryo-larvae were exposed to a series of hypotonic solutions (seawater : 0.56% potassium chloride solution) mixed in the following ratios:
(a) 2:1    (b) 1:1    (c) 1:2    (d) 1:3

The samples were exposed to each hypotonic solution for 10 minutes in sequential order starting from (a) passing through each hypotonic solution through to (d).

The embryo-larvae were then transferred to glass centrifuge tubes using disposable plastic pasteur pipettes and then centrifuged for 5 minutes at 1000 rpm, to form a pellet. The supernatant was carefully removed without disturbing the pellet and replaced drop by drop with cold (4°C) Carnoy’s fixative (methanol and glacial acetic acid, 3:1 ratio). This fixative was removed and replaced with fresh fixative a further two times before preparation of the metaphase spreads.
Figure 3.1. Outline of the timings for each stage in the exposure of embryo-larval material to BrdU.
To prepare the metaphase spreads, embryo-larvae were resuspended in the Carnoy's fixative by gentle pipetting and a few drops of the suspension transferred onto labelled glass microscope slides using glass pasteur pipettes. A few drops of 60% (v/v) glacial acetic acid (diluted with distilled water) were then added, whilst simultaneously rotating the slide to ensure even spreading of the chromosomes as the acetic acid burst open the cells. The slides were then placed onto a hot plate maintained at 40°C and intermittently rotated until all of the acetic acid had evaporated and the slide was left dry. There were sufficient embryo-larvae from each treatment (period of exposure to BrdU and temperature) to produce 10 slides from each, therefore providing 10 replicates. The spreading of the chromosomes was checked with a phase-contrast light microscope (Leica, DMR) at a magnification of x400.

3.2.2.3 Fluorescence plus Giemsa (FPG) differential staining of the chromosomes

The sister chromatid differential (SCD) staining of the chromosomes was carried out following a procedure developed by Goto et al. (1975) with some modifications introduced by Jha et al. (1996).

A 0.025% (w/v) of Hoechst 33258 solution was prepared in phosphate-buffered saline (PBS) (1 tablet dissolved in 200 ml distilled water) in an amber glass 250ml bottle and stored in a dark cupboard until ready for use. The slides with the chromosome spreads were arranged in glass coplin jars and rinsed twice with distilled water to remove any dust or artefacts which may have attached to the surface of the slides. The distilled water was drained away, replaced with 0.025% Hoechst solution and the slides incubated in the dark for 20 minutes at room temperature (20°C). The slides were then removed from the Hoechst solution and rinsed twice with distilled water and allowed to air dry. A few drops of PBS were added to each slide and coverslips applied. The slides were then placed onto a hot plate maintained at 55°C and simultaneously exposed to blacklight (F18W-BLB lights, Sylvania, UK) for 25 minutes. The slides were rinsed a further two times allowing
the coverslips to slide off in the process and air dried. The slides were then stained with 10% Giemsa (v/v) diluted with Giemsa buffer solution, for 15 minutes. The slides were then rinsed with distilled water twice, allowed to air dry and then coverslips were mounted using DPX mountant.

3.2.2.4 Analysis of metaphase spreads

Metaphase spreads were examined using a bright field microscope (Olympus, Polvar), at a magnification of x1000 (with oil immersion). In order to determine the cell cycle kinetics, at least 100 cells per replicate were examined and classified as first (M1), second (M2) or third or subsequent (M3+) division cells. The cells were classed by taking note of the pattern of staining for the metaphase cell being examined. Cells in M1 consisted of chromosomes which were stained dark throughout the spread, with the staining of the sister chromatids of each chromosome being of equal depth of staining. Cells in M2 consisted of chromosomes which were differentially stained and of a "harlequin" appearance. Cells in M3 consisted of a mixture of chromosomes which were differentially stained and chromosomes which comprised of sister chromatids which were both lightly stained. The PRI was calculated using the following equation:

\[
PRI = \frac{(1 \times M1) + (2 \times M2) + (3 \times M3)}{\text{number of cells scored}}
\]

This is based on the method proposed by Lamberti et al. (1983). Once the PRI had been calculated, the generation time (GT) could be calculated using the following equation, as proposed by Ivett & Tice (1982):

\[
GT = \frac{\text{hours in BrdU}}{PRI}
\]

3.2.3 Results

Plate 3.1 illustrates the appearance of the metaphase cells and the different staining patterns observed in the different cell cycles described in section 3.1.2.4. Figure 3.2
presents the effect that temperature had upon the PRI of the embryo-larval cells. As expected, there was an increase in the rate at which the cells divided in the treatments where the samples were allowed to grow at 20°C compared to those incubated at 15°C, with the exception of those incorporated with BrdU for only 4 hours. Figure 3.3 presents the generation time (GT) which was calculated from the PRI at each time period that the samples were incubated with BrdU, the trend is reversed, with the GT being lower at the higher temperature 20°C, compared with those at 15°C (with the exception of those incorporated with BrdU for only 4 hours).

From the GT calculated for each treatment, the average generation time (AGT) could be calculated from the 4 treatments at each temperature regime. The AGT for the embryo-larval cells grown at 15°C was therefore 3.82 hours and 3.41 hours for those grown at 20°C. These data are summarised in table 3.1.

The ASTM (1997b) recommends that the temperature for *M. edulis* does not exceed 19°C. Therefore, embryo-larvae were incubated at 15°C for all studies following from here on. From the information collated from this study, one cell cycle was assumed to be approximately 4 hours. Therefore in studies involving the detection of chromosome aberrations, cells were harvested following $\frac{1}{2}$ cell cycles following BrdU incorporation (6 hours); and in studies where sister chromatid exchanges were examined, cells were harvested following 2 cell cycles (8 hours) following BrdU incorporation, therefore allowing a maximum crop of cells arrested during the second cell cycle.
Plate 3.1 Metaphase spreads from cells of embryo-larval *M. edulis* in (a) M1, (b) M2 and (c) M3. Photographs taken at x1000 magnification (oil immersion).

Table 3.1 Summary of data obtained for the analysis of PRI, GT and AGT of *M. edulis* embryo-larval cells.

<table>
<thead>
<tr>
<th>Temperature (°)</th>
<th>Period in BrdU (hours)</th>
<th>PRI</th>
<th>GT (hours)</th>
<th>AGT (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>4</td>
<td>1.48</td>
<td>2.70</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>6</td>
<td>2.15</td>
<td>2.79</td>
<td>3.82</td>
</tr>
<tr>
<td>15</td>
<td>8</td>
<td>2.16</td>
<td>3.70</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>12</td>
<td>1.97</td>
<td>6.09</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>1.19</td>
<td>3.36</td>
<td>3.41</td>
</tr>
<tr>
<td>20</td>
<td>6</td>
<td>2.31</td>
<td>2.60</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>8</td>
<td>2.38</td>
<td>3.36</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>2.77</td>
<td>4.33</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.2 The effect of temperature upon the proliferation rate index of *M. edulis* embryo-larval cells (calculated from 100 cells per treatment).

Figure 3.3 The effect of temperature upon the generation time of *M. edulis* embryo-larval cells (calculated from 100 cells per treatment).
3.3 *Mytilus edulis*: Validation of cytogenetic and developmental endpoints by exposure to Methyl Methane Sulfonate.

3.3.1 Aims & Objectives

Following the determination of the cell cycle kinetics of the embryo-larval cells of *M. edulis*, it was necessary to validate the sensitivity of these target cells and test their amenability to cytogenetic assays. The direct-acting mutagen methyl methane sulfonate (MMS) was used at a range of doses (1.0x10^{-7}M to 1.0x10^{-3}M) and exposed to the target organisms. Cytogenetic observations were based upon the analysis of chromosomal aberrations (CAbs) and sister chromatid exchanges (SCEs). The assessment of cytotoxicity was based upon the determination of the proliferation rate index (PRI).

3.3.2 Materials & Methods

3.3.2.1 Exposure of embryo-larvae to methyl methane sulfonate

Methyl methane sulfonate (MMS) was selected as a representative direct-acting mutagen. Previous work carried out by Jha *et al.* (1996) had also used this chemical for validating the responses of the embryo-larval stages of the polychaete worm *Platynereis dumerilii*. The concentrations used in this study were based upon a similar range to that used by Jha and his co-workers, that being 1.0x10^{-3}, 1.0x10^{-4}, 1.0x10^{-5}, 1.0x10^{-6} and 1.0x10^{-7}M MMS. Since MMS was observed to be readily miscible with seawater, exposure vessels (1 litre glass beakers) were prepared, producing a final volume in each vessel of 500 ml of the test solutions at the relevant concentrations. These were prepared by adding liquid MMS directly to 600 ml seawater to produce a 1.0x10^{-3}M working concentration of MMS in seawater. Once this had been mixed thoroughly using a glass stirring rod, a 10 fold dilution was prepared by transferring 60 ml of this 1.0x10^{-3}M MMS solution into a second vessel and adding 540 ml seawater. Once again this was mixed thoroughly before transferring a volume of 60 ml seawater from the 1.0x10^{-4}M MMS solution into a third vessel and adding a 540 ml volume of seawater. This was continued until each of the five different concentrations of MMS were produced. Then from each
vessel, the relevant volume was then removed and disposed of to produce a final volume of 500 ml in each vessel.

*M. edulis* embryo-larvae were collected by the controlled spawning of adult animals, as described in section 2.1 and the treatment of the gametes and subsequent embryos were carried out in the same manner as that of the experiment described in section 3.1.2.1. When the embryos were 12 hours old, BrdU was added to the exposure vessels to produce a working concentration of $1.0 \times 10^{-5} \text{M}$ BrdU solution and embryos were transferred to the exposure beakers containing 500 ml of test solution with BrdU at a density of 15 embryos per ml. The embryo-larvae were exposed to the MMS and BrdU for approximately one and a half cell cycles (6 hours) and approximately two cell cycles (8 hours), for the detection of CAbs and SCEs respectively. The samples were incubated in the dark to prevent photolysis of the chemicals and maintained at $15 \pm 1^\circ \text{C}$ for the growing embryo-larvae as recommended by the ASTM standards (1997a).

At the end of the exposure periods (6 and 8 hours) the embryo-larvae were treated with 0.025% colchicine solution and hypotonic solutions and metaphase spreads were prepared as previously described in section 3.1.2.2. At the end of the exposure period, sub-samples of the embryo-larvae were transferred to clean seawater and allowed to grow until 48 hours (at $15 \pm 1^\circ \text{C}$). They were then analysed for survival and development effects. The embryos which were either dead or had not reached the typical prodissococonch (D-shell) structure at this time (48 hours) were considered to be abnormal, as per the criteria recommended by the ASTM (1997b); His & Beiras (1995); and His et al. (1997).

3.3.2.2 Staining of metaphase spreads

Slides prepared for the examination of CAbs in which the embryo-larvae had been allowed to grow for one and a half cell cycles (6 hours) post-12 hours of age, were stained with 10% (v/v) Giemsa solution diluted with Giemsa buffer solution, for 15 minutes. The slides were then rinsed twice with distilled water, allowed to air dry and coverslips were
mounted with DPX mountant. These slides were labelled with the letter “G” to indicate that they had been stained with Giemsa only.

Slides which were prepared from embryo-larvae which had passed through approximately 2 cell cycles (8 hours) were differentially stained using the FPG method described in section 3.2.2.3. These slides were labelled with the letters “FPG” to indicate that they had received fluorescence plus Giemsa staining.

3.3.2.3 Analysis of metaphase spreads

Metaphase spreads were examined using a bright field microscope, at a total magnification of x1000 (with oil immersion). Slides labelled with “G” were examined for CAbs, where at least 100 cells per treatment were scored and data collected and presented as the percentage of cells containing CAbs and the total number of CAbs. Chromosome and chromatid gaps were not considered to be true aberrations and were recorded but not included in the collation of the data.

In order to determine the PRI, 100 cells were classified into first (M1), second (M2) and third or subsequent (M3+) cell divisions and the PRI calculated as described in section 3.2.2.4. For the examination of SCEs, at least 30 cells in their second cell division were scored for the frequency of SCEs. The mean number of SCEs per cell was then calculated.

3.3.3 Results

Figure 3.5 presents the effects of MMS on the percentage of aberrant cells and total aberrations. There was a dose-related response with an increasing percentage of aberrant cells ($R^2 = 0.8949\%$) as well as with the total frequency of aberrations ($R^2 = 0.9125\%$). This indicates that MMS has a significant dose-related genotoxic effect upon the embryo-larval cells of *M. edulis*. 
Figure 3.4 The effect of MMS on the induction of chromosomal aberrations in *M. edulis* embryo-larvae (measured in 100 cells per treatment).

Figure 3.5 The effect of MMS on the induction of sister chromatid exchanges (>30 cells per treatment) and proliferation rate index (100 cells per treatment) in *M. edulis* embryo-larvae.
Table 3.2 Range of SCE frequency in M2 metaphase cells in *M.edulis* embryo-larval cells exposed of MMS.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>RANGE OF SCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW control</td>
<td>1-9</td>
</tr>
<tr>
<td>1.0 x 10^{-7}M</td>
<td>1-6</td>
</tr>
<tr>
<td>1.0 x 10^{-5}M</td>
<td>1-8</td>
</tr>
<tr>
<td>1.0 x 10^{-5}M</td>
<td>1-6</td>
</tr>
<tr>
<td>1.0 x 10^{-4}M</td>
<td>1-6</td>
</tr>
<tr>
<td>1.0 x 10^{-3}M</td>
<td>3-31</td>
</tr>
</tbody>
</table>

Figure 3.6 The effect of MMS upon the normal development of early life stages of *M.edulis* based upon external morphology.

\[ R^2 = 0.9215 \]
Figure 3.6 presents the PRI and the rate of induction of SCEs in the embryo-larvae. There was a dose-dependent decrease in the PRI ($R^2 = 0.77\%$), indicating that MMS has a cytotoxic effect, slowing the cell cycle kinetics and therefore increasing the period taken to pass through each cell cycle. There was also a slight increase in the induction of SCEs with increasing dose ($R^2 = 0.51\%$), with particularly high mean frequencies of SCEs in the highest concentration of MMS ($1.0 \times 10^{-3} \text{M}$). The range of SCE frequency in each treatment has been summarised in table 3.2.

3.4 *Mytilus edulis*: Validation of the Classification method for the Comet assay – Comparison of this method vs. the Image analysis automated method (Kinetic Imaging).

3.4.1 Aims and Objectives

The aim of this study was to identify a correlation between the visual classification of the levels of DNA damage of the Comet assay and automated measurements of damage using the image analysis software Komet 5.0 (Kinetic Imaging) for validation of each method. Correlation of these techniques would allow the use of either method for measuring DNA damage in cells treated with the Comet assay, with the confidence that each method was as reliable and robust as the other. Hydrogen peroxide ($\text{H}_2\text{O}_2$) was selected as a DNA damaging compound, since it has been reported to be the most prevalent mutagen to which human DNA is exposed (Green et al., 1996). Furthermore,
this agent is known to efficiently induce DNA strand breaks and has been used extensively as a positive control in the comet assay (Nacci et al., 1996; Henderson et al., 1998; Mitchelmore et al., 1998).

3.4.2 Materials & Methods

3.4.2.1 Sampling of haemolymph

*Mytilus edulis* adults collected from Sharrow Point, Whitsand Bay, Cornwall (detailed in section 2.1.1.1.) were used for this study. Following an acclimatisation period of 48 hours in aquaria, 16 individuals of similar size (3.5-4.0 cm) were selected. From each animal, 0.2 ml haemolymph was extracted from the posterior adductor muscle into 0.2 ml physiological saline, as described in section 2.3.1. The blood samples were then pooled by transferring the samples from 8 animals into a siliconised (5 ml) centrifuge tube and repeating this for the remaining set of 8 animals (thus providing duplicates). For the duration of the blood extraction process, the haemolymph samples were maintained on ice to minimise cellular stress.

3.4.2.2 In vitro exposure of haemolymph samples

Each of the two pooled haemolymph samples were then vortexed to ensure adequate mixing of the cells and then divided into 8 labelled, siliconised microcentrifuge tubes (for both samples). The samples were then centrifuged at 2.3 rpm (or 0.5 xg) for 2 minutes at room temperature. Following centrifugation, the supernatant was carefully removed from each sample by pipetting and replaced by 300 μl H₂O₂ solution (diluted with physiological saline) of final concentrations 0, 100, 500 and 1000 μM H₂O₂. The samples were incubated for 1 hour on ice, held in the dark by covering the ice container with aluminium foil.
3.4.2.3 Eosin Y cell viability assay

Following the incubation period, 20 μl from each sample was transferred to a microscope slide and the viability of the cells assessed using the Eosin Y assay. This is necessary to determine the viability of the cells before carrying out the Comet assay to ensure that DNA damage being detected was caused by the chemical in question (H₂O₂) rather than due to poor condition of the cells. 1 μl Eosin Y solution (2 mg/ml physiological saline solution) was added to the haemocyte sample on each slide and mixed using the micropipette tip. A coverslip was then applied and the sample examined under a light microscope at a total magnification of x400. The quantity of viable cells, recognised by their green staining and the non-viable cells, recognised by their red staining was recorded. The proportion of viable cells was then calculated as a percentage of the total number of cells examined. The accepted level of viability was 90% for the continuation with the Comet assay, in any cases where the viability of the cells was <90%, the experiment would be aborted before applying the comet assay. In the samples for this experiment, the mean viability of the cells was 92.8% (S.D. 2.5).

3.4.2.4 Comet assay

The formulations for each of the reagents and solutions used for the comet assay are detailed in Appendix I. Reagents were supplied by Sigma-Aldrich, Poole, UK, unless otherwise specified.

During the in vitro incubation period of the cells, microscope slides were prepared for the comet assay by first applying the base layer of agarose. Plain microscope slides were dipped into molten 1.5% normal melting point (NMP) agarose (dissolved in Tris-Acetate-EDTA solution), the lower side of the slides were wiped clean with tissue paper and then placed into an oven pre-heated at 40°C and allowed to dry for a minimum of 30 minutes. Each slide was discreetly labelled on the underside using a diamond pen.
Following the incubation period, the samples were centrifuged again for 2 minutes at 2.3 rpm. The supernatant was discarded and replaced with 200 µl 0.5% low melting point (LMP) agarose (dissolved in Kenny’s salt solution), which was held at 40°C in a water bath, to ensure that the agarose did not heat-damage the cells. The cells were mixed with the agarose by gentle pipetting, then 85 µl of the cell-agarose suspension was applied to the NMP agarose base coat and immediately covered with a 22x22 mm coverslip. This was repeated with a further 85 µl of the same cell-agarose suspension which was applied to a second area on the slide thus preparing a replicate for each sample. The slides were placed onto a metal tray held on ice to set the agarose for approximately 10 minutes, whilst the other slides had the cell-agarose suspension applied. Each slide being placed systematically onto the cold metal tray, following application of the cell-agarose suspension.

1 litre lysis solution was then prepared by adding Triton X-100 and dimethylsulfoxide (DMSO) to produce a final concentration of 1 and 10% respectively. The coverslips were then removed from each slide and discarded and the slides placed horizontally in a staining trough. The working lysis solution was then carefully poured into the staining trough, ensuring that the gels were not disturbed and each slide was completely covered with solution. This was then incubated for 1 hour in the dark at 4°C.

After the one hour lysis period, the slides were carefully removed from the lysis solution, gently rinsed with distilled water and then placed into an electrophoresis chamber (Pharmacia Biotech, model GNA200) containing electrophoresis buffer held at room temperature. The slides were left in this solution for 20 minutes to allow the DNA to unwind. The electrophoresis power supply (Pharmacia Biotech, model EPS 300) was consequently started ensuring the current was set to 15V and amperage was set to 320 mA, by adjusting the volume of the electrophoresis buffer as necessary and allowed to run for 30 minutes. The electrophoresis was carried out with a water re-circulating system to
minimise changes of the temperature during electrophoresis and maintained the temperature within the chamber to within 2°C of room temperature (20°C ± 2°C).

The slides were carefully removed from the electrophoresis chamber, rinsed with distilled water, then immersed in neutralisation buffer for 5 minutes. Following this, excess neutralisation buffer was drained off the slides and 40 μl of ethidium bromide solution applied to each replicate, coverslips applied and slides stored in humid airtight containers in the dark at 4°C for up to 24 hours before analysis.

By moving the microscope stage while simultaneously observing the microscope images on the image processing monitor in ‘live’ mode, a minimum of 50 cells per replicate (100 cells per slide) were selected at a total magnification of x200. These were first visually scored and classed according to their level of DNA damage as described in section 1.4.1.1. and illustrated in figure 1.8 and the data recorded by hand. Simultaneously, the same cells were measured by video capture and image analysis and the data recorded automatically recorded by the computer.

Of the various parameters that are measured automatically by the image analysis program, the most widely used measurements which are described in the literature are:

1. Tail length (Singh et al., 1988; Olive et al., 1990a, 1990b; Hellman et al., 1995),
2. Tail moment (Olive et al., 1990), and
3. Head-to-tail ratio (Östling and Johanson, 1984; Olive, 1989; Müller et al., 1994; Böcker et al., 1997).

Since the head-to-tail ratio measurements have not been well defined by previous authors (Böcker et al., 1997), the tail moment and tail length measurements were selected for comparison with the visual classification method.
Plate 3.2 (a) Viable and (b) Non-viable *M. edulis* haemocytes stained with Eosin Y solution. Viable cells are easily distinguished by their green colouration and non-viable cells by their red colouration. Photographs taken at x400 magnification.

Figure 3.7 The relationship between visual classification of DNA damage (by class) and automated image analysis measurements of (a) tail moment and (b) tail length. (* denotes a statistically significant difference from the control (p<0.0005)).
3.4.3 Results

Plate 3.2 illustrates the appearance of viable and non-viable *M. edulis* haemocytes, stained with Eosin Y. The differentiation in the colour of the staining provided an immediate indication of the viability of the cells.

A comparison was made between the visual classification method and the tail moment and tail length parameters. Since the data was not non-parametric and could not be normalised by transformation, the non-parametric Kruskall-Wallis test was applied to the test the data. Figure 3.7a presents a box-and-whisker plot which shows that with increasing class ranking, there was a statistically significant increase (p<0.0005) in the tail moment measured by image analysis. A similar trend was observed with the measurements of tail length. This indicates that there is a distinct increase in DNA damage estimated by the visual classification method and this method is comparable to the automated image analysis measurements.

3.5 *Cerastoderma edule*: Evaluation of the sensitivity of haemocytes for the Comet assay.

3.5.1 Aims and Objectives

The aim of this study was to evaluate the potential for the Comet assay to be used as a method of detecting genetic damage in the common cockle, *Cerastoderma edule*. Haemocytes were used as the target cell type and were exposed *in vitro* to a series of increasing doses of the oxidising agent, hydrogen peroxide (H₂O₂), ranging from 0 – 5000 μM. The response of the cells in terms of DNA damage was determined with automated measurements using the image analysis software Komet 5.0 (Kinetic Imaging Ltd., UK).
3.5.2 Materials & Methods

3.5.2.1 Sampling of haemolymph

*Cerastoderma edule* adults collected by hand at low tide from Bantham, located on the river Avon, Devon were used for this study. Following an acclimatisation period of 48 hours in aquaria, 10 individuals of similar size (3.5-4.0 cm) were selected. From each animal 0.2 ml haemolymph was extracted from the posterior adductor muscle into 0.2 ml physiological saline, as described in section 2.3.2. The blood samples were then pooled by transferring the samples from the 10 animals into a siliconised (5 ml) centrifuge tube. For the duration of the blood extraction process, the haemolymph samples were maintained on ice to minimise cellular stress and metabolic activity within the cells.

3.5.2.2 *In vitro* exposure of haemolymph samples

The pooled haemolymph sample was then vortexed to ensure adequate mixing of the cells and then 500 µl samples were aliquoted into 10 labelled, siliconised microcentrifuge tubes. The sub-samples were then centrifuged at 2.3 rpm (or 0.5 xg) for 2 minutes at room temperature. Following centrifugation, the supernatant from each sub-sample was carefully removed by pipetting and replaced by 500 µl H₂O₂ solution (diluted with physiological saline) of final concentrations 0, 100, 500, 1000 and 5000 µM H₂O₂. The sub-samples were incubated for 1 hour on ice and maintained in the dark during the incubation period by covering the ice container with aluminium foil.

3.5.2.3 Eosin Y cell viability assay

Following the incubation period, 20 µl from each sample was transferred to a microscope slide and the viability of the cells assessed using the Eosin Y assay. This was carried out in the same manner as that for *M. edulis* haemocytes, described in section 3.4.2.3. In this study the viability of the cells was found to be 92%.
3.5.2.4 Comet assay

As per section 3.4.2.3, the formulations for each of the reagents and solutions used for the comet assay are detailed in Appendix I. Once again, reagents were supplied by Sigma-Aldrich, Poole, UK, unless otherwise specified.

Once the viability of the cells had been checked and confirmed to exceed 90%, the samples were centrifuged, the supernatant discarded, the cells resuspended in LMP agarose and the microgels prepared as per *M. edulis* cells described in section 3.4.2.3. The samples were then electrophoresed for 30 minutes running at a voltage of 15V and a current of 320 mA.

Following electrophoresis, the microgels were rinsed with distilled water, then immersed in neutralisation buffer for 5 minutes. Following neutralisation, excess neutralisation buffer was drained off the slides and each microgel stained by applying 40 µl of ethidium bromide solution, coverslips were then applied and slides stored in humid airtight containers in the dark at 4°C for up to 24 hours before analysis.

A minimum of 50 cells per replicate (100 cells per slide) were randomly selected and measured by video capture and image analysis using the Kinetic Imaging Komet 5.0 software. The cells were examined at a final magnification of x200 using a fluorescent microscope (Leica, DMR) fitted with a rhodamine filter.

### 3.5.3 Results

When the samples were examined, it was noted that there were very few cells in the samples treated to 5000 µM which were amenable to measurement by image analysis. A majority of the cells in these treatments were apoptotic, with the ‘tail’ of the comets disconnected from the ‘head’ and a relatively low proportion of DNA remaining in the head. Due to this factor, the results presented are for treatments ranging from 0-1000µM H₂O₂, with the disproportionate number of cells measured in samples exposed to 5000 µM being omitted.
Figure 3.8 DNA damage effects following in vitro exposure of *C. edule* haemocytes to H$_2$O$_2$ measured with automated image analysis software (a) Tail length; (b) tail moment; and (c) % tail DNA. (* denotes a statistically significant difference from the control, p<0.05).
Since the data is non-parametric and could not be transformed, the non-parametric Kruskall-Wallis test was used to analyse the data. These data are presented in figures 3.8a, b and c as box-and-whisker plots. There was a significant increase (when comparing the medians) between all the samples treated with H$_2$O$_2$ compared with the seawater control (p<0.005) where tail length and tail moment were used as the parameters for determining DNA damage. A dose-dependent increase was detected with (a) tail length ($R^2 = 0.90\%$) and (b) tail moment ($R^2 = 0.97\%$) measurements. However, the relationship between DNA damage measured as % tail DNA and H$_2$O$_2$ concentration was weak ($R^2 = 0.42\%$), with a statistically significant increase in DNA damage observed only in samples exposed at the highest concentration of 1000 µM.

3.6 Discussion & Conclusions

The studies described here and some other previous studies indicate that the assays included in the experiments were sensitive, robust and suitable for detecting genotoxicity. The incorporation of BrdU into the cells, in combination with the FPG technique successfully differentially stained the sister chromatids and allowed the determination of the cell cycle kinetics of *M.edulis*. The average generation time of *M.edulis* was found to be lower than that of *P.dumerilii*. In the study detailed in section 3.1, one cell cycle of *M.edulis* was calculated to be approximately 3.8 hours at 15°C, whereas for *P.dumerilii* it was reported to be approximately 4.5 hours at 20°C (Jha *et al.*, 1996) indicating that the cells of the early embryo-larval stages of *M.edulis* proliferate at a faster rate than those of *P.dumerilii* of a similar age.

Following on from the calculation of the cell cycle kinetics for the embryo-larval cells of *M.edulis* the validation of the various cytogenetical techniques could be applied (described in section 3.2). In this study, MMS was used to evaluate the genotoxic response of *M.edulis* embryo-larval cells and it was proven that *M.edulis* embryo-larval cells were a sensitive and useful cell type for cytogenetic analyses. A dose-related increase was observed in the rate of induction of CAbs and SCEs and there was a dose-related decrease
in the PRI and the proportion of embryo-larvae with normal development. A similar dose-related response was observed in *P. dumerilii* embryo-larvae exposed to comparable doses of MMS (Jha *et al.*, 1996).

The comet assay had been established and used at the University with the visual classification method based upon the categories described by Anderson *et al.* (1994), Collin *et al.* (1997) and Wilson *et al.* (1998) to determine the level of DNA damage. The method was successfully applied to the single cells of marine organisms, adapting the methods from those carried out on cultured human lymphocytes. The study described in section 3.3, where the visual classification method was compared with the automated image analysis methods when taking tail moment or tail length as endpoints into account for the comparison. In contrast to the study by Collins *et al.* (1997) however, here the correlation between visual scoring and image analysis assessed scores of percent DNA in tail was weak. The results where tail moment and tail length were used, indicated that since there was a discreet and statistically significant difference between cells classed at one level of DNA damage with another. This allowed either method to be used, with the confidence that the results could be compared and interchanged.

The study described in section 3.4 involved the adaptation of the comet assay to haemocytes collected from *C. edule*. In this experiment, the cells were found to be sensitive and reproducible as a function of H$_2$O$_2$ concentration for the determination of DNA damage. In a study by Wilson *et al.* (1998), the comet assay was carried out in *M. edulis* gill cells, which were exposed to H$_2$O$_2$. They found that in *in vitro* exposures, there was a dose-dependent increase in DNA damage, comparable with that observed in the current study with *C. edule* haemocytes. A similar trend was reported by Mitchelmore *et al.* (1998b) when *M. edulis* digestive gland cells were exposed to H$_2$O$_2$. The results of this study with *C. edule* haemocytes will be compared with a similar dose-range of H$_2$O$_2$ where haemocytes from *M. edulis* are used, in chapter 9. As well cells from invertebrates,
the comet assay has been applied to cells from fish haemocytes which have been exposed to H₂O₂ (Nacci et al., 1996), which also induced increasing levels of DNA damage.

In conclusion, these studies, along with others carried out previously, have indicated that the cytogenetic endpoints are repeatable and robust, so long as suitable internal negative controls are used during each experiment. The comet assay is a useful method for determining DNA damage, so long as the viability of the cells being tested are of a high enough proportion. Furthermore, the comet assay can be applied to cells other than the haemocytes from *M. edulis* in which the assay was originally optimised. *Cerastoderma edule* appeared to be a suitable alternative species for *in vitro* as well as *in vivo* exposures.
Chapter 4

Genotoxic effects of ultraviolet radiation in the early life stages of marine invertebrates
Hypothesis: Ultraviolet radiation exposure causes genotoxic, cytotoxic and developmental effects detectable in the early life stages of marine invertebrates.

4.1 Introduction

The consequences of exposure to elevated levels of ultraviolet radiation (UVR), in particular UV-B, in the early life stages of a number of aquatic species have been well documented. The literature often focuses upon the early life stages since a large number of species have a pelagic life stage that feed in the upper layers of the water column, where biologically active UV-B is able to penetrate to significant depths and therefore is more environmentally relevant (Lesser et al., 2001). Their vulnerability is influenced by their near surface distribution, relative inactivity, low level of protective integuments or pigments and the fact that the sensitive process of organogenesis and periods of active growth are taking place (Bidigare, 1989; Jeffrey, 1990; Karentz & Lutze, 1990; Berghahn et al., 1993). Furthermore, smaller organisms may have an epidermis of only a few layers resulting in a lower level of epidermal protection compared with larger individuals (Barron & Ka’Aihue, 2001). In bacterioplankton and phytoplankton, there have been reports that the amount of UVB-induced DNA damage correlates with the size of the individual species. These reports suggest that in smaller organisms, the shortened light path reduces the amount of refraction and absorption by cytoplasmic components increasing the susceptibility of smaller organisms to UVR effects (Karentz et al., 1991; Bothwell et al., 1993; Zellmer, 1995; Jeffrey & Mitchell, 1997; McNamara & Hill, 1999).

Since DNA readily absorbs UV-B radiation, much of the biological activity of sunlight is dependent upon the photochemistry of DNA (Mitchell, 1995). The photoproducts of UVR exposure of biological systems are described in greater detail in section 1.2.2. Cellular targets of UVR include nucleic acids, proteins, membrane lipids and UV-A and UV-B are known to inhibit growth and productivity of phytoplankton and bacterioplankton, much of this inhibition being caused by DNA damage. Much of the lethality of UVR is related to the inhibition of DNA and RNA synthesis, with different
types of UVR causing damage in DNA with different cytotoxic potentials due to their ability to block DNA replication. In other words, genetic lesions which block semiconservative DNA replication are lethal, whereas those that allow polymerase bypass are potentially mutagenic and may increase the genetic instability of the target organism (Brash et al., 1987; Jeffrey & Mitchell, 1997). It has been suggested that UV-induced DNA damage is more serious for cultured cells, planktonic gametes, embryos and larvae in which gene expression is more active (Adams & Schick, 1996; Naganuma et al., 1997; Au et al., 2002).

With the depletion of the ozone layer, the predicted increase in daily UV-B irradiance within the surface layers of water, have been found to cause a significant increase in mortality rate in many zooplankton species (Hardy & Gucinski, 1989). Some species are actually attracted to and subsequently killed by higher levels of UV-B (Damkaer & Dey, 1983). Copepods have been studied in depth, with observations on their activity and distribution in the water column, egg hatching success, development, fecundity and survival all showing an altered or detrimental effect following irradiation by UV-B (Karanas et al., 1979, 1981; Bollens & Frost, 1990; Chalker-Scott, 1995; Zagarese et al., 1997; Aarseth & Schram, 1999; Nozais et al., 1999; Browman et al., 2000; Rodriguez et al., 2000; Lacuna & Uye, 2001). Similar responses in feeding activity, motility, fecundity and mortality rate have been observed in amphipods (Ankley et al., 1994), the larvae of other crustaceans including shrimps, crabs and lobsters (Damkaer et al., 1981; Morgan & Christy, 1996; Hovel & Morgan, 1999; Rodriguez et al., 2000a; Wübben, 2000), Antarctic krill (Jarman et al., 1999) and Daphnia species (Hessen, 1994, 1999; Siebeck & Böhm, 1994; Zagarese et al., 1994; Zellmer, 1995; de Lange et al., 1999; Leech & Williamson, 2001) particularly those species which lack or have fewer darkly pigmented chromatophores (Morgan & Christy, 1996; Hurtubise et al., 1998).

The pelagic early life stages of other species such as the coral Agaricia agaricites and sea urchin Sterechinus neumayeri have also been shown to be sensitive to UVR, with
results of cellular damage (measured as the number of cells per embryo), cytogenetic (measured as anaphase aberrations) and developmental (morphological abnormalities) being reported, as well as reduced survivorship following UVR exposure (May, 1974; Anderson et al., 1993; Gleason & Wellington, 1995). Corals also have exhibited sublethal effects following elevated UV-B exposure, including decreases in photosynthetic rate, respiration, calcification and planula release (Shick et al., 1996). Chiang et al. (2003) also found that environmentally realistic levels of UV-B radiation induced ocular damage in barnacle larvae, impairing the phototactic behaviour of naupliar larvae and reducing the settlement success of cypris larvae. Fish larvae have also been shown to be sensitive to UVR and it was found that the radiation wavelength dependence of DNA damage was similar to that of the mortality rates for the crustacean Calanus finmarchicus and of the Atlantic cod Gadus morhua, suggesting that the UV-induced death of these species was caused as a direct result of DNA damage (Browman et al., 2000). The sensitivity of fish to UVR exposure varies with species, however, it is observed that young fish are more sensitive than their adult counterparts (Hunter et al., 1982; Berghahn et al., 1993). Furthermore, the type and level of pigmentation influences the level of damage incurred by UV exposure (Armstrong et al., 2002). The dermal tissues have been found to be the most sensitive tissue in fish, with sunburn being reported in juvenile rainbow trout (Onchorynchus mykiss), and plaice (Pleuronectes platessa) which have rendered the fish susceptible to fungal infection (Siebeck et al., 1994).

It has been reported that, as well as direct effects upon individual organisms, inter-trophic impacts have been measured in aquatic food webs. Van Donk & Hessen (1995) reported that microalgae exposed to UVR were less efficiently assimilated by Daphnia magna compared with non-irradiated algae, due to physiological changes in the cellular structure of the algal cells. This reduced digestibility of algal cells could significantly alter trophic interactions and potentially reduce the transfer of energy between primary and secondary consumers in aquatic systems. Furthermore, it has been suggested that the
transfer of UV-absorbing mycosporine-like amino acids from algae to primary consumers may affect the UV-protective capacities of organisms such as krill (Newman et al., 2000).

In addition, symbiotic organisms such as corals or sea anemones and their associated zooxanthellae may be affected. Numerous reports of depressed photosynthesis in zooxanthellae have been made resulting in the bleaching of host organisms (Harriot, 1985; Fisk & Done, 1985; Goenaga et al., 1989; Gleason & Wellington, 1993; Brown et al., 1994; Fitt & Warner, 1995; Anderson et al., 2001).

4.2 Aims and objectives

In this chapter, an integrated approach was adopted to evaluate the genotoxicity, cytotoxicity and developmental effects of UVR exposure, in the early life stages of *Mytilus edulis* and *Platynereis dumerilii*. The aim was to examine the hypothesis that UVR causes genetic damage in the early life stages of marine invertebrates and that these changes in genetic integrity could be detected using biomarkers at different levels of biological organisation. Genotoxicity was measured by assessing the induction of chromosomal aberrations and sister chromatid exchanges, in metaphase cells; cytotoxicity was based upon the use of sister chromatid differential (SCD) staining and determined with the measurement of the proliferation rate of the cells; and developmental effects were determined by embryo morphological development assays and measurements of growth in larvae.

4.3 Materials and Methods

4.3.1 UV exposure of embryo-larvae

*Mytilus edulis* embryo-larvae were collected by the stimulated spawning of adult animals, under controlled laboratory conditions, as described in section 2.2.1. *Platynereis dumerilii* embryo-larvae were collected by placing together at least two pairs of mature
adults (males with females) which had emerged from their tubes and allowed to spawn under controlled conditions, as described in section 2.2.2.

The fertilisation success of the eggs was determined by microscopic examination. First, the volume of the gamete suspension was condensed by filtering through a nylon mesh (mesh size 30 μm for M. edulis and 80 μm for P. dumerilii) to reduce the volume to 100ml. Then 5 replicates of 20 μl volumes of the gamete suspension were pipetted onto glass microscope slides, before examination of the eggs for evidence of fertilisation (the presence of polar bodies or cell cleavage as described in section 2.2.1), using a compound microscope (Leica, DMR) at a magnification of x400. The fertilisation rate was determined by calculating the proportion of fertilised eggs as a percentage of the total number of eggs produced. Following fertilisation of the females' gametes, the embryo-larvae were allowed to develop in sterile, pyrex glass evaporating dishes containing 200ml filtered seawater incubated (15±1°C for M. edulis; 20±1°C for P. dumerilii) at a density not exceeding 30 embryos per ml. The details of the number of adults used, numbers of eggs produced and the fertilisation rates of the gametes for each experiment are presented in Appendix II.

Following fertilisation, the vessels were covered with aluminium foil and the growing embryos maintained in the dark in incubators (Sanyo Gallenkamp) at a constant temperature (15°C for M. edulis; 20°C for P. dumerilii) for at least 12 hours, until the start of the UV exposures. To make provision for differential staining of the sister chromatids, 5-Bromo-2-deoxyuridine (BrdU) solution was added immediately prior to the start of the UV exposure, to produce a working concentration of 1.0x10⁻⁵M in each of the sample vessels, as had been carried out in previous validation studies. This differential staining would allow the determination of proliferative rate index (PRI) and the induction of sister chromatid exchanges (SCEs). No BrdU solution was added to samples which were to be used for determining the induction of chromosomal aberrations (CAbs).
The vessels containing the embryo-larvae were then transferred from the dark incubating cabinets and placed in the UV exposure cabinet described in section 2.2.1. The samples were exposed for 84, 96, 132 and 228 minutes which correspond to approximately 10, 18, 32 and 56% levels of ozone depletion in south-west England during spring. This is a semi-logarithmic scale, routinely used in ecotoxicological experiments (Duodroff et al., 1952). Measurements of UVR within the exposure cabinet and calculations of the doses and corresponding levels of ozone depletion were calculated as described in section 2.3.1. Table 4.1 shows the calculations carried out to quantify the time periods of exposure, the doses received by the samples and the equivalent levels of ozone depletion. The exposure period was terminated for each dose simultaneously, to allow treatment of the embryo-larvae for chromosome preparation at the same time.

Two replicate vessels were used per irradiation dose and cytogenetic endpoint. Each exposure vessel was rotated to an adjacent position within the exposure cabinet at regular intervals during the exposure period, to ensure that the samples received a comparable dose and therefore negating any potential hot-spots where higher levels of irradiation might be present. This is represented in figure 4.1.

4.3.1.1 Metaphase preparation and Chromosome Aberration analysis

The methods of slide preparation, chromosome staining and chromosome analysis were adopted from earlier studies by Harrison & Jones (1982) and subsequently modified by other workers (Dixon et al., 1985; Jha et al., 1995, 1996, 2000; Hutchinson et al. 1998). A summary of the experimental protocol is presented as a flow diagram in figure 4.2.

Following the exposure of the embryo-larvae, each sample was collected on a nylon sieve (30μm for M.edulis; 80μm for P.dumerilii) and exposed to 0.025% colchicine solution (w/v) dissolved in seawater for 30 minutes. The colchicine treatment prevents the
Table 4.1. Measurements of unweighted and Setlow weighted UV-B levels (measured with a Macam spectroradiometer, model SR9910-V7) and subsequent calculations carried out to quantify the time periods of exposure, the doses received by the biological samples and the equivalent levels of ozone depletion (using the model proposed by Björg & Murphy 1985).

<table>
<thead>
<tr>
<th>Level of Unweighted UV-B (Wm^-2)</th>
<th>Level of Setlow Weighted UV-B (Wm^-2)</th>
<th>Period of Exposure (mins)</th>
<th>Unweighted Dose (kJm^-2)</th>
<th>Weighted Dose (kJm^-2)</th>
<th>Equivalent Level of Ozone Depletion (approx.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.43 E^-01</td>
<td>3.04 E^-03</td>
<td>84</td>
<td>2.23</td>
<td>0.015</td>
<td>10%</td>
</tr>
<tr>
<td>4.43 E^-01</td>
<td>3.04 E^-03</td>
<td>96</td>
<td>2.55</td>
<td>0.018</td>
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<td>4.43 E^-01</td>
<td>3.04 E^-03</td>
<td>132</td>
<td>3.51</td>
<td>0.02</td>
<td>32%</td>
</tr>
<tr>
<td>4.43 E^-01</td>
<td>3.04 E^-03</td>
<td>228</td>
<td>6.06</td>
<td>0.04</td>
<td>56%</td>
</tr>
</tbody>
</table>

Figure 4.1. Schematic representation of the inside of the Fisons UV irradiation cabinet used for exposing samples to UVR.
Obtain ♂ and ♀ gametes

Fertilise eggs

Collect newly fertilised embryos

Incubate for 12 h

15°C for M.edulis
20°C for P.dumerilii

Start UV Exposures

Expose for 84, 96, 132 or 228 mins

Add BrdU solution

Terminate Exposure

- Treat samples with colchicine
- Hypotonic solutions and fix
- Prepare metaphases

- Collect subsample at 24h old

- Allow subsample to grow until 48h old

Cytogenetic Endpoints (CAbs, PRI, SCEs)

Embryo-larval Development & Growth Endpoints

Figure 4.2. Flow chart representing the protocol for the exposure of marine invertebrate larvae to UVR, the collection of chromosomes for cytogenetic endpoints and embryo-larvae for developmental and growth endpoints.
formation of the spindles during anaphase, thus arresting the maximum number of cells possible in metaphase to ensure a maximum harvest of cells in this phase of mitosis. Following this treatment, the embryo-larvae were exposed to a series of hypotonic solutions (seawater:0.075M KCl) for 10 minutes for each treatment, comprising of seawater and 0.075M potassium chloride (dissolved in distilled water) in the following ratios: (a) 2:1 (b) 1:1 (c) 1:2 (d) 1:3

The samples were then transferred to glass centrifuge tubes using disposable plastic pasteur pipettes and then centrifuged for 5 minutes at 1000 rpm, to form a pellet. The supernatant was carefully removed without disturbing the pellet of embryo-larvae and replaced drop by drop with cold Carnoy's fixative (methanol and glacial acetic acid, 3:1 ratio, held at 4°C). The Carnoy's fixative was removed and replaced with fresh fixative twice before preparation of the metaphase spreads.

To prepare the metaphase spreads, embryo-larvae were resuspended in the Carnoy's fixative by gentle pipetting (with siliconised glass pasteur pipettes) and a small quantity of the fixed larvae transferred onto labelled glass microscope slides. Drops of 65% glacial acetic acid were then added, whilst simultaneously rotating the slide to ensure even spreading of the chromosomes as the acetic acid burst open the cells. The slides were placed onto a hot plate maintained at 40°C and intermittently rotated until all of the 65% acetic acid had evaporated and the slide was dry. The spreading of the chromosomes was then checked with a phase-contrast light microscope at a magnification of x400.

4.3.1.2 Staining and Scoring of chromosomes for Chromosomal Aberration Analysis

Following preparation of the chromosomes, the slide were rinsed with distilled water and then stained by immersion into 10% (v/v) Giemsa solution (BDH, UK) in
Giemsa buffer (pH 6.8) (BDH, UK), for 25 minutes. The slides were then rinsed twice in distilled water and allowed to air dry. Coverglasses were then mounted using DPX mountant (BDH, UK) and allowed to dry overnight for at least 24 hours before analysis.

Prior to analysis, the slides were coded in a manner which prevented the investigator from knowing the treatment of the slide, randomised and then scored blind to avoid any bias during scoring. 100 metaphase spreads per replicate were examined using a bright-field microscope at a final magnification of x1000 under oil immersion. Only metaphases containing 28 ± 2 chromosomes were scored. CAbs were expressed as the percent aberrant cells and total aberrations (chromosome and chromatid type, excluding gaps), as per the criteria recommended by the United Kingdom Environmental Mutagen Society (Scott et al., 1996).

4.3.1.3 Differential Staining and Scoring for Proliferation Rate Index (PRI) and Sister Chromatid Exchanges (SCEs)

Following preparation of the chromosome spreads, the slides were rinsed with distilled water. The slides were then differentially stained following a procedure developed by Goto et al. (1975) and subsequently adopted by Jha et al. (1996) for application of the method to the embryo-larvae of marine invertebrates. The slides were incubated in 0.025% Hoechst 33258 solution (w/v) dissolved in phosphate-buffered saline (PBS) for 20 minutes, maintained in the dark at room temperature. They were then rinsed twice with distilled water and allowed to air dry. A few drops of PBS were added to each slide and a coverglass applied. The slides were then placed on a hot plate held at approximately 55°C, for 25 minutes whilst simultaneously exposed to blacklight (F18W-BLB lights, Sylvania, UK). The slides were then rinsed again in distilled water, allowing the coverglasses to slide off and air dried. The slides were then stained with 10% Giemsa
stain, diluted with Giemsa buffer solution (BDH) for 15 minutes, before rinsing twice in distilled water. Once the slides had dried, coverglasses were mounted using DPX mountant. The DPX was allowed to dry for at least 24 hours before analysis of the chromosomes.

As with the analysis of the chromosomes for CAbs, slides were coded, randomised and scored blind for the classification of metaphases into 1st, 2nd and 3rd (3rd+) cell divisions for the calculation of PRI and of the induction of sister chromatid exchanges (SCEs). 100 metaphase spreads were scored for PRI (per replicate), of which at least 25 cells (per replicate) in 2nd division were selected for the determination of SCEs. Only complete metaphases (containing 28 ±2 chromosomes) were analysed for cell division and SCE determination.

PRI was calculated with the following equation:

\[
PRI = \frac{(1 \times M1) + (2 \times M2) + (3 \times M3)}{\text{number of cells scored}}
\]

4.3.1.4 Development and Growth Assessments

The effects of UVR on the developmental of the embryo-larval stages were assessed at 48 hours post-fertilisation. A sub-sample from each replicate was collected at this time and fixed in 10% formalin before examination under a compound microscope. Embryo-larvae were examined for morphological abnormalities in their development (approximately 50 larvae for \textit{M.edulis} and 20 larvae for \textit{P.dumerilii}). Features such as irregular morphology, undeveloped soft tissue or the presence of necrotic tissue was examined and recorded in both species. In \textit{P.dumerilii} also the lack of, or abnormal development of eyes and or chaetae was classed to be abnormal development.

By 24 hours post-fertilisation, normal embryo-larvae were expected to have reached the trochophore stage, with cilia around the margin and symmetrical development. In \textit{M.edulis}, at 48 hours post-fertilisation, a majority of the larvae were expected to have
reached the prodissococonch (D-shell) stage, when allowed to grow at 15°C. Abnormal larvae were undeveloped or showed developmental abnormalities to the soft tissue or velum, or to the shell or its hinge, as described by ASTM (1997). In *P.dumerilii*, normal larvae were expected to have reached the metatrochophore stage at 48 hours post-fertilisation, when allowed to grow at 20°C. Abnormal larvae in this species were underdeveloped or showed developmental abnormalities to the head, parts of the segmented body, the eyespots and or the chaetae, as described by Hutchinson *et al.* (1995). The frequency of dead embryo-larvae was also determined in both species, which was characterised by the appearance of a mass of degenerating cells.

The growth of larvae was assessed at 48 hours post-fertilisation. Random samples of 50 larvae were collected and individually measured for maximum length (along the hinge of the D-shell in *M.edulis* and from head to the end of the abdomen in *P.dumerilii*). Larval samples were placed upon a glass microscope slide and measured by video capture and image analysis using a Quantimet 570 image analyzer (Cambridge Instruments, Cambridge, UK). Data was automatically recorded and recovered with the Microsoft software Excel 97.

4.3.2 Statistical Analyses

Data were treated with the one-way analysis of variance (ANOVA) multivariate technique, wherever sufficient data points permitted and where the distribution was parametric. Non-parametric data was normalised by transformation (by log or square-root) and treated with ANOVA, assuming the variance between populations was not statistically significant (*p*<0.05), as suggested by Scott & Clarke (2000). In these cases, the data were presented as plots of the mean (with standard deviation or 2 times the standard error where applicable). Where data could not be normalised, the non-parametric Kruskall-Wallis test
was applied to compare the medians between populations and in these cases, the data were presented as box-and-whisker plots as proposed by French & Lindley (2000).

Chromosomal aberration data were treated with the z-test to provide an indication of the level of significance between populations. The same test was applied to % M2 cells containing SCEs and for PRI data as proposed by Lovell et al. (1989). Regression analyses were applied wherever dose-dependent responses were observed as proposed by Smith & Anderson-Cook (2000).

Statistical analyses were carried out using the statistical package Statgraphics Plus, Version 4.0 and Microsoft Excel XP. Bar graphs were constructed using Microsoft Excel XP, box and whisker plots were constructed using Statgraphics Plus 4.0.

4.4 Observations

When the metaphases were examined for PRI and SCEs, it was found that none of the embryo-larvae had survived the period of the exposure, in vessels which contained the 1.0x10^{-5}M BrdU including the UV-B free, white light control treatments. It appeared that light had reacted with the BrdU and caused the toxicity to be elevated beyond the tolerance of the organisms. This led to a repetition of the experiments, described in section 4.3.1, with an amendment to the concentration of the BrdU. The working concentration of the BrdU was reduced by a ten-fold dilution (1.0x10^{-6}M).

When the chromosomes were examined following exposures to UVR with 1.0x10^{-6}M BrdU, it was found that there was no significant contrast between the staining of sister chromatids. Furthermore, the chromosomes were observed to contain a high level of aberrations and many of the cells were classed as "highly damaged" or "pulverised" metaphases. Plate 4.1 shows a typical example of the metaphase spreads observed from _M. edulis_ embryo-larvae which had been exposed to UVR with 1.0x10^{-6}M BrdU.
Plate 4.1. A partial metaphase spread showing typical chromosomes observed from *M. edulis* embryo-larvae following UVR exposure, in the presence of $1.0 \times 10^{-6}$ BrdU solution. Magnification x1000.

Figure 4.3. Flow diagram for the amended protocol for the differential staining of sister chromatids.
Due to the lack of differential staining in the sister chromatids and evident genotoxicity of the BrdU, a further modification of the protocol was introduced. To incorporate the BrdU into the DNA, a solution of $1.0 \times 10^{-5} \text{M}$ BrdU was added to the samples following the UV irradiation of the samples, in the absence of light, rather than simultaneous exposure with the UVR. Figure 4.3 shows the amended protocol for the differential staining of chromatids.

4.5 Results

4.5.1 Effects of UVR on the Induction of Chromosomal Aberrations (CAbs)

4.5.1.1 *Mytilus edulis*

Plate 4.2 illustrates a normal and aberrant metaphase spread showing chromosomal aberrations from *M. edulis* in control and exposed samples. The frequency of chromosomal aberrations was determined in at least 100 cells per sample (excluding chromatid and chromosome gaps) as per the criteria described by Scott *et al.* (1990). The number of cells exhibiting aberrations has been expressed as the percentage of aberrant cells and the total number of aberrations per sample.

A dose-dependent increase was observed in both the frequency of % aberrant cells and the total number of aberrations, (figures 4.4a and 4.4b). This relationship was statistically significant ($R^2 = 0.9402$ for percentage aberrant cells; $R^2 = 0.879$ for total frequency of aberrant cells). When the Z-test was applied to the data, there was no statistically significant difference from the control in samples treated to radiation levels equivalent to 10% ozone depletion, however there was a significant difference in samples exposed to UVR equivalent to 18% ozone depletion and above ($p<0.05$). There was a four-fold increase in the incidence of aberrations in the cells from *M. edulis* exposed to the highest dose of UVR (equivalent to 56% ozone depletion) when compared with the control, this being a strong statistically significant difference ($p<0.001$) from the control.
Plate 4.2. *M. edulis* (a) normal metaphase chromosome spread and (b) metaphase spread with chromosomal aberrations (indicated with arrows). Magnification x1000.

Plate 4.3. *P. dumerilii* (a) normal metaphase chromosome spread and (b) metaphase spread with chromosome type break (indicated with the arrow). Magnification x1000.
Figure 4.4. (a) The effect of UV exposure on the percentage of cells containing chromosomal aberrations in *M. edulis* embryo-larvae; (b) The effect of UVR on the total frequency of chromosomal aberrations in the embryo-larvae of *M. edulis*. Error bars = SD; • denotes a statistically significant difference from the control (p<0.05); ** denotes a strong statistically significant difference from the control (p<0.001).
Figure 4.5. (a) The effect of UV exposure on the percentage of cells containing chromosomal aberrations in *P. dumerilii* embryo-larvae; (b) The effect of UVR on the total frequency of chromosomal aberrations in the embryo-larvae of *P. dumerilii*. Error bars = SD; * denotes a statistically significant difference from the control (p<0.05); ** denotes a strong statistically significant difference from the control (p<0.001).
4.5.1.2 *Platynereis dumerilii*

Plate 4.3 illustrates chromosomal type breaks in a metaphase spread from *P. dumerilii* in UVR exposed samples, compared with a normal metaphase spread. The frequency of chromosomal aberrations was determined in at least 100 cells per sample and the number of cells exhibiting aberrations has been expressed as the percentage of aberrant cells and the total number of aberrations per sample (figures 4.5a and 4.5b). There was a general dose-dependent increase in the induction of CAbs with increasing UVR (*R^2* = 0.81 for percentage aberrant cells; *R^2*=0.8168 for total frequency of aberrant cells). A statistically significant difference was determined at doses equivalent to 32% ozone depletion (*p*<0.05) and at 56% ozone depletion (*p*<0.001), when analysed with the Z-test.

4.5.2 Effects of UVR on the Induction of Sister Chromatid Exchanges (SCEs)

4.5.2.1 *Mytilus edulis*

When considering the mean frequency of total SCEs in the *M. edulis* embryo-larvae exposed to UVR, there was a significant increase in all of the treatments when compared to the control (*p*<0.05). However, there was no apparent dose-dependent response as illustrated in figure 4.6a. When the data was analysed for the proportion of cells containing SCEs as a percentage of the total number of cells analysed in M2 for SCEs, a weak dose-dependent increase was observed (*R^2* = 0.5315), which is presented in figure 4.6b.

4.5.2.2 *Platynereis dumerilii*

In *P. dumerilii* embryo-larvae, a weak dose-related increase in the mean induction of SCEs per cell was observed (*R^2*=0.5043). However, this was only statistically
Figure 4.6. (a) Mean frequency of SCEs per cell following UVR exposure in *M. edulis* embryo-larvae; (b) Effect of UVR on the proportion of M2 cells in which SCEs are induced from *M. edulis* larvae. Error bars = SD; * denotes a statistically significant difference from the control (p<0.05).
Figure 4.7. (a) Mean frequency of SCE per cell UVR exposure in \textit{P.dumerilii} embryo-larvae; (b) Effects of UVR on the proportion of M2 cells in which SCEs are induced in \textit{P.dumerilii} embryo-larvae. Error bars = SD; * denotes a statistically significant difference from the control (p<0.05).
significantly different from the control in the sample exposed to UVR at a dose relating to 56% ozone depletion (figure 4.7a). When the data was analysed for the proportion of cells containing SCEs as a percentage of the total number of cells analysed in M2 for SCEs, a more significant dose-related trend could be detected ($R^2 = 0.8063$). Significant increases in SCE affected cells could be detected in samples treated to 18% ozone depletion and 56% ozone depletion ($p<0.05$); in samples exposed to 32% ozone depletion, the distribution of the data resulted in the increase being not significantly different from the control ($p>0.05$), the data has been presented in figure 4.7b.

4.5.3 Effects of UVR on the Proliferative Rate Index (PRI)

4.5.3.1 *Mytilus edulis*

The results of the analysis of proliferation rate index (PRI) by classifying the cells into 1st, 2nd and 3rd/3rd+ division, showed that there was no significant impact upon the PRI. Although there was a slight decrease in the PRI between the control and the highest dose (UVR levels equivalent to 56% ozone depletion), this was not statistically significant following ANOVA of the mean of each treatment. This indicated that the rate of cellular turnover was not significantly impacted by the increases in UVR levels. The samples within the negative control were of the highest variability. Figure 4.8 illustrates the mean PRI observed.

4.4.3.2 *Platynereis dumerilii*

The mean results of the analysis of PRI, showed that there was a significant impact caused at the higher UVR levels relating to 32% and 56% ozone depletion (ANOVA, $p<0.05$), but insignificant impact upon the PRI at doses corresponding to 10 and 18%
Figure 4.8. The Effect of UVR on PRI in *Mytilus edulis* embryo-larvae. Error bars = SD.

Figure 4.9. The effect of UVR on PRI in *P. dumerilii* embryo-larvae. Error bars = SD; * denotes a statistically significant difference from the control (p<0.05).
ozone depletion. Figure 4.9 illustrates the effect of UVR on the mean PRI.

4.5.4 Effects of UVR on Development

4.5.4.1 *Mytilus edulis*

When the larvae aged 48 hours, were analysed for normal morphological development. Plate 4.5 illustrates the appearance of (a) a normal trochophore larva and (b) an abnormal trochophore larva. Plate 4.5 illustrates the appearance of (a) a normal prodissococonch (D-shell) larva and (b) an abnormal prodissococonch larva. A dose-dependent response was observed in the proportion of larvae which showed normal development with no morphological abnormalities. Using one-way ANOVA, a statistically significant decrease was observed in samples exposed to UV levels equivalent to 18% or more ozone depletion (p=0.0168) compared with the control and 10% ozone depletion treatments. The mean proportions of normal larvae exposed to the various treatments are illustrated in figure 4.10.

4.5.4.2 *Platynereis dumerilii*

When the metatrochophore larvae aged 48 hours, were analysed for normal morphological development, a dose-dependent response was observed in the proportion of larvae which showed normal development with no morphological abnormalities. Plate 4.5 illustrates the appearance of (a) a normal metatrochophore larva and (b) an abnormal metatrochophore larva.

Since the data were non-parametric and could not be normalised by transformation, the Kruskall-Wallis test was applied to compare the medians between one treatment and
Figure 4.10. The effect of UVR exposure upon the normal development of *Mytilus edulis* larvae aged 48 hours (trochophores and prodissococonch) grown at 15°C. Error bars = 2SEM; * denotes a statistically significant difference from the control (p<0.05).
Plate 4.4. (a) Normal and (b) Abnormal trochophore *M. edulis* larvae following UVR exposure, note the irregular shape of the larva, enlarged peripheral cells and the irregular distribution of the peripheral cilia.

Plate 4.5. (a) Normal and (b) Abnormal prodissococonch ‘D-shell’ *M. edulis* larvae following UVR exposure, note the protruding velum which is unable to be protected by the deformed shell.
another. The effect of UVR exposure upon the proportion of larvae which developed normal morphology, was statistically different in all the treatments when compared with the control (p=0.004). The distribution of the data is illustrated in the box-and-whisker plot presented in figure 4.11.

4.5.5 Effects of UVR on Growth

4.5.5.1 Mytilus edulis

The measurement of the maximum length of prodissococonch (D-shell) larvae, provided an indication of the effect that UVR had upon the rate of growth. Figure 4.12 presents the results of the measurements, with a clear dose-dependent decrease in the size of the larvae.

Using the non-parametric Kruskall-Wallis test, to compare the medians of different treatment groups, it was found that there was a statistically significant difference between the samples treated with UV levels equivalent to 32% and 56% ozone depletion and the control, 10% and 18% ozone depletion treatments (p= $2.4879 \times 10^{-7}$).

4.4.5.2 Platynereis dumerilii

The Kruskall-Wallis test indicates that there is a statistically significant difference between the median size of normal larvae treated to the equivalent of 32% ozone depletion, when compared to the median size of the larvae from the control group (p=0.00004). There was no significant difference between the control group in relation to the other UV treatments. The distribution of the data is illustrated in the box-and-whisker plot presented in figure 4.13.
Plate 4.6. (a) Normal and (b) Abnormal 48 hour old metatrochophore *P. dumerilii* larvae following UVR exposure, note the lack of distinct parapodia and chaetae and the enlarged mid-thoracic organ development.

Figure 4.11. The effect of UVR exposure upon the normal development of *Platynereis dumerilii* larvae aged 48 hours (metatrochophores). (Error bars = 2SEM; * denotes a statistically significant difference from the control (p<0.05)).
Figure 4.12. Mean *M. edulis* D-Shell larvae length aged 48h following UVR exposure (* denotes a statistically significant difference from the control (p<0.05)).

Figure 4.13. *P. dumerilii* metatrochophore larvae length aged 48h following UVR exposure (* denotes a statistically significant difference from the control (p<0.05)).
4.6 Discussion

Previous authors have indicated that the potential impacts from ozone depletion and consequently the increase in biologically damaging UV-B, can affect organisms from various trophic levels of the aquatic ecosystem (Damkaer & Dey, 1983; Gleason & Wellington, 1995; Morgan & Christy, 1996; Licht & Grant, 1997; Zagarese et al., 1997; Lacuna & Uye, 2001). Such studies carried out in both the field and in laboratory conditions, have indicated that zooplankton including the larval stages of fishes and invertebrates are extremely sensitive to UV-B radiation (Smith & Baker, 1989; Berghahn et al., 1993; Kuhn, 2000; Wubben, 2000).

In the work presented in this chapter, it can be seen that genotoxic, cytotoxic and developmental effects of UVR exposure, can be detected in the early life stages of the commercially and ecologically important invertebrate species, *Mytilus edulis* and *Platynereis dumerili*. using cytogenetic and larval development assays.

4.6.1 Chromosomal aberrations

The data collated from these studies suggests that *M. edulis* was more sensitive to UVR exposure in terms of genotoxic responses, when compared with *P. dumerili*. In *M. edulis* increased levels of UVR equivalent to 18% or more, caused a statistically significant increase in the induction of CAbs in relation to control samples, whereas levels of UVR equivalent to 32% ozone or more, were required to produce significant increases in CAbs in *P. dumerili*. Anderson et al. (1993) reported a dose-dependent increase in anaphase chromosomal aberrations in sea urchin embryos, which correlates with the observations made in the current studies. Furthermore, when CAbs are induced by UV radiation, these lesions tend to be long-lived (Wolff, 1982). One cause for the difference in sensitivity may be the difference in embryo-larval size. As mentioned in section 4.0,
previous authors have indicated that smaller cells and organisms have a higher level of susceptibility to UV radiation compared with larger organisms (Karentz et al., 1991; Bothwell et al., 1993; Zellmer, 1995; Jeffrey & Mitchell, 1997; McNamara & Hill, 1999). In the studies described here, embryo-larvae aged 24 hours from *M. edulis* were approximately 40 µm in diameter, whereas *P. dumerilii* embryo-larvae of the same age were approximately 100 µm. At 48 hours of age, there is still a significant difference in size between the two species, with *M. edulis* larvae being approximately 80 µm in length and *P. dumerilii* being approximately 200 µm in length.

4.6.2 Sister Chromatid Exchanges

When considering the induction of SCEs, *M. edulis* showed a significant increase in the mean number of SCEs per cell and the proportion of M2 cells in which manifestations of SCEs were observed, at UVR levels corresponding to 10% ozone depletion and above. In *P. dumerilii* levels of UVR at a dose of 18% ozone depletion and above were required to induce significant increases in SCEs. Previous authors have found relatively higher frequencies of SCEs induced by chemical contaminants in each of these species (Jha et al. 1996, 1997, 2000). However, it should be noted that the frequencies of SCEs induced in the current studies were indications of the residual SCEs since the cells were exposed to BrdU after the UVR exposure due to the photoactivation of BrdU. This showed that the residual unrepaired SCEs could be detected with the differential staining technique and it was possible to incorporate BrdU post-UV exposure, for the employment of differential staining of sister chromatids. There may potentially have been a delay in the cell cycle progression due to UV damage, and perhaps the responses observed were not true reflections of the SCE induction levels. By increasing the fixation time it may have been possible to therefore allow any damaged cells to arrive at metaphase, and any increased levels of SCEs could have then been detected.
Cytotoxic responses were measured with the analysis of PRI where the effect of UVR upon cellular proliferation kinetics was assessed. The PRI reveals the average number of times the cells have divided during the period following incorporation of 5-bromodeoxyuridine and cell harvesting (Lamberti et al., 1983). In *M. edulis*, there was a slight decrease in the PRI with increasing UV dose, though there was no statistically significant difference when compared with the control. In *P. dumerilii*, a similar trend was observed with a statistically significant decrease in PRI in samples exposed to UV levels corresponding to 32% ozone depletion and above. When these results are compared with similar studies carried out by Jha et al. (1996, 1997, 2000) and Hutchinson et al. (1998) with chemical contaminants, it can be seen that PRI as an endpoint was more sensitive in these other studies compared with the responses to UVR exposure. The relative insensitivity of this endpoint observed in the current studies may have been due to the amendment made to the protocol, required to prevent photoactivation of the BrdU and subsequent increased toxicity. Since incorporation of BrdU was carried out subsequent to the UVR exposure, rather than in simultaneous exposure, any detrimental effects upon the cell cycle kinetics during UV exposure would not be detected by the PRI. Another cytotoxic assay was carried out, which also examines the cellular proliferation rate in sea urchins, by Anderson et al. (1993), where the number of cells were counted in the embryos following exposure to UVR. They observed a dose-dependent decrease in the number of cells per embryo indicating that UVR suppresses the rate of cell proliferation.

Suggestions have been made that in the early life stages of aquatic organisms the cell division cycle during the cell cleavage is relatively short (Epel et al., 1999). The authors also stated that these cell division cycles often did not have mitotic checkpoints, which would explain the lack of dose-dependent effect of UVR upon PRI.
4.6.4 Developmental responses

Organism responses showed a different level of sensitivity when comparing the two species. When considering the proportion of larvae which were of normal morphological form at 48 hours of age, *P. dumerilii* was significantly affected at doses of UV corresponding to 10% ozone depletion. In contrast, *M. edulis* had a higher proportion of normal larvae at this dose and significant impacts upon the morphology of larvae were not observed until they were exposed to UV levels relating to 18% ozone depletion and above. There was no comparative difference in the effect of UVR upon the rate of growth of larvae exhibiting normal morphological growth between the two species, both showing significant effects on size following exposure to UV levels equivalent to 32% and above. The relatively high level of UVR which caused a significant decrease in size, may suggest that should individual larvae develop with normal morphological form, then no significant impact is made on the energetic budget of the larvae for normal rates of growth. A parallel response was observed in previous work, where UVR also caused a dose-dependent increase in the frequency of abnormal morphological development of the early life stages of the sea urchin, *Sterechinus neumayeri* (Anderson *et al.*, 1993). Due to the difficulties in identifying the frequency of individuals which were dead (often indeterminate masses of degrading organic material were observed which consisted of numerous individuals), the proportion of “dead” embryo-larvae was not presented. It is common for high frequencies of mortalities in the early life stages of invertebrates due to the high proportion of reproductive excess produced (Gosselin & Qian, 1997).

4.6.5 Conclusions

Total ozone levels over the UK vary with the seasons, in winter the change may be very rapid due to natural as well as anthropogenic reasons. Much of the research to date
has concentrated on the Polar regions because the greatest reductions in stratospheric ozone have been measured over the Antarctic. However, with decreases also occurring in the Arctic, there are potential impacts on the UK and Europe. For a few days in March 1996, ozone column concentrations in this region were reduced to 50% of the normal values, this being due to unusual meteorological conditions and the passage overhead of Arctic lower stratospheric air in which ozone had been depleted (Pearce, 1996; www.met-office.gov.uk, 2002). This was an extreme case, with typical reductions of ozone normally being closer to a 12% reduction in ozone levels (WMO, 1995).

Taking into consideration the historical and predicted levels of ozone depletion, the observations in the experiments carried out in the current studies suggest that *M. edulis* and *P. dumerilii* would not develop significant increases in genotoxic lesions at environmentally realistic (approximately 12%) levels of ozone depletion. However, should the normal morphological development be affected due to sudden increases of ozone depletion (observed at 10% ozone depletion in *P. dumerilii* and 18% ozone depletion in *M. edulis*), particularly during the spring when the spawning of these animals is at its peak, impacts upon the long-term survival could be compromised. For *P. dumerilii* embryo-larvae, morphological abnormalities such as underdeveloped eyes and appendages would render them more susceptible to predators and avoidance measures for survival may be jeopardised. In the case of *M. edulis* embryo-larvae, a delay in the transition from the trochophore stages to the prodissococonch, or the poor development of the shell, would increase the period of time that the larvae were at their most vulnerable to predators, since they would not have a well developed protective shell.

Although UV exposure could negatively impact invertebrate zooplankton populations, the lethal effects are likely to be minimal within the context of all other environmental factors that produce the very high levels of mortality typically observed in their planktonic life stages. As implicated by Browman *et al.* (2000), the impact of
sublethal effects, which may be of more significant importance, remains to be evaluated. The differences in sensitivity between species may be due to the different abilities for the species to repair genetic damage. Furthermore, the employment of protective mechanisms such as the production of mycosporines or pigments on the surface of the larvae was not assessed in the species of interest. These factors were not measured in these studies and should be investigated further.
Chapter 5

PAHs & UVR: An investigation of the interactive toxic effects in the early life stages of marine invertebrates
Hypotheses:

(i) Polycyclic aromatic hydrocarbons cause genotoxic, cytotoxic and developmental effects detectable in the early life stages of marine invertebrates.

(ii) Ultraviolet radiation interacts with polycyclic aromatic hydrocarbons causing alterations in the toxicity to the early life stages of marine invertebrates.

5.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are present in a wide variety of environmental samples including terrestrial, aquatic and atmospheric phases. Many PAHs are known to act as potent carcinogens and/or mutagens and are therefore considered to be of high environmental relevance (Malström et al., 2000). The various sources of PAHs in the marine environment include deposition from the atmosphere, municipal and industrial wastes, surface runoff and riverine inputs. However, an accurate estimate of the total PAH input from these sources is difficult to establish due to the diffuse nature of the source and influence of aperiodic events such as extreme weather conditions, or sporadic oil disasters (McElroy et al., 1987). Ultimately, the aquatic environment acts as a sink for many chemical contaminants and it is therefore important to assess the potential genotoxic impact of such contaminants upon aquatic biota.

In the marine environment, the eggs and larvae of many species are planktonic for weeks or even months, during which time they float close to the water surface. The highest concentrations of dissolved or dispersed oil are found in the upper water layers and the early life stages of marine organisms are particularly sensitive to contaminants (Falk-Peterson & Lønning, 1984; Roy et al., 2002), therefore they can be considered as relevant target organisms for toxicity testing with hydrocarbons. The lethal toxicity of single PAH compounds to aquatic invertebrates have been the subject of a number of investigations (Neff, 1985; Kennish, 1992; Lee et al., 2001). In aquatic invertebrates sublethal effects of PAHs have included decreased growth rates, development time, reproductive output, rate of feeding and oxygen uptake (Geiger & Buikema, 1981, 1982; Neff, 1985; Donkin et al., 1989). Studies investigating the effects of refined petroleum derivatives rich in PAHs,
such as diesel fuel, have found impaired egg hatching, growth, development and behaviour of invertebrate species such as crabs, molluscs and the larvae of the grass shrimp *Paleonectes pugio* (Laughlin *et al.*, 1978; Nagarajah *et al.*, 1985; Fisher & Foss, 1993). Harpacticoid copepods have demonstrated adverse responses to petroleum hydrocarbons in field and mesocosm studies, with inhibition of feeding rates, decreased nauplii production and changes in behaviour (Coull & Chandler, 1992; Carman & Todaro, 1996; Lotufo, 1997).

In previous studies of chemical contaminant effects, it has been found that the early life stages of sea urchins can be used for the detection of genotoxic effects. Anaphase chromosome aberrations have been reported in the purple sea urchin (*Strongylocentrotus purpuratus*) and detrimental effects upon embryo development and survival in other species such as the Mediterranean sea urchin, *Paracentrotus lividus*, as well as other marine invertebrates such as the hard clam, *Mercenaria mercenaria* and fish species including the Atlantic mackerel, juvenile turbot *Scophtalmus maximus* and larval Pacific herring *Clupea pallasi* (Longwell & Hughes, 1980; Stiles *et al.*, 1991; Anderson *et al.*, 1994; Trieff *et al.*, 1995; Garman *et al.*, 1997; Baussant *et al.*, 2001; Carls *et al.*, 2001).

The larval stages of other invertebrates such as chironomids have also been recognised as sensitive biomonitoring organisms for toxicity testing, where decreased survival and growth and the development of morphological deformities have been used as endpoints to assess the toxicity of environmental samples (Hudson & Ciborowski, 1995; De Bisthoven, 1999). The first developmental stages of bivalves have been shown to be highly sensitive to contaminants including a number of organic compounds, as well as metals such as mercury and copper (Bieras & His, 1995; His *et al.*, 1999; Troncoso *et al.*, 2000). Cytogenetic damage has also been demonstrated in the early life stages of mussels, following exposure to arsenic, organotin and other organic contaminants (Jha *et al.*, 2000, 2000a).
The cytotoxicological testing of PAHs using cultured fish cell lines have become useful tools in the initial screening of aquatic pollutants and prediction of toxic effects (Alguacil et al., 1991; Araújo et al., 2000). Following in vivo exposure of embryos of the pink salmon Oncorhynchus gorbuscha to crude oil, the growth rates and subsequent survival rates of hatching juvenile fish were also reduced, although the animals appeared to be healthy (Heintz et al., 2000). Due to the relative small size of early life stages, methods of detecting body burdens of organic contaminants in the embryo-larval stages are not routinely available. Hence, in the larvae of Atlantic tomcod (Microgadus tomcod), the induction of CYP1A1 was used to provide an index of relative exposure of the bioavailability of the organic compounds 3,3', 4,4'-tetrachlorobiphenyl and benzo(a)pyrene (Roy et al., 2002).

Benzo(a)pyrene (B[a]P) is considered to be the most prevalent carcinogenic PAH and is often used as a model PAH (Miller et al., 1988; Suero et al., 2000; Nacci et al., 2002). The chemical structure of B(a)P and some other commonly occurring PAHs have been presented in figure 1.7. It is well established that metabolic activation is required before many PAHs are able to interact with DNA and the cytochrome P-450-dependent monoxygenase reactions in many species are involved in the metabolism of PAHs (Stegeman, 1985; Gonzalez, 1989; Araújo et al., 2000). In addition to the metabolic activation of B(a)P to DNA binding derivatives, oxidation pathways which generate reactive oxygen species are also capable of directly damaging DNA (Mauthe et al., 1995). The metabolites of B(a)P are known to induce a number of different types of lesions including DNA adducts (Venier & Canova, 1996), micronuclei (Vernier et al., 1997), strand breaks (Mitchelmore et al., 1998), chromosomal aberrations (Matsuoka et al., 1998), oxidative damage (Canova et al., 1998) and mutations (Rodriguez & Loechler, 1993).

Considering the ubiquitous nature of chemical contaminants, with particular reference to polycyclic aromatic hydrocarbons (PAHs) and the parallel universal presence
of ultraviolet radiation (UVR), exposure of aquatic organisms to the two simultaneously is inevitable. There is a growing body of research investigating the chemical interaction between UVR and toxicants and the potential alterations in the toxic capacity of such contaminants. Photolysis is one of the main photo-degradation pathways followed by PAHs in the environment particularly in aquatic ecosystems (Chen et al., 1996). Toxicity associated with the exposure of biological material to UV-B radiation (such as DNA mutation, oxidative stress and so on) resembles that of PAH toxicity such that, in addition to direct effects on PAH chemistry, UV-B radiation may also significantly enhance PAH toxicity through formation of oxygen-free radicals which can lead to oxidative damage and detrimental effects on cellular protection mechanisms. Furthermore, UV-B and PAH exposure can lead to the inactivation of critical proteins or adduct formation in DNA (Steevens et al., 1999). The chemical structure of PAHs allows the easy absorption of sunlight by the molecule, making them easily activated by ultraviolet radiation (Afsten et al., 1996). Indications are that the more water-soluble bicyclic and tricyclic PAHs are not carcinogenic, but transformation of some of these contaminants by UVR into products that are acutely toxic to a variety of aquatic organisms can occur (Chen et al., 1996). In cases where the PAHs have accumulated in biota, absorption of UVR photoactivates the chemicals. These photoactivated chemicals can induce damage to cellular membranes and potentially lead to detrimental effects to biological mechanisms and death (McDonald & Chapman, 2002).

A number of previous investigations have been documented describing the photoactivation or photodegradation of PAHs by UV-B in water and sediment phases (Bowling et al., 1983; Kagan et al., 1985; Newsted & Giesy, 1987; Oris & Giesy 1987; Davenport & Spacie, 1991; Ankley et al., 1994; Afsten et al., 1996). These reactions have resulted in a change in toxicity of some PAHs (either an increase or decrease) to a variety of aquatic species including Daphnia (Holst & Giesey, 1989; Davenport et al., 1994; Johnson et al., 1994), and Ceriodaphnia dubia (Burton & Turley, 1995; Ireland, 1996).
Swartz et al. (1997) reported an enhancement of toxic effects in the marine amphipod *Rhepoxynius abronius* exposed to the PAHs fluoranthene and pyrene, but no significant change in toxicity of acenaphthene and phenanthrene when UVR was simultaneously applied.

As mentioned earlier, the early life stages of many species are particularly susceptible to UVR due to their small size and pelagic mode of life. When considering the input of organic contaminants into the aquatic environment, much of this occurs at the water surface when deposited from aerosols. Furthermore, oil spills float at or near the surface of the water (Falk-Peterson & Lønning, 1984; Roy et al., 2002). PAH exposure in the aquatic environment is therefore likely to be simultaneous to the presence of UVR. It has been suggested that the potential hazard of photoenhanced toxicity may be greatest for the early embryo and larval stages of aquatic organisms that are relatively translucent to UV and inhabit the photic zone of the water column and intertidal areas (Barron & Ka’Aihue, 2001; Barron et al., 2003). A number of studies have reported the photoactivation of contaminants which had been taken up by the early life stages organisms and then subsequently exposed to UVR. The larvae of the northern leopard frog *Rana pipiens* which were exposed to the PAH fluoranthene and then subsequently exposed to ultraviolet light, were reported to have a significantly reduced time to death compared with larvae which were exposed to fluoranthene and then allowed to depurate (Monson et al., 1999).

A comprehensive study carried out by Pelletier et al. (1997) where the larvae and juveniles of the bivalve *Mulinia lateralis* and the mysid shrimp *Mysidopsis bahia* were exposed to individual PAHs (anthracene, fluoranthene and pyrene) and several water-accommodated fractions of petroleum products containing PAHs, indicated changes in toxicity following photoactivation. The results of their experiments provided evidence that ultraviolet light can dramatically increase the toxicity of these PAHs when compared to fluorescent light exposures in both single chemical and petroleum product tests. Increases
ranging from 12 fold to more than 50,000 fold in toxicity measured by rates of mortality of these species, were observed in the single chemical tests. The findings of their experiments suggest that low levels of PAHs in the environment could potentially cause significant toxicity in the presence of ultraviolet light.

In addition, investigations have been carried out to examine the potential for UV-B to increase the sensitivity of organisms such as the rotifer Brachionus calyciflorus to contaminant toxicity, independent of the action of UV-B on the toxicants (Preston et al., 1999). Studies by Choi & Oris (2000) in which PLHC-1 (Poeciliopsis lucida) cells were co-exposed to PAH and UV radiation, provided evidence that photoactivated PAH contaminants generate reactive oxygen species (ROS) resulting in oxidative stress. Furthermore, these studies demonstrated that the mode of toxic action is primarily disruption to the cell membranes via lipid peroxidation.

5.2 Aim and objectives

In this chapter, the genotoxicity, cytotoxicity and developmental effects of a cocktail of PAHs, on the early life stages of Mytilus edulis and Platynereis dumerilii, were evaluated. These data were compared with findings of previous studies where B(a)P was exposed to the embryo-larvae of the target species (Jha et al., 2000a). Furthermore, a series of studies was carried out to evaluate the genotoxicity, cytotoxicity and developmental effects of UVR exposure in combination with PAH contaminants.

The aim was to examine the hypothesis that PAHs cause genetic damage in the early life stages of marine invertebrates and the changes in genetic and cytologic integrity could be detected using biomarkers at different levels of biological organisation. In addition, experiments were conducted to evaluate the hypothesis that UVR interacts with chemical contaminants and alters the toxic potential of the xenobiotics concerned in the early life stages of marine invertebrates.
Genotoxicity was measured by assessing the induction of chromosomal aberrations and sister chromatid exchanges in metaphase cells; cytotoxicity was based upon the determination of proliferation rate of the cells; and developmental effects were determined by embryo-larval morphological development assays.

5.3 Materials & Methods

The experimental protocol for the determination of the effects of PAH exposure is summarised in figure 5.1. An additional step of photoactivation by UVR of the PAH stock solutions prior to exposure of the embryo-larvae of *M. edulis* and *P. dumerilii* was carried out in experiments investigating the interactive effects of UVR and PAH as described in section 5.3.2.

5.3.1 Collection of embryo-larvae

*Mytilus edulis* embryo-larvae were collected by the stimulated spawning of adult animals, under controlled laboratory conditions, as described in section 2.2.1. *Platynereis dumerilii* embryo-larvae were collected by placing together at least two pairs of sexually mature adults (males and females) which had emerged from their tubes and allowed to spawn under controlled conditions, as described in section 2.2.2.

For each spawning and fertilisation of female gametes, the fertilisation success of the eggs was checked microscopically, the total number of embryos calculated and the ensuing growth of embryo-larvae was carried out by maintaining the organisms at suitable conditions in incubators, as described in section 4.1.1 before the start of the exposure of the animals. Further details of the number of adults used, number of eggs produced, the fertilisation rates for each experiment can be found in Appendix II.
Figure 5.1. Flow chart representing the protocol for processing of invertebrate embryo-larvae for cytogenetic and developmental endpoints following exposure to PAHs.
5.3.2 Preparation of PAH solutions

A complex mixture of PAHs was used in the exposure of the early life stages of *M. edulis* and *P. dumerilii*. Details of the concentrations of each individual PAH in the complex mixture are presented in 5.1. The PAHs present in the mixture are all listed as reference contaminants routinely monitored as part of the National Monitoring Plan (NMP) programme. The NMP is a survey of the quality of UK coastal waters carried out by the Marine Pollution Monitoring Management Group (Marine Pollution Monitoring Management Group, 1998). A stock solution of the complex mixture of PAHs was predissolved in dimethyl sulfoxide (DMSO) which acted as the solvent carrier for the stock solutions, due to the low solubility in seawater of the individual compounds. For non-irradiated treatments, a stock solution of the PAH mixture was supplied (by a co-worker from CEFAS) ready dissolved in 1 ml DMSO, at a total PAH concentration of 5800 μg ml⁻¹. This in turn was further diluted with DMSO in 1.5 ml amber vials to provide final concentrations of 100 and 1000 ng l⁻¹ PAH when 100 μl of the stock solutions were added to a final volume of 200 ml diluent in each exposure vessel. Each stock solution was covered with aluminium foil to shield the contaminants from light and prevent further photo-degradation or activation. These stock solutions were stored in refrigerators at 4°C (± 2°C) until ready for use.

In parallel to the preparation of the PAH stock solutions, UV light-exposed PAH stock solutions were prepared in the same manner, however, they were prepared in 1.5 ml clear glass vials and were exposed to UVR at doses equivalent to a mid-summer’s day in the SW England (unweighted dose of 2.5 kJ m⁻²) within the UV exposure cabinet (described in section 2.2.1) at 20°C (±2°C). Following UV exposure, the irradiated stock solutions were covered with aluminium foil and all the stock solutions were stored in the dark at 4°C until ready for use.

Stock solutions of PAH (100 μl volumes), which were either irradiated and non-irradiated, were first added to 100 ml seawater held in glass evaporating dishes and mixed...
Table 5.1. Quantities of individual PAHs (µg/ml) in the mixed stock solution used in the experiments (dissolved in dimethyl sulfoxide) (source - CEFAS, UK).

<table>
<thead>
<tr>
<th>PAH</th>
<th>Quantity (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acenaphthene</td>
<td>1000</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>2000</td>
</tr>
<tr>
<td>Anthracene</td>
<td>100</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td>100</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>100</td>
</tr>
<tr>
<td>Benzo(b)fluoranthracene</td>
<td>200</td>
</tr>
<tr>
<td>Benzo(k)fluoranthene</td>
<td>100</td>
</tr>
<tr>
<td>Benzo(ghi)perylene</td>
<td>200</td>
</tr>
<tr>
<td>Chrysene</td>
<td>100</td>
</tr>
<tr>
<td>Dibenzo(a,h)anthracene</td>
<td>200</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>200</td>
</tr>
<tr>
<td>Fluorene</td>
<td>200</td>
</tr>
<tr>
<td>Indeno(1,2,3-cd)pyrene</td>
<td>100</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>1000</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>100</td>
</tr>
<tr>
<td>Pyrene</td>
<td>100</td>
</tr>
</tbody>
</table>
thoroughly with a glass rod. 50 ml of 5-Bromo-2-deoxyuridine (BrdU) solution (in seawater) was then added to the contaminant-seawater solutions, to produce a final working concentration of $1.0 \times 10^{-5}$ M in each of the sample vessels, for differential staining and therefore determination of proliferative rate index (PRI) and sister chromatid exchanges (SCEs), as described in section 4.1.1. On addition of 50 ml of the embryo-larvae suspension (in seawater) final concentrations of 0, 100 and 1000 ng l$^{-1}$ PAH complex mixture were produced. These concentrations were selected since they were found to be within environmentally realistic levels, previously measured in and around waters of England and Wales by Law et al. (1997). The final volume in each of the exposure vessels totalled 200 ml. The addition of 100 μl of the stock solutions ensured that the nominal concentration of solvent was not greater than 0.05% of the dilution water, as recommended by the American Society for Testing Materials (ASTM, 1997). Solvent controls (DMSO) were also run in parallel to the seawater controls and PAH treatments to determine any toxic effects of the solvent carrier.

5.3.3 Exposure of embryo-larvae

The embryo-larvae were added to the exposure vessels containing the contaminants at a density of 25-30 embryos per ml, as per ASTM recommendations. Each treatment was carried out in duplicate. During the period of exposure, the samples were shielded from light by covering the vessels with aluminium foil, to prevent potential photoactivation of the PAHs or the BrdU necessary for sister chromatid differential staining. They were maintained at a constant temperature by keeping them in incubators (Sanyo Gallenkamp, UK) for the duration of the exposures (15°C ± 2°C for M.edulis and 20°C ± 2°C for P.dumerilii).

The cell cycle kinetics had been previously determined as described in chapter 3, whereby one cell cycle was assumed to be 3.8 hours for M.edulis and 4.7 hours for
P.dumerilii. From these data the optimum exposure periods were calculated, therefore when fixing the embryo-larvae for the preparation of chromosomes for the evaluation of the induction frequency of sister chromatid exchanges (SCEs), two cell cycles were allowed to pass post-incorporation of BrdU to ensure the maximum frequency of cells in their second cell cycle.

5.3.3.1 Metaphase preparation and Chromosome Aberration (CAb) analysis

The methods of slide preparation, chromosome staining and chromosome analysis were adopted from studies by Harrison & Jones (1982) and described previously in section 4.1.1.1. In brief, after one and a half cell cycles (6 hours for M.edulis; 7 hours for P.dumerilii) each of the embryo-larvae samples were collected on nylon sieves (mesh size 30μm for M.edulis and 80μm for P.dumerilii), then exposed to 0.025% colchicine solution (w/v) dissolved in seawater for 30 minutes. The samples were then treated to a series of hypotonic solutions at a ratio of 2:1, 1:1, 1:2 and 1:3 (seawater : 0.075M KCl) for 10 minutes in each treatment. Following this, they were then fixed in cold Carnoy's fixative, which was removed and replenished a further two times before preparation of the metaphase spreads.

The metaphase spreads were prepared as previously described in section 4.1.1.2. Briefly, the fixed embryo-larvae were resuspended and a few drops of the sample transferred onto microscope slides. A few drops of 65% acetic acid were added to burst open the cells and continuously rotated to ensure even distribution of the chromosomes. The slides were dried over a hot plate held at 40°C and the chromosomes checked with a phase-contrast microscope using a x40 objective before staining of the chromosomes. From each sample, 4-5 slides were prepared.

5.3.3.2 Staining and scoring of chromosomes for CAb analysis

Metaphases were stained as previously described in section 4.1.1.2. In brief, following rinsing in distilled water, the slides were stained with 10% (v/v) Giemsa
solution in Giemsa buffer (pH 6.8) for 25 minutes. The excess stain was then rinsed twice from the slides by gently rinsing the slides in distilled water and then allowed to air dry. This was followed by the mounting of coverslips with DPX, the slides were labelled randomly and then scored blind. 100 metaphases per replicate were examined for aberrations using a bright-field microscope (Leica, DMR) at a total magnification of x1000 using oil immersion. Only complete metaphases were scored (2n = 28 ± 2). Chromosomal aberrations (CAbs) were expressed as the percent aberrant cells and the total frequency of aberrations (chromosome and chromatid type, excluding gaps), as previously described.

5.3.3.3 Differential staining and scoring for Proliferation Rate Index (PRI) and Sister Chromatid Exchanges (SCEs)

Embryo-larvae which had BrdU incorporated into the DNA were allowed to grow for two cell cycles (8 hours for *M. edulis*; 10 hours for *P. dumerilii*), before they were treated with colchicine, hypotonic solutions and fixed in Carnoy's fixative. Chromosomes were prepared from the samples in the same manner as that for chromosomal aberrations in section 5.2.3.1. The chromosomes were then treated for differential staining. Briefly, the slides were rinsed in distilled water, then immersed in 0.025% Hoechst 33258 solution (w/v) dissolved in phosphate buffered saline (PBS) solution (1 tablet dissolved in 200 ml distilled water) at room temperature (20 ± 1°C) for 20 minutes in the dark. This was followed by a double rinsing in distilled water and air drying. A few drops of PBS were applied to each slide, a coverslip added and the samples were then exposed to blacklight for 25 minutes whilst being held on a hotplate maintained at 55°C (±2°C). Following rinsing with distilled water, the coverslips were removed and the chromosomes on the slides were stained with 10% Giemsa stain for 15 minutes, before rinsing in distilled water, air drying and subsequent coverslip mounting with DPX mountant.
As executed previously, the slides were randomised, coded and scored blind for classification into 1st, 2nd and 3rd / 3rd + cell divisions for the calculation of PRI. Metaphases in the 2nd cell cycle displaying differential staining and harlequin chromosomes were scored for the induction of SCEs. A more detailed description of the individual steps in the methodology is described in section 4.1.1.3.

5.3.4 Development of embryo-larvae

Sub-samples of the embryo-larvae, not used for the preparation of chromosomes (for cytogenetic analyses) were allowed to grow until they reached 48 hours of age. Surviving larvae were then assessed for developmental effects following exposure to photoactivated PAHs. At 48 hours of age, the embryo-larvae were collected on a nylon mesh (30 μm for *M. edulis*; 80 μm for *P. dumerilii*), fixed in 10% formalin and transferred to 2.0 ml microcentrifuge tubes.

Sub-samples of the larvae were examined for morphological abnormalities in their development. Approximately 300 individuals per treatment were examined for *M. edulis*, however there were insufficient surviving larvae from *P. dumerilii* for viable statistical analyses to be performed.

5.3.5 Statistical analyses

Data were treated with the one-way analysis of variance (ANOVA) multivariate technique, wherever sufficient data points permitted and where the distribution was parametric. Non-parametric data was normalised by transformation (by log or square-root) and treated with ANOVA, assuming the variance between populations was p<0.05, as suggested by Scott & Clarke (2000). In these cases, the data were presented as plots of the mean (with standard deviation or 2 times the standard error where applicable). Where data could not be normalised, the non-parametric Kruskall-Wallis test was applied to compare
the medians between populations and in these cases, the data were presented as box-and-whisker plots as proposed by French & Lindley (2000).

Where the number of samples was limited, such as data collected for chromosomal aberrations, the data were treated with the Z-test to provide an indication of the level of significance between populations as proposed by Lovell et al. (1989). Regression analyses were applied wherever dose-dependent responses were observed as proposed by Smith & Anderson-Cook (2000). Statistical analyses were carried out using the statistical package Statgraphics Plus, Version 4.0 and Microsoft Excel XP. Bar graphs were constructed using Microsoft Excel XP, box and whisker plots were constructed using Statgraphics Plus 4.0.

5.4 Results

5.4.1 Effects of PAHs on the induction of chromosomal aberrations

5.4.1.1 Mytilus edulis exposed to PAH (photoactivated and non-photoactivated)

The frequency of CAbS was determined in at least 100 cells per replicate, with the number of cells exhibiting aberrations being expressed as the percentage aberrant cells and the total number of aberrations per treatment. For the non-photoactivated treatments, an increase in the percentage of aberrant cells was observed in embryo-larvae exposed to DMSO, 100 ng/l and 1000 ng/l compared with the seawater control, although neither PAH treatment showed a statistically significant increase compared with the seawater or solvent controls when the Z-test was applied to the data (p>0.05). There was a drop in the proportion of cells containing CAbS and the total frequency of CAbS at the highest dose of PAH (1000 ng/l), compared with the samples exposed to 100 ng/l. No dose-dependent relationship in the percentage of cells containing aberrations, or the total number of aberrations was observed, as illustrated in figures 5.2a and 5.2b respectively.

The dose-response was much more significant in samples exposed to photoactivated PAHs and increases in the percentage of cells containing CAbS were
observed in all the treatments compared with the seawater controls. However, when the Z-test was applied there was no statistically significant difference from the control.

When comparing the effect of photoactivated PAHs with non-photoactivated treatments, there was consistently an increase in the genotoxicity in samples exposed to photoactivated PAHs indicated by increases in the total frequency and proportion of cells with CAbs. However, there was only a statistically significant increase in the highest concentration (1000 ng/l PAH) for both the percentage of cells containing CAbs and the total frequency of CAbs (p<0.05).

5.4.1.2 Platynereis dumerilii exposed to PAH (photoactivated and non-photoactivated)

The frequency of CAbs was determined in the same manner as that for M.edulis in section 5.3.1.1. In contrast to the mussels, a significant dose-dependent increase in the frequency of CAbs was observed in P.dumerilii embryo-larval cells exposed to 100 ng/l and 1000 ng/l (R² = 0.9542). However, when the Z-test was applied to the data, there was no significant difference between the treatments when compared with the control samples (p>0.05). These results are presented in figure 5.3a. On the other hand, when considering the total frequency of aberrations per 100 cells, a statistically significant difference was detected in embryo-larvae exposed to 1000 ng/L PAH (p<0.02) when compared with the seawater control (figure 5.3b). For the total aberrations scored a dose-dependent increase was detected (R² = 0.9302).

In the samples exposed to photoactivated PAHs, there was a significant dose-response correlation (R²=0.9818) identified with an increasing proportion of cells containing CAbs with increasing photoactivated PAH dose (figure 5.3a) which matched the trend observed in samples exposed to non-photoactivated PAHs. When the Z-test was applied to the data, there was a statistically significant increase in the percentage of aberrant cells in the samples exposed to the 1000 ng/l photoactivated PAH treatments. A
Figure 5.2. The effect of photoactivated and non-photoactivated PAH exposure on (a) the proportion of cells containing chromosomal aberrations; and (b) the total frequency of aberrations in *M. edulis* embryo-larvae. (Error bars = SD; # denotes a statistically significant difference of the photoactivated treatments from non-photoactivated treatment at the same PAH concentration (p<0.05).
Figure 5.3. The effect of photoactivated and non-photoactivated PAH exposure on (a) the proportion of cells containing chromosomal aberrations; and (b) the total frequency of aberrations in *P. dumerilii* embryo-larvae. Error bars = SD; asterisks denote a statistically significant difference from the seawater control (* = p<0.05; **= p<0.02; ***= p<0.01).
similar tendency was observed in the total aberrations (figure 5.3b) scored. A very strong
dose-dependent correlation was observed for the samples exposed to photoactivated PAHs
($R^2=0.9987$) and highly significant increases in the total aberrations were observed in
samples exposed to $1000 \text{ ng l}^{-1}$ photoactivated PAH compared with the seawater controls
($p<0.01$).

Although there were consistently increased levels of chromosomal damage in
samples exposed to photoactivated PAHs compared with non-photoactivated PAHs, when
the data was treated with the Z-test to compare the effects of photoactivation, none of the
treatments displayed a statistically significant increase ($p>0.05$). The observed increases in
genetic toxicity in photoactivated PAH treatments were on par with those observed in
*M. edulis* embryo-larvae.

### 5.4.2 Effects of PAHs on the induction of sister chromatid exchanges (SCEs)

#### 5.4.2.1 *Mytilus edulis* exposed to PAHs (photoactivated and non-photoactivated)

For the SCE data, there were sufficient data replicates for comparison of the means
to be measured. The data was normally distributed and therefore ANOVA could be
applied. There was a statistically significant difference between all the treatments
(including the solvent control) and the seawater control ($p=0.031$). In the samples exposed
to non-photoactivated PAHs there was no significant difference between the different
concentrations of PAH and the DMSO control. A dose-dependent increase in the mean
frequency of SCEs induced per cell ($R^2 = 0.7716$) was observed (figure 5.4).
The dose-related response was not significant in the samples exposed to photoactivated
PAHs ($R^2=0.4877$) and a drop in the frequency of SCEs was observed in the samples
exposed to photoactivated $100 \text{ ng l}^{-1}$ PAH.

When assessing the effect of photoactivation of the PAHs (compared with non-
photoactivated PAHs), there was only a slight increase in the frequency of SCEs in the
Figure 5.4. The effect of photoactivated and non-photoactivated PAH exposure on the induction of sister chromatid exchanges (SCEs) in *M. edulis* embryo-larvae. Error bars = SD; * denotes a statistically significant difference from the seawater control (p<0.05). There was no significant increase in the induction of SCEs in photoactivated treatments compared with non-photoactivated treatments.

Figure 5.5. The effect of photoactivated and non-photoactivated PAH exposure on the induction of sister chromatid exchanges (SCEs) in *P. dumerilii* embryo-larvae. Error bars = SD; * denotes a statistically significant difference from the seawater control (p<0.05). There was no significant increase in the induction of SCEs in photoactivated treatments compared with non-photoactivated treatments.
seawater, DMSO and 1000ngl\textsuperscript{-1} PAH treatments. However, analyses with one-way ANOVA showed that this was not statistically significant (p>0.05).

5.4.2.2 Platynereis dumerilii exposed to PAHs (photoactivated and non photoactivated)

In \textit{P.dumerilii} embryo-larval cells exposed to non-photoactivated PAHs, there was a statistically significant increase in the induction of SCEs in all of the treatments when compared with the seawater control (p<0.00001), reflected by similar observations in \textit{M.edulis}. A dose-dependent increase in the mean number of SCEs induced per cell (\(R^2 = 0.8378\)) was observed, as can be seen in figure 5.5.

Samples exposed to photoactivated PAHs were significantly higher in all treatments compared with the photoactivated seawater control. The induction of SCEs in photoactivated PAH treatments (100 and 1000 ngl\textsuperscript{-1} PAH) were significantly higher than the photoactivated DMSO treatment (p<0.05), with a strong dose-dependent relationship being observed (\(R^2 = 0.9982\)).

When comparing the effects of photoactivated and non-photoactivated PAHs on the induction of SCEs in \textit{P.dumerilii} embryo-larval cells, there was no measurable increase in the level of genotoxic effect indicated by this end-point (figure 5.5).

5.4.3 Effects of PAHs on the proliferative rate index (PRI)

5.4.3.1 Mytilus edulis exposed to PAHs (photoactivated and non-photoactivated)

A dose-dependent decrease in the PRI value was observed from \textit{M.edulis} embryo-larvae which had been exposed to DMSO, 100 and 1000 ngl\textsuperscript{-1} PAH (\(R^2 = 0.9892\)), all of which were statistically significantly different from the seawater control (p<0.002). However, since there was a significant decrease in PRI between the seawater control and the solvent (DMSO) control, one-way ANOVA was carried out on the data to identify any significant differences between the solvent control and the PAH exposed samples. There
was a statistically significant difference between the PRI values in the DMSO control and the samples exposed to 1000 ng/l PAH (p<0.05). These data have been presented in figure 5.6.

A dose-dependent decrease in the PRI value was observed in the *M. edulis* samples exposed to photoactivated PAHs ($R^2=0.7746$), with all of the treatments (including the photoactivated DMSO) being significantly different from the photoactivated seawater control (p<0.05). However, the photoactivated PAH treatments were not statistically significant from the solvent control.

When comparing the effect of photoactivated PAHs with non-photoactivated PAHs, there was a significant decrease in the PRI values of photoactivated treatments compared with their non-photoactivated counterparts (with the exception of the seawater treatments).

### 5.4.3.2 *Platynereis dumerilii* exposed to PAHs (photoactivated and non-photoactivated)

The PRI in *P. dumerilii* embryo-larval cells did not exhibit the same response as those from the *M. edulis* embryo-larval cells. A decrease in the PRI was observed only in the cells from *P. dumerilii* embryo-larvae which had been exposed to 1000 ng/l PAH, however this was not statistically significant when tested with the z-test when comparing the means. The z-test was applied in this case due to the number of replicates per treatment being insufficient for ANOVA for comparison of the means. There was not a significant correlation between the dose and the PRI measured ($R^2=0.3255$). The mean results are presented in figure 5.7.

A dose-dependent decrease in the PRI value was observed in the *P. dumerilii* samples exposed to photoactivated PAHs with a strong correlation between dose and PRI value ($R^2=0.9747$), with all of the treatments (including the photoactivated DMSO) being significantly different from the photoactivated seawater control (p<0.05). However, the
photoactivated PAH treatments were not statistically significant from the solvent control when tested with the Z-test.

When comparing the effect of photoactivated PAHs with non-photoactivated PAHs, there was a significant decrease in the PRI values of photoactivated treatments compared with their non-photoactivated counterparts (with the exception of the seawater treatments) (p<0.05).
Figure 5.6 The effect of PAH exposure on PRI in *Mytilus edulis* embryo-larvae. (Error bars = SD; * denotes a statistically significant difference from the control; # denotes a statistically significant difference of the photoactivated treatments from non-photoactivated treatment at the same PAH concentration, p<0.05).

Figure 5.7 The effect of PAH exposure on PRI in *Platynereis dumerillii* embryo-larvae. (Error bars = SD; * denotes a statistically significant difference from the control; # denotes a statistically significant difference of the photoactivated treatments from non-photoactivated treatment at the same PAH concentration, p<0.05).
Plate 5.1. An abnormal prodissoconch *M. edulis* larva with a ‘pinched’ hinge (indicated with an arrow), observed in samples exposed to PAHs. Photograph taken at x400 magnification.

Figure 5.8. The effect of PAH exposure on the normal development of *Mytilus edulis* larvae aged 48 hours (trochophores and prodissoconch). Error bars = SD; * denotes a statistically significant difference from the control; # denotes a statistically significant difference of the photoactivated treatments from non-photoactivated treatment at the same PAH concentration, p<0.05).
5.4.4 Effects of PAHs on the development of embryo-larvae

5.4.4.1 *Mytilus edulis* exposed to PAHs (photoactivated and non-photoactivated)

The effects of PAH exposure on *M. edulis* larval morphology were determined in the same manner as that described in section 4.1.1.5. When the trochophore and prodissoconch larvae aged 48 hours were analysed for normal morphological development, a dose-dependent decrease in the proportion of larvae which showed normal development and no morphological abnormalities was observed ($R^2 = 0.6727$). It was noted that some prodissoconch larvae had abnormalities to the hinge of the shell, in addition to other deformities to the shell morphology and soft tissue which were observed in abnormal larvae in previous experiments where the embryo-larvae were exposed to ultraviolet radiation (section 4.1.1.5). Plate 5.1 illustrates an example of a prodissoconch larva with a 'pinched' hinge following exposure to PAHs. Using one-way ANOVA, a slight but statistically non-significant decrease in the proportion of normal larvae was observed in the sample exposed to 100 ng l$^{-1}$ PAH ($p > 0.05$) and a significant decrease in the proportion of normal larvae was observed in the samples exposed to 1000 ng l$^{-1}$ ($p < 0.00001$). This data has been presented in figure 5.8.

For samples which were exposed to photoactivated PAHs, there was not a strong dose-dependent relationship between the dose and the proportion of larvae which showed normal morphology, although all of the treatments (including the photoactivated solvent control) showed significantly decreased proportions of larvae with normal morphology. When comparing the effects of photoactivated with non-photoactivated PAH, a significant decrease of normal larvae were observed in samples exposed to DMSO and 100 ng l$^{-1}$ PAH treatments (figure 5.8).
5.4.4.2 *Platyneris dumerilii* exposed to PAHs (photoactivated and non-photoactivated)

The development of *P. dumerilii* embryo-larvae was not scored due to the insufficient numbers of larvae surviving for 48 hours post-exposure, making statistical analysis invalid.

5.5 Discussion

The life-stages at which exposure to a contaminant occur are highly relevant to the level of damage which is observed. A number of authors have reported the embryonic and larval stages as being the most sensitive life stages (Roy *et al.*, 2002). In the studies included in this chapter, there was evidence that the cocktail of PAHs exposed to the early life stages of the two target invertebrate species, *M. edulis* and *P. dumerilii*, were genetically, cytologically and developmentally detrimental, particularly at the higher concentration of exposure (1000 ng l\(^{-1}\) PAH). Furthermore, the photoactivation of PAHs prior to exposure of the early life stages of marine invertebrates can alter the genotoxic and cytotoxic capacity of the contaminants. Jha *et al.* (1995) indicated that the cytochrome P-450 enzyme systems may be present in the early life stages of *P. dumerilii*, although there is a paucity in reports of this enzyme system in the embryo-larval stages of invertebrate species (Livingstone, 1991). Therefore the present studies suggest that perhaps the lower concentrations of PAH contaminants used in these studies are not sufficient to induce the transforming enzymes in the early life stages of marine invertebrates, whereas higher concentrations have a more significant impact.

Benzo(*a*)pyrene (B[*a*]P) was one of the PAHs included in the mixture for exposures. As mentioned earlier in the introduction, this compound has often been used as a model PAH for toxicity studies. It has been reported that B(*a*)P induces aneuploidy in Chinese hamster cells, by causing incomplete mitotic spindle formation (Matsuoka *et al.*, 1998). In the studies described here, the chromosome number for each metaphase cell was not
measured for aneuploidy, however, this may have been a useful endpoint to have taken into account to identify the mechanisms of PAH activity on chromosomal integrity in marine bivalve cells.

5.5.1 Chromosomal aberrations (CAbs)

In the studies of this chapter, the induction of genotoxic responses in the form of CAbs was not distinctly related to the concentration of the PAH mixture and no dose-dependent relationship was observed. In contrast, when Jha et al. (2000a) exposed *M. edulis* embryo-larvae to B(a)P, a significant dose-dependent increase for the induction of CAbs was observed, with no significant increase caused by the solvent control. A similar trend was observed when *P. dumerilii* were exposed to B(a)P (Jha et al., 1996). In each of these studies by Jha et al. the carrier solvent used was dimethylformamide (DMF). Although, in the current studies, there was an increase in the frequency of CAbs in treated samples compared with the control, there also appeared to be a genotoxic effect being caused by the solvent DMSO. This effect was not expected since the quantity of the solvent in each sample did not exceed the critical 0.05% concentration implicated by the ASTM (1997). Other solvent carriers have also been used by authors investigating the effects of B(a)P including DMSO (Fossi et al., 1996; Matsuoka et al., 1998; Araújo et al., 2000), Tween 80 (Fossi et al., 2000) and methanol (Miller et al., 1988) and it was therefore not unusual for DMSO to be used as the solvent carrier for B(a)P. It is possible that the embryo-larvae of the two invertebrate species investigated here were sensitive to the solvent as well as the PAHs dissolved in it. However, although the statistical analyses did not indicate a difference between the induction of CAb induction in the PAH-treated samples and the solvent controls, there was nonetheless an increase at the higher dose of PAH (1000 ng/l) suggesting that at these concentrations of PAH, the embryo-larval stages of invertebrates were able to transform the PAHs into active xenobiotics, as evidenced by studies by Dixon et al. (1985), Hose et al. (1985) and Jha et al. (1996, 2000a).
The disparity between the induction of CAbs and dosage of the PAH mixture in *M. edulis* samples, may have been due to low concentrations of the contaminants being insufficient to induce DNA damage detectable with CAbs. On the other hand, when the response to non-photoactivated 1000 ng/l PAH is taken into consideration, there was in fact a reduction in both the proportion of CAb-containing cells and the total frequency of CAbs when compared with the response observed in samples exposed to the lower dose of 100 ng/l PAH in *M. edulis*. This may have been due to the contaminants causing a toxic effect which caused death of the cells and therefore fewer surviving cells could be found with CAb. Another cause may have been an inhibition of the cell cycle due to toxic effects and therefore fewer daughter cells reaching the metaphase stage during the first subsequent division, this theory is supported by the decrease in PRI with increasing dose. The absence or reduction in the cells would result in a score of CAbs lower than that which would be expected from lesions which had formed during the interphase part of the cell cycle. Galloway *et al.* (1985) emphasised the importance of taking into account the potential of a test chemical to cause cell cycle delay and recommended the harvesting of cells later than the first mitosis after treatment. In the current experiments, the cells were harvested after one and a half cell cycles for CAb analysis as recommended by Scott *et al.* (1990).

However, a strong dose-dependent relationship was observed in photoactivated PAHs for percentage aberrant cells ($R^2=0.8909$) (figure 5.2a), a less significant relationship being observed for the total aberrations induced ($R^2=0.663$). These data suggest that perhaps some experimental disparity could have occurred when exposing the samples to non-photoactivated 1000 ng/l PAH. Significant increases in CAb induction were observed in both the proportion of aberrant cells and the total aberrations, correlating well with previous work by Jha *et al.* (2000a).

In the experiments conducted with *P. dumerilii* embryo-larvae, a strong dose-dependent correlation was observed for both the proportion of aberrant cells and the total
frequency of aberrations in each of the non-photoactivated and photoactivated PAH treatments. These responses correlate with work undertaken on this species by Jha et al. (1996, 1997, 2000b) and Hagger et al. (2002) where the embryo-larval stages were exposed to organic contaminants or varying concentrations of sewage.

5.5.2 Sister chromatid exchanges (SCEs)

The induction of SCEs appeared to be a sensitive endpoint in these experiments. In both species, a dose-dependent increase was observed for the frequency of SCEs that were induced per cell. The intercellular range of the frequency of SCEs induced also increased with the dose of PAH, with a maximum frequency of 9 SCEs in cells at the highest dose (1000 ng/L PAH). In studies by Jha et al. (2000a) where M.edulis embryo-larvae were exposed to B(a)P, a parallel trend was observed with a significant increase for the induction of SCEs was observed with a strong dose-dependent relationship. The maximum frequency of SCEs induced at the highest concentration of 10 x10^-5 M B(a)P in this study was 18. Furthermore, a study by Harrison & Jones (1982) produced dose-dependent increases of SCEs when M.edulis were exposed to various mutagens.

When P.dumerilii were exposed to a range of doses of B(a)P, a similar dose-dependent increase to that seen in M.edulis was observed (Jha et al., 1996). Once again, an increase in the intercellular range of the frequency of SCEs with increasing B(a)P concentration was noted.

Since the mechanisms of CAb and SCE formation are different (SCEs being a S-phase dependent phenomenon), it is possible that the SCEs are induced by PAHs more readily than CAbs. SCEs come from unremoved adducts, whereas CAbs are most likely the result of double-stranded breaks in DNA (Wolff, 1982; Natarajan et al., 1993) and it is well-known that a number of PAH contaminants induce DNA adducts.

In the studies included in this chapter and others already mentioned where the SCEs were investigated in M.edulis and P.dumerilii (Jha et al., 1996, 2000a, 2000b), there
was some variability in the baseline levels of SCEs measured in the negative controls. For example, in *M. edulis*, the mean frequency of SCEs in control samples ranged from 0.5 to 2.34 per cell. This emphasises the importance of running a negative control within each study and limits the appropriateness of comparing directly the induction frequencies of SCEs between different studies. The baseline levels from each study should always be taken into account. The potential for high levels of variability in the induction of SCEs was emphasised by Jones and Harrison (1987), where *M. edulis* larvae were collected and the frequency of SCEs per chromosome were measured over a two year period. Although the animals were collected from the same field site and maintained in the same manner throughout the experimental period, there were significant variances in the frequency of SCEs between sample periods. The authors suggested that the adults which produce the offspring may have had an influence upon the frequencies of SCEs in the larvae and perhaps exposure of different adults caused the differences in SCE induction. Furthermore, Pasantes *et al.* (1996) reported an increase in the induction of SCEs in the gill cells of *M. galloprovincialis* where cells were exposed to BrdU for 2 consecutive cycles of labelling. Also, they found that significant differences in the frequencies of SCEs were detected between animals collected from field sites during different months. This implies that seasonal factors may also affect the level of SCEs induced. The concentration of the BrdU is also an important factor since BrdU itself is mutagenic (Kato, 1974) and has been shown to cause a reduction in the development rate of mussel embryos (Dixon & Pascoe, 1994).

5.5.3 Proliferation Rate Index (PRI) and Development effects

It is well known that the different phases of the cell cycle are delayed following treatment with clastogens and arrest at different stages of the cell cycle allows for the repair of DNA damage (Palitti, 1998). The maintenance of genomic integrity is vital for cell survival and proliferation, if the damage to the cell is too severe to be adequately
repaired the cell is likely to undergo apoptosis or enter an irreversible state resembling senescence (Shackelford et al., 1999). The PRI in the current studies were reduced in both species investigated when exposed to the higher dose of 1000 ng/L PAH, indicating the contaminants had a cytotoxic effect upon the embryo-larval cells and delayed the cell cycle kinetics. This correlates with reports of B(a)P and other PAH carcinogens, which generate bulky DNA adducts and apurinic sites and have been shown to result in the inhibition of DNA synthesis and the induction of S phase cell cycle arrest in mammalian cells (Kaufmann, 1995; Shackelford et al., 1999). In studies of B(a)P exposure (Jha et al., 2000a) and organotin compounds (Hagger et al., 2002), M.edulis and P.dumerilii showed dose-dependent decrease in PRI values respectively. This endpoint was originally proposed by Lamberti et al. (1983) for application to actively proliferating and highly differentiated mammalian cells in vitro. The PRI for embryo-larvae cells in vivo represents an average figure based upon a mixed population of differentiating cells with differing mitotic rates over the exposure period described (Jha et al., 1996).

The cell kinetics are directly linked to cell division and hence the rate of growth (Raven & Johnson, 1996). Impacts on the rate of growth of aquatic invertebrates by B(a)P exposure, have been implicated to be a buffering effect of contaminant exposure, where reproductive maturity of Daphnia magna have been noted to be attained at a smaller body size, compared with control animals (Lynch, 1985). As well as influencing the rate of growth, environmental pollutants including genotoxins have been reported to induce malformations in the early life stages of invertebrates and vertebrates (Hose et al., 1982, 1984; Kubota et al., 1992; Sundelin & Eriksson, 1998; Brown et al., 1999). Fish embryos exposed to weathered crude oil containing PAH contaminants resulted in malformations, genetic damage, mortality, decreased size and inhibited swimming (Carls et al., 1999). In the current studies, M.edulis showed a dose-related decrease in the proportion of larvae which exhibited normal morphology and development. A significant decrease was
observed at the higher dose of PAH (1000 ng/L PAH), which could have significant detrimental effects on the chances of survival of the individuals to adult stages.

In terms of survival, the larval stages (glochidia) of the freshwater mussel *Utterbackia imbecillis* were found to have significantly reduced median lethal times, when exposed to fluoranthene and UV simultaneously, compared with samples exposed to the contaminant on its own. The author of this work (Weinstein, 2001, 2002) also reported that the ability to repair photoactivated polycyclic aromatic hydrocarbon damage, was not influenced by the period of darkness which followed the exposure period.

Unfortunately, insufficient surviving embryo-larvae from *P.dumerilii* were present at 48 hours post-exposure for the results to be collated and statistical analyses to be performed. This shows how the number of eggs produced by each female can limit the use of *P.dumerilii* as test species compared with *M.edulis*, the females of which can produce 3-4 times as many eggs as the polychaete. When the studies of this chapter were undertaken multiple endpoints were measured and the cytogenetic endpoints were prioritised over the measurements of morphological development. Therefore the frequency of fertilised eggs was a limiting factor upon the number of endpoints which could be measured from the same batch of biological material.

5.5.4 Photoactivation of PAH contaminants

Traditionally the toxicological effects of PAHs have focused upon the metabolic activation of these compounds to metabolites which have been found to be more toxic than the parent compounds (Arfsten *et al.*, 1996). However, the studies described in this chapter and of numerous other authors, provide evidence for an increase in toxicity of PAHs when UVR is able to photoactivate the contaminants (as described earlier in section 1.5.3). Aquatic organisms face a relatively high risk of the acute effects of PAH phototoxicity, compared to terrestrial animals, since they are continually in contact with the aqueous phase with relatively higher concentrations of PAHs. Episodic events such as oil spills, or
dredging of sediments may cause a sudden release of large quantities of associated PAHs into the water column and this in conjunction with ultraviolet radiation could potentially have a significant impact on marine biota (Arfsten et al., 1996).

Here, in both *M. edulis* and *P. dumerilii* species an elevation in the genetic toxicity of the PAH mixture was observed when examining chromosomal aberrations as the endpoint. SCEs on the other hand did not show any significant changes in genotoxicity between the photoactivated and non-photoactivated treatments. It would be necessary to investigate any transformations of the chemical structure of the PAHs involved to fully understand the mechanisms involved with the genotoxic lesions measured. When both of these endpoints have been measured in the past, indications have been that SCEs are a more sensitive endpoint compared with CAbs (Dixon, 1983). There is a paucity of literature where the genetic toxicity of photoactivated contaminants in the early life stages of aquatic invertebrates has been investigated, although genotoxic endpoints have been measured in adult stages. In a study carried out by Steinert *et al.* (1998), mussels were attached to different vertically orientated lines at different depths at two field locations, one being a reference site, the other at a location known to have high concentration of PAHs. At each location half the deployed population was arranged so that it was exposed to sunlight, the other half placed in the shade. The results of this study showed a consistently higher level of DNA damage in animals exposed at the PAH-contaminated site with simultaneous sunlight exposure. The interactive effects of UVR and PAH contaminants in adult stages will be discussed in further detail in chapter 6.

In contrast to genotoxic endpoints, survival as a measure of toxicity has been measured in the early life stages of several invertebrate species by other authors (Pelletier *et al.*, 1997; Weinstein, 2002). However, due to the high juvenile mortality rates in many benthic marine invertebrate species noted by Gosselin & Qian (1997), the normal morphological (anatomical) development of embryo-larval stages of the target species was used as an endpoint to identify whole organism responses to exposure to agents, as
opposed to survival rates (as previously mentioned in section 1.7.4.1). The normal morphology of larvae aged 48 hours was measured in *M. edulis* and indicated that photoactivated PAHs had an increased detrimental effect on the normal development of the D-shell larvae compared with samples exposed to PAH in the absence of light.

The level of impact caused by the introduction of PAH contaminants into the aquatic environment (e.g. by oil spills) can be influenced by the type of oils present and the sink for the contaminants. Contaminants which gather in sediments have the potential of accumulating in benthic organisms. Such benthic organisms in turn often produce pelagic larvae (Pelletier *et al.*, 1997), hence the impact of PAH exposure may affect later generations.

### 5.6 Conclusions

The studies in this chapter in addition to those carried out previously, indicate that a mixture of PAHs can cause genotoxic effects detectable by increases in CAbs and SCE induction; cytotoxic effects detected by cell cycle kinetics’ studies; and developmental effects in the early life stages of marine invertebrates. Also, as supported by other work, this provides evidence that UVR can increase the toxicity of PAH contaminants measured by cytogenetic endpoints as well as morphological parameters.

The findings of this chapter suggest that the embryo-larval stages of the two species investigated here were sensitive to the solvent carrier and it would be useful to identify the level of toxicity caused by DMSO and compare this to other solvent carriers. Regardless, there is clear evidence that PAH contaminants cause detrimental effects upon the DNA and other cellular components of the marine invertebrates studied here. There is a need to clearly identify the link between the types of lesions formed within the DNA and the mechanisms that are then manifested as CAbs or SCEs, due to PAH contaminant exposure in the early life stages. There is evidence here that the embryo-larval stages are capable of activating promutagens to genotoxic metabolites, as have been indicated by
previous authors (Dixon *et al.*, 1985; Hose, 1985; Jha *et al.*, 1996, 2000). Chemical analyses of the biological material post-exposure and measurements of the enzyme levels would further complement these studies.

Although high levels of variability have been identified for the induction of SCEs, this assay is nonetheless a useful and sensitive method for detecting DNA damage at the chromosomal level of organisation (Dixon & Clarke, 1982; Harrison & Jones, 1982; Tucker & Preston, 1996). In this chapter, indications are that SCEs are induced more readily than CAbs at lower concentrations of PAHs and therefore this cytogenetic endpoint is more sensitive.

Indications are that the interaction of UVR with chemical contaminants should always be considered in toxicity testing, particularly when carrying out laboratory based exposures under fluorescent lighting. Such studies are particularly pertinent when the results may be used to predict responses of organisms to pollutant exposure in the natural environment.
Chapter 6

PAHs & UVR: An investigation of the interactive toxic effects in the adult life stages of marine organisms
Hypotheses:
(i) Polycyclic aromatic hydrocarbon exposure causes genotoxic effects detectable in adult marine organisms.
(ii) Ultraviolet radiation interacts with chemical contaminants causing alterations in their toxic potential to adult marine invertebrates.

6.1 Introduction

The bioaccumulation mechanisms of benzo(a)pyrene (B[a]P) and other carcinogenic polycyclic aromatic hydrocarbons (PAHs) in marine organisms are of high relevance to aquaculture and in the utilisation of marine resources for human consumption. It is therefore imperative that the fate of such organic contaminants within these organisms is understood (Okay et al., 2000). While some organic (and non-organic) contaminants are directly mutagenic, others such as B(a)P and some other hydrocarbons are found to be mutagenic only after they are activated to mutagenic derivatives by monooxygenases or conjugative enzymes.

Such xenobiotics are considered to be “procarcinogens”, whereby they have been shown to induce malignant transformation only when metabolised within the endoplasmic reticulum of the cells (Gricuite, 1979; Jacob & Grimmer, 1983; López-Barea & Pueyo, 1998). Figure 6.1 summarises the four possible pathways that Canova et al. (1998) proposed for the metabolic activation of B(a)P in mussels. Pathway 1, is the most widely accepted activation pathway and is based on ring oxidation to diolepoxides. More specifically, the (+)-anti-B(a)P-7,8-diol-9,10 epoxide which forms stable DNA adducts has been widely studied for its mutagenic and carcinogenic potential (Buening et al., 1978; Stegeman, 1981; Wei et al., 1998). It was once thought that the hydration of PAH epoxides was a ‘detoxification’ mechanism of PAHs within the organism, however, it is now known that dihydrodiols may serve as substrates for monooxygenases, some of which are regarded as the actual effective carcinogen (Jacob & Grimmer, 1983). This pathway also leads to the production of phenols, diols and tetrols (Sims et al., 1974). Quinones are regularly found as metabolites of PAHs and following exposure to B(a)P, benzo(a)pyrene-1,6-, 3,6- and 6,12-quinone have been identified as metabolic products (Jacob & Grimmer,
Figure 6.1. Possible pathways for the activation of B(a)P resulting in lesions in vivo. (DD = dihydrodiol dehydrogenase; ROS = reactive oxygen species). Re-drawn from Canova et al., 1998.
These are formed when pathway 2 is followed, whereby free radicals are produced which cause oxidative damage and DNA fragmentation (Garcia Martinez & Livingstone, 1995; Devanesan et al., 1996; Flowers et al., 1996). Pathway 3 involves the formation of radical cations, possibly mediated by the cytochrome P450 or by peroxidative reactions (Cavalieri & Rogan, 1995). Alternatively, pathway 4 may be followed, which results in the formation of the ultimate DNA-reactive metabolite, benzylic carbenium ion due to endogenous methylation of B(a)P (Stansbury et al., 1994).

A combination of the lipophylic nature of many organic contaminants, the binding of many such contaminants to organic matter and the filter-feeding life-style of bivalve molluscs renders such animals suitable target organisms for organic contaminant studies. In work undertaken by Tenore et al. (1973), the feeding rates of three bivalve mollusc species, the mussel *Mytilus edulis*, the American oyster *Crassostrea virginica* and the hard clam *Mercenaria mercenaria* were compared. Of these three species, the mussel was found to have the highest feeding rate, indicating the high level of filtration of suspended organic particulates from the water and therefore its high potential for bioaccumulation of PAH contaminants. The ability for filter-feeding molluscs to bioaccumulate PAHs is of concern should they be used for human consumption. PAHs with high molecular weights tend to have higher octanol-water partition coefficients and therefore have correspondingly higher bioaccumulation potential and bioconcentration factors (Law et al., 1997).

Benthic organisms are readily exposed to sediment-bound contaminants via the interstitial water (Kaag et al., 1998) and marine organisms including both vertebrates and invertebrates appear to receive most PAH exposure directly from the water (Law et al., 1997). Fish from areas where sediments are contaminated with high levels of PAHs have been found to contain high incidences of liver cancer, as well as severely modulated immune responses, altered leukocyte counts, suppressed chemotactic, phagocytic and
chemoluminescent responses (Weeks & Warinner, 1986; Moore et al., 1989; Myers et al., 1992; Faisal & Huggett, 1993; Baumann & Harshbarger, 1995; Holladay et al., 1998). Furthermore, observations of reduced numbers of macrophage aggregates, altered lymphoproliferative responses to mitogens and aberrant cytotoxic cell activity have been reported (Payne & Fancey, 1989; Faisal et al., 1991, 1991a). Fish respond in a manner comparable to mammalian test species to chemicals that induce peroxisome proliferation and oxidative damage in hepatocytes. Furthermore cytochrome P-450 monooxygenases may metabolise carcinogens into more toxic metabolites (Al-Sabti & Metcalfe, 1995).

In crabs (Carcinus aestuarii) exposed to benzo(a)pyrene and other organic contaminants, a number of biomarkers were used to detect exposure effects by Fossi et al. (1996, 2000). DNA damage was found to be elevated in all exposure groups using the alkaline unwinding assay in the hepatopancreas tissue and significant increases in the frequency of micronuclei was detected in samples from the highest treatment group (1ppm B[a]P). Monoxygenase activity was significantly increased in hepatopancreas and gill tissue and porphyrin concentrations were significantly elevated in the hepatopancreas tissue from exposed samples. Furthermore, significant differences were observed in the heart rate of animals exposed to 1ppm B(a)P compared with the control group. This study provides an indication of the types of physiological responses which can be detected in adult invertebrates, following exposure to PAH contaminants.

A number of studies have examined the induction of micronuclei (MN) for the testing of compounds as well as waste waters. The MN test is used to detect genetic damage in cells by visual scoring of the number of cells that contain micronuclei. A significant number of aquatic invertebrate studies have featured the use of the MN assay in both laboratory and field conditions and have been comprehensively reviewed by Dixon et al. (2002). The incidence of micronucleated cells have been found to increase in the gill tissue and haemocytes of marine and freshwater mussels treated to various metal and organic compounds (Majone et al., 1987; Brunetti et al., 1988; Scarpato et al., 1990;
Wrisberg & Rhemrev, 1992; Venier et al., 1997; Bolognesi et al., 1999; Pavlica et al., 2000), although it has been suggested that the technique requires further improvement before being applied to environmental monitoring (Burgeot et al., 1995, 1996).

The MN assay has been applied to cultured rainbow trout gonadal (RTG-2) cells for evaluating the genotoxic potential of industrial waste waters (Kohlpoth et al., 1999). Furthermore, the assay has been used in in vivo studies in various fish species. For example, erythrocytes from *Clarias gariepinus* have been reported to contain elevated levels of MN following exposure to γ-radiation and methyl methane sulfonate (Bahari et al., 1994) and in *Heteropneustes fossilis* following exposure to mitomycin C and paper mill effluents (Das & Nanda, 1986). However, some species have been found to be insufficiently sensitive from field exposures, such as the wild white croaker *Genyonemus lineatus* (Carrasco et al., 1990). PAHs have been reported to have a significant genotoxic effect detected with the MN assay in larvae of the amphibian *Pleurodeles waltl*, with particularly strong effects observed in animals exposed to B(a)P compared with effects caused by naphthalene exposure (Djomo et al., 1995; Békaert et al., 1999).

The single cells gel electrophoresis (SCGE) or ‘comet’ assay has been applied to the adult stages of both aquatic invertebrates and vertebrates. Steinert (1996) investigated the potential for the comet assay to identify apoptosis caused by in vitro exposure of haemocytes to copper, B(a)P and UVR collected from the bivalve species *M. edulis*. In the same species, the comet assay has been used to quantify DNA damage in the gill cells (Wilson et al., 1998) and digestive gland cells (Mitchelmore et al., 1998a, 1998b). In addition to laboratory exposures, field studies have also been undertaken to identify a relationship between DNA damage and the exposure of marine mussels to environmental contaminants (Steinert et al., 1998a). In vivo exposures of other bivalve species such as oysters (*Crassostrea virginica*), scallops (*Pactunopecten yessoensis*), and clams (*Tapes japonica*) also have been conducted where the animals were exposed to PAH spiked sediments, or organic contaminants and the comet assay subsequently carried out on...
haemocytes or gill cells collected from the animals (Nacci et al., 1996; Sasaki et al., 1997).

In addition, fish species have also been used with the comet assay. A number of studies have been carried out using different cell types of freshwater fish such as bullheads (*Ameiurus nebulosus*) and carp (*Cyprinus carpio*), brown trout (*Salmo trutta fario*), zebra danio (*Brachydanio rerio*), and rainbow trout (*Onchorynchus mykiss*) (Pandrangi et al., 1995; Belpeame et al., 1996; Deventer, 1996; Devaux et al., 1997). However, relatively few studies involving marine fish have been documented. Belpeame et al. (1998) undertook a series of validation studies of the comet assay in various tissue types (blood, gill, liver and kidney) in the marine flatfish turbot (*Psetta maximus*) and found the assay to be a simple and sensitive method for the detection of DNA damage. Furthermore, Nacci et al. (1996) carried out a comprehensive study where blood cells from the flounder *Pleuronectes americanus* were exposed *in vitro* to hydrogen peroxide and *n*-methyl-*n*-nitro-nitrosoguanidine (MNNG), in addition to *in vivo* exposures of the same species using spiked food and sediment, in this work three different tissue types were used (blood, liver and intestine). Flatfish are particularly pertinent for monitoring purposes since these species reside close to the sediments which are known sinks for contaminants (Cotelle & Férard, 1999).

Haemocytes are distributed throughout the open vascular system and are considered to be the main target for neoplastic diseases in mussels (Mix, 1986). It is relatively uncomplicated to collect haemocytes from bivalves, as well as blood from fish, for these reasons this tissue type was the subject of analysis for this chapter. The use of haemocytes is advantageous over that of other tissues in that extraction of this cell type can be carried out in a non-destructive manner, consequently individuals can be returned to the population following haemolymph extraction. This is particularly significant where populations are small or sensitive.
The adult stages of many aquatic species are not as significantly affected by ultraviolet radiation (UVR) exposure compared with early life stages, due to their ability to move away from areas of high irradiation into shaded areas, the presence of protective pigments and well developed carapace or skin. Benthic species in particular are unlikely to be at risk from UVR, due to the attenuation of UVR through the water column, and in water with high suspended organic material UVR will not penetrate to any significant depths (Siebeck et al., 1994; Fabacher & Little, 1995; Steeger et al., 2001).

As mentioned in the previous chapter the photoactivated increase in toxicity to aquatic organisms of a number of polycyclic aromatic hydrocarbons (PAHs) has been well documented and it has been shown that some PAHs are acutely toxic at concentrations below their aqueous solubilities when exposed to ultraviolet radiation (UVR) (Newsted & Giesy, 1987). The impact of photo-induced toxicity in the early life stages of aquatic organisms have been reviewed in chapter 5. The effects in the adult life stages of aquatic organisms are also of relevance and increases in PAH toxicity have been reported in a number of aquatic species.

A number of investigations have been made to examine photo-altered toxicity in invertebrate species. Studies by Steinert et al. (1998a, 1998b) indicated that sunlight increased the level of DNA damage induced in mussels exposed to PAHs in laboratory and field conditions when compared with mussels exposed to PAHs only. The oligochaete Lumbriculus variegatus was found to have an increased rate of mortality when placed under UV light following exposure to water-borne anthracene. The mortality rates were found to be a function of the intensity of the light as well as the dose of the PAH (Ankley et al., 1994, 1995; Monson et al., 1995). In the same study the midge Chironimus tentans was found to be relatively insensitive to simultaneous UV and PAH exposure, the authors suggested that this may be due to this species being able to metabolise and excrete the PAHs that cause phototoxicity more efficiently. Furthermore, it was noted that C.tentans has an integument that decreases the penetration of UV light. A further postulation was
that this species has relatively efficient systems for managing oxidative stress induced by photoactivation of PAHs.

Photoactivation of PAHs has also been reported in aquatic vertebrates. Increased reactive oxygen species production and lipid peroxidation were observed in the bluegill sunfish *Lepomis macrochirus* when they were exposed simultaneously with UVR and anthracene (McCloskey & Oris, 1991; Choi & Oris, 2000). It has also been suggested that a decline in the catch of the Atlantic salmon *Salmo salar* might be attributed to the photo-induced toxicity of PAHs (Scott, 2001).

In a study by Fernandez & L'Haridon (1992), newt larvae (*Pleurodeles waltl*) were exposed to a variety of PAHs with or without near ultraviolet light (UV-A). Genotoxicity was evaluated using the micronucleus assay in erythrocytes, however, there was no detectable change in toxicity in animals treated with contaminants with simultaneous UV-A. In contrast, a series of experiments were carried out whereby PAH solutions were pre-exposed to UV-A for 24 hours, then exposed to the newt larvae, and in these experiments the samples proved to be genotoxic.

### 6.2 Aims and objectives

The objectives of the studies present in this chapter were to evaluate the genotoxic and cytotoxic potential of water-borne benzo(a)pyrene in adult *Mytilus edulis* and a cocktail of PAHs administered via food to *Platichthys flesus* respectively. The aim was to examine the hypothesis that PAHs cause genetic damage in the adult life stages of marine organisms and these changes in genetic integrity could be detected using the micronucleus and comet assays. These techniques were applied to haemocytes extracted from *M. edulis* which had been exposed to water-borne benzo(a)pyrene for 6 days and *P. flesus* which had been exposed to a mixture of PAHs, exposed via their food for 11 months. Cytotoxicity was determined in the haemocytes of *M. edulis* with the neutral red lysosomal retention (NRR) assay.
Furthermore, a series of studies were carried out with adult *M. edulis* to evaluate the genotoxic or cytotoxic effects of UVR exposure in simultaneous combination with benzo(*a*)pyrene. The aim of this was to examine the hypothesis that UVR interacts with chemical contaminants altering their toxic potential in adult invertebrates. To achieve this aim, genotoxicity was measured by assessing the induction of micronuclei and determining the levels of DNA damage using the comet assay in the haemocytes of *M. edulis*; cytotoxicity was determined by measuring the lysosomal retention time of the supravital dye, neutral red in the haemocytes of *M. edulis*. The data were then compared with that collated from the studies undertaken where benzo(*a*)pyrene exposure was examined in isolation, to determine whether or not any changes in toxicity had occurred.

The penetration of biologically active UV-B radiation through the shell of adult *Mytilus edulis* was also evaluated, by measuring UV-B levels passing through an empty shell. Furthermore, chemical analyses were undertaken to determine the level of change in the chemical structure of benzo(*a*)pyrene following exposure to UVR.

### 6.3 Materials & Methods

The experimental protocol for the determination of the effects of B(*a*)P and simultaneous B(*a*)P and UVR exposure on adult *M. edulis* is summarised in figure 6.2. Haemolymph samples were collected on day 0, prior to the commencement of the exposure of the mussels, for the micronucleus, comet and neutral red retention assays. Following three days of exposure, haemolymph samples were collected and the micronucleus and comet assays were conducted, and water changes and replenishment of the chemical contaminants were carried out. Further haemolymph samples were collected for the micronucleus and neutral red retention assays on the sixth day. Each of the three assays could not be conducted at each sampling time due to restrictions imposed by simultaneous management of all three assays by one investigator.
Figure 6.2 Flow chart representing the protocol for the exposure of adult marine mussels (*M. edulis*) to B(a)P or B(a)P + UVR with simultaneous UVR and the endpoints measured at each stage.
6.3.1 Exposure of adult *M. edulis* to B(a)P

Adult *M. edulis* were collected from their natural beds and allowed to acclimatise in laboratory conditions for a minimum of 24 hours, as described in section 2.1.1.1 prior to the commencement of the experiments. Random animals of approximately similar shell size (approximately 4 cm in length) were selected and numerically labelled by attaching plastic Dymo™ labels to the outer surface of the shell with superglue® (Bostik, UK) adhesive.

Test solutions for the exposures were prepared from stock solutions of B(a)P at a concentration of 1mg per ml dimethyl sulfonate (DMSO). Following preparation, the stock solutions were stored in amber glass vials, covered in foil and maintained at 4°C prior to use. Both B(a)P solutions and DMSO solvent controls were added to seawater at a volume of 100 μl DMSO per litre seawater, ensuring a concentration well below that of the 0.05% maximum percentage of solvent in dilution water recommended by the American Society for Testing Materials (ASTM, 1997).

Once acclimatised, adult mussels were introduced to 2 litre glass beakers at a density of 3 animals per beaker, containing 1.5 litre filtered (10 μm carbon filter) seawater held at 15°C (±2°C) which the DMSO or B(a)P solutions had been added to produce final doses of 1μg/l and 10μg/l B(a)P. The beakers were covered with aluminium foil to shield the test water from light. The test solution in each beaker was continuously aerated via plastic airlines, to the ends of which glass pasteur pipettes were attached to prevent adsorption of the B(a)P to the plastic airlines. Two beakers per treatment (i.e. 6 animals per treatment) were set up for the exposures. After 3 days of exposure, haemolymph samples were collected from the mussels, the test water discarded and replenished with new seawater re-dosed with DMSO or B(a)P as appropriate and the animals returned to the beakers.
6.3.2 Exposure of adult *Mytilus edulis* to B(a)P and simultaneous UVR

Adult *M. edulis* were collected from Sharrow Point, labelled and prepared for exposure as described in per section 6.2.1. The test solutions for the exposures were prepared from stock solutions of B(a)P in the same manner as section 6.2.1. Once acclimatised, mussels were introduced to 2 litre glass beakers at a density of 3 animals per beaker, containing 1.5 litre filtered (10 μm carbon filter) seawater held at 15°C, to which the DMSO or B(a)P solutions had been added. The test solution was aerated via plastic airlines to the ends of which were attached glass pasteur pipettes. Each beaker was covered with a circle of filter material, cellulose diacetate, which allows UV-B light to pass through but excludes light of wavelengths within the UV-C range for simultaneous exposure to UVR. Each circle of filter film was held in place on the beaker with small pieces of Blu Tack® (Bostik, UK) in a similar manner as that of the early life stages exposed to UVR described in section 2.4.4.1. Two beakers per treatment (i.e. 6 animals per treatment) were set up for the exposures. The samples were simultaneously exposed to ultraviolet (UV) radiation by placing the vessels containing the target organisms in the exposure cabinet described in 2.4.1. The UV and white lamps were automatically switched on and off by timer switch with illumination periods of 12 hours on, and 12 hours off to represent daily fluctuations of light illumination comparable to outdoor conditions, providing a UV dose equivalent to a mid-summer’s day in SW England. The animals were exposed for 3 days before haemolymph samples were collected from them.

6.3.3 Measurements of UV-B penetration through the shells of adult *M. edulis*

It was assumed that the pigmentation and the thickness of the shells of adult marine mussels (*M. edulis*) would provide protection to the sensitive soft tissues and would not allow the penetration of UV-B. This hypothesis was tested by sacrificing a set of 10 adult mussels measuring 4.5-5.5 cm in length, the outer surface of which had been cleaned of epibionts. The maximum shell length, breadth and width was measured for each
individual using graduated callipers and recorded. The soft tissue from each animal was then excised from the shell using a scalpel, taking care not to damage the shell. Each shell was then placed within the UVR exposure cabinet in a position which covered the spectroradiometer sensor surface. The lamps were then switched on and allowed to burn for at least 30 minutes before measurements of UV-A and Setlow weighted UV-B were taken using the spectroradiometer (Macam, UK) with and without each shell covering the UVR detector.

6.3.4 Chemical analyses

It is known that the exposure of aromatic compounds to light results in the production of partially oxidised intermediates which are more susceptible to biodegradation than the parent compounds (Lehto et al., 2000). Fluorescence spectrophotometry was used in a preliminary study to determine the concentration of B(a)P in the seawater samples to identify any changes in concentrations of B(a)P following UVR exposure and to test the hypothesis that UVR changes the chemical structure of B(a)P. Fluorescence spectrophotometry involves generating an emission spectrum by scanning the emission wavelength (λ_em) while the sample is irradiated at a single excitation wavelength (λ_ex) (Rodriguez & Sanz, 2000).

A series of 2 litre glass beakers were prepared, in the same manner as that for exposure of adult *M. edulis* described in section 6.2.1. In brief, 1.5 litre of filtered seawater (10 μm carbon filter) was transferred to each beaker held at 15°C (±2°C) to which the DMSO or B(a)P solutions had been added to produce final doses of 1μg/l and 10μg/l B(a)P. One series of treatments was covered in aluminium foil to prevent photodegradation or photoactivation of the contaminants, the other series of treatments were covered with circles of cellulose diacetate to prevent evaporation and exposed to UVR for 3 consecutive days with a 12 hours light, 12 hours dark regime, in the same manner as that described in section 6.2.1.1. Each treatment was prepared in duplicate.
Plate 6.1 Photograph of the three-tier flow-through tank system where each 200 litre tank contained 15 fish (*P. flesus*).
Immediately prior to the measurements of the samples, a series of standard B(a)P solutions were prepared at concentrations 0, 0.1, 0.5, 1.0, 5.0, and 10.0 μg/l B(a)P in seawater. Following the measurements of the standards, at the end of the three-day UVR exposure period, samples were collected from each beaker in triplicate and fluorescence analyses performed. The analyses were performed using a Hitachi F-4500 fluorescence spectrophotometer coupled to a software package operated by Windows 3.1. This was done by transferring each sample to a 3.5 ml quartz fluorescence cuvette and the fixed fluorescence detected with excitation and emission slit widths of 5.0 nm for the evaluation of B(a)P concentrations. The wavelength pair used to measure B(a)P was 380/430 nm as stated by a number of previous authors (Krahn et al., 1984; Maccubbin et al., 1988; Johnston & Baumann, 1989; Lin et al., 1996; Cormier et al., 2000; Aas et al., 20001).

The resulting fixed fluorescence spectra were smoothed and the fluorescence intensity measured at 380 nm (λex) and 430 nm (λem) for milliQ water blanks, standard solutions and samples. The mean of the emission peaks detected at approximately 430 nm were then calculated and a comparison made between irradiated and non irradiated B(a)P (in seawater) samples.

6.3.5 Exposure of Platichthys flesus to PAHs

A stock of healthy 0-group P. flesus were obtained by researchers at The Centre for Environment, Fisheries and Aquaculture Science (CEFAS) Burnham-on-Crouch site, from Port Erin Marine Laboratory and grown to their second year prior to the commencement of the experiments. Two weeks prior to the commencement of the exposures, 15 fish were introduced to a 200 litre glass fibre tank, containing seawater which was set up as a flow-through and pumped return system. There were 3 tanks for each treatment, giving a total of 45 fish per treatment, as illustrated in plate 6.1.

The fish were fed a diet of nutra marine pellets (Trouw aquaculture, Northwich, UK) daily at 0.9% of their body weight per day into which a combination of four PAHs
were administered, using hexane as a carrier solvent. The PAHs used were benzo(a)pyrene, benzantracene, phenanthrene and pyrene (Sigma, Poole, UK), with each PAH being equally represented in the mix by weight to produce doses of 0.1 and 5 mg/kg of PAH. The PAHs were dissolved into 50ml of hexane by ultrasonication and added to the solvent reservoir of the dosing apparatus. 400 g of pelleted food was placed in the mixing jar and rotated at 80 rpm and under a vacuum pressure of 10 bar, the PAH mixture dissolved in hexane coated the food. The pellets were then allowed to evaporate to dryness and the PAH contaminants remained in the feed due to their lipophylic nature. To account for losses of PAH during the food mixing process, 20% extra of the nominal dose of PAH was added to each concentration.

6.3.6 Sampling of haemolymph

Haemolymph samples were collected from the posterior adductor muscle from each adult *M. edulis* as described in section 2.3.1. For the analysis of MN in the cells, samples were extracted on the first day, prior to the commencement of the contaminant exposure, on the third day and also the last (sixth) day post-exposure. Previous work (Wrisberg *et al.*, 1992) has indicated that the maximum induction of MN occurs approximately 48 hours post-exposure. Furthermore, allowing periods in between each sampling prevented the induction of excessive stress on the animals by repeated sampling. Previous work (unpublished) where mussels were sampled repeatedly on a daily basis suffered from the mechanical damage caused by the intrusion of hypodermic needles and increased mortality rates. For the determination of DNA damage with the comet assay, samples were collected on the third day following exposure. For the determination of cytotoxicity with the neutral red retention assay, haemolymph samples were extracted on the first day prior to the start of the exposure and the sixth day following the start of exposure to identify maximum cytotoxic effects.
Samples from 10 *P. flesus* were collected by extracting approximately 1 ml of haemolymph from the caudal vein from each fish by Home office-licensed staff from CEFAS, as described in section 2.3.3 at the end of the 11 month exposure period. Approximately 0.5 ml of each sample was then provided for the genotoxic assays described in this chapter. The fish were then sacrificed by licensed staff from CEFAS and samples taken by them for the analysis of other biological endpoints such as the induction of DNA adducts and ethoxyresorufin O-deethylase (EROD) activity and sections of various organs fixed for later histopathological analyses.

6.3.7 Micronucleus Assay

6.3.7.1 Slide Preparation

To increase the adhesion of the cells to the surface of the microscope slides, the microscope slides were treated with poly-L-lysine (Sigma, Poole, UK) 2 days in advance of the extraction of haemolymph or blood samples. 10% poly-L-lysine solution (2 µl, 1:10 ratio poly-L-lysine and distilled water) was applied to each slide smeared over the surface of the slide with the edge of a coverslip and allowed to air dry.

50 µl of the haemolymph or blood samples (from both species) were transferred to labelled, poly-L-lysine coated slides and smeared with the edge of a clean glass coverslip. Two replicate slides per animal were prepared. The slides were then incubated in a light-proof humidity chamber for 30 minutes. Following this period, any excess haemolymph was allowed to drain off the edge of the slide and then allowed to air dry at room temperature (approximately 20-30 minutes). Once the slides were dry, the samples were fixed in methanol by immersing the slides for 15 minutes, allowed to air dry and then stored until ready for staining (up to 48 hours).
6.3.7.2 Staining and scoring of Micronuclei (MN)

To stain the cells, the slides were first rinsed in distilled water to remove any dust or debris which may have adhered to the surface of the slides, then immersed in 5% (v/v) Giemsa stain diluted with Giemsa buffer solution (BDH, UK) for 20 minutes. The slides were then rinsed twice with distilled water and then allowed to air dry. Once dried, glass coverslips were mounted using DPX mountant (Sigma, UK).

Slides were randomly coded and scored blind before decoding. The nucleus within 1000 cells per replicate was examined and classed according to whether they were normal or micronucleated. The cells were examined at a total magnification of x400 and features which appeared to be MN were confirmed following examination at a magnification of x1000. Although artefacts which resemble MN do sometimes occur, it is rare that they are barely distinguishable from true MN. In micronucleated cells, the frequency of MN per cell was also recorded. True micronuclei were scored when they fitted the criteria (Schmid, 1975; Tates et al., 1980):

(i) Their chromatin structure and colour intensity was similar to, or weaker than that of the main nucleus.

(ii) Their borders were distinctly recognisable, suggesting the presence of a nuclear membrane.

(iii) They were not greater than one third of, or less than one tenth of the size of the main nucleus.

(iv) Their morphology was round in shape.

(v) They were included in the cytoplasm of the cell.

(vi) They were completely detached from the main nucleus.

Abnormalities in the nuclear structure were also recorded (e.g. the presence of chromatin bridges, or binucleated cells), although these were not included in the 1000 cells recorded in the observations.
6.3.8 Eosin Y

Before the haemolymph samples collected from *M. edulis* were used for the comet assay, a sub-sample of 20 μl was transferred to a microscope slide and the viability of the cells assessed using the Eosin Y assay. This was carried out as described in section 3.2.2.3. The viability of the cells in these samples ranged from 89.7% and 92.1%.

6.3.9 Trypan Blue

For the blood samples collected from *P. flesus*, the viability of the cells was assessed using Trypan Blue stain, since the red colouration of the blood masked the red colour of viable cells detected with the Eosin Y stain. In brief, a cell suspension was prepared in a buffered salt solution (1:1000 by volume) in a siliconised microcentrifuge tube. The buffered salt solution was prepared as per Nacci *et al.* (1996), the recipe for which is detailed in Appendix I. 0.5ml of a 0.4% Trypan Blue solution (w/v) was transferred to a test tube and 0.3 ml buffered salt solution and 0.2ml of the cell suspension added and mix by vortexing. This suspension was allowed to stand for 5 minutes. A drop of this suspension was then transferred to both chambers of a haemocytometer using a pasteur pipette and the number of cells were then counted at x400 magnification. Non-viable cells were stained blue, viable cells appeared colourless. From the counts under the haemocytometer, the density of the cells could be calculated as well as the viability. In these samples, the viability exceeded 90%.

6.3.10 Comet Assay

All reagents and solutions were prepared up to a week in advance of the procedures for running the Comet assay, as defined in Appendix I. Haemolymph samples were stored on ice immediately after extraction, before their use for the Comet assay.
6.3.10.1 Slide preparation

Fully frosted slides were labelled on the underside with a diamond pen whilst 2 ml aliquots of low melting point (LMP) and normal melting point (NMP) agarose were being brought to 60°C in a water bath. In the meantime, 250 μl of haemolymph collected from *M. edulis* was centrifuged for 3 minutes at 200 x g and then the supernatant was removed and discarded. For *P. flesus* samples centrifugation was not necessary due to the high density of cells per volume of blood extracted, compared with the density of cells in samples collected from *M. edulis*.

Once molten, two drops of 85 μl of NMP agarose were applied in two different areas on the slide (to produce two replicates), 22x22 mm coverslips were applied to each drop of agarose immediately to give rise to thin base layers of agarose. The slides were then placed onto a metal tray which was held on ice to chill the slides and allow the base layers of agarose to set. Each slide was treated systematically in this manner to ensure an even distribution of the NMP base layer of agarose on each area. The base layer was allowed to set for approximately 10 minutes before application of the LMP agarose with the cells. Each haemolymph sample (approximately 10,000 cells per sample) was then resuspended in 170 μl of LMP agarose, ensuring the temperature was not too hot before addition (i.e. not hot to the touch) to prevent heat damage of the cells due to the agarose.

The slides were systematically removed from the chilled metal tray, the coverslips carefully removed from the surface of the microgel, 85 μl of the cell-agarose suspension applied to the each microgel base layer on each slide and a clean 22x22 mm coverslip applied on top. The slides were then replaced onto the cold metal tray to allow the second layer of agarose to set.

6.3.10.2 Cell lysis

Meanwhile the working lysing solution was prepared by adding Triton X-100 and dimethylsulfoxide (DMSO) to the stock lysing solution (2.5 M NaCl, 100 mM Na₂EDTA,
10mM Tris Base, 1% Na Sarconisate, dissolved in distilled water, pH 10), to produce a final concentration of 1% and 10% respectively, in a total volume of 250 ml. The coverslips were carefully removed from each microgel on the slides and the slides placed horizontally into a nalgene® staining tray. The lysing solution was then carefully poured into the tray to cover all the slides. The samples were then incubated in the lysing solution for 1 hour at 4°C in the dark to lyse the cells.

6.3.10.3 Alkaline DNA Unwinding and electrophoresis

Following the period of lysis, 2 L of electrophoresis buffer was prepared by mixing 600 ml 1N sodium hydroxide (NaOH), 10 ml 200 mM ethylene-diaminetetra-acetic acid (EDTA) and 1390 ml of distilled water. This was poured into an electrophoresis chamber (Pharmacia Biotech, UK), around which a re-circulating water flow was established to stabilise the temperature of the electrophoresis buffer within the chamber. The slides were then removed from the lysis buffer, rinsed carefully with distilled water and transferred to the electrophoresis chamber. They were arranged in a manner which ensured the microgels on each slide were completely submerged in the buffer, each slide was placed parallel to one another length-wise across the chamber and the labelled end of the slides was consistently at the same position. The slides were allowed to incubate in this alkaline electrophoresis buffer for 20 minutes during which time the DNA unwinds within the cells. After this period, the power supply was switched on and a voltage of 15V and a current of 320 mA was delivered, for a period of 25 minutes.

6.3.10.4 Neutralisation and staining of slides

Once the electrophoresis had been completed, the power supply was stopped and the slides were removed systematically from the electrophoresis chamber. Each slide was carefully rinsed by applying a few drops of distilled water with a plastic pipette, then placed into a clean, dry nalgene® staining trough. A few drops of neutralising buffer were
then applied to each microgel using a plastic disposable pipette. This was left to incubate for approximately 3 minutes and a repetition of the application of the neutralising buffer carried out a further two times to neutralise the strongly alkaline electrophoresis buffer and allow safe handling of the slides.

Each slide was then drained of excess neutralising buffer and to each microgel on each slide (two per slide), 40 µl 0.02 mg/ml ethidium bromide solution was applied and a 22mm x 22mm coverslip was then laid on to the microgels. The slides were then placed in airtight containers containing damp tissue paper (to maintain the humidity during storage of the slides) and maintained at 4°C in the dark until ready for analysis (within 48 hours).

6.3.10.5 Scoring of comets

50 cells per microgel replicate (100 cells per slide) were scored visually, by classifying the level of damage observed of each cell into one of Class 0, I, II, III, IV depending upon the amount of migration of DNA out of the nucleus head. Figure 1.8 (in chapter 1) illustrates the different classes of DNA damage into which the cells were categorised. Once the slides had been randomly scored, the results were decoded and an arbitrary score applied to each set of cells from the slides. This was calculated, by the following equation:

Comet score = ((0 x cells in class 0)+(1 x cells in class I)+(2 x cells in class II)+(3 x cells in class III)+(4 x cells in class IV)

This produced an arbitrary value where the score could range from 0 (where all of the cells showed negligible levels of damage) to 400, in a case where all 100 cells were classed as showing class IV damage as described by Collins et al. (1997a, 1997b) and Horváthová et al. (1998).

6.3.11 Neutral Red Retention (NRR) Assay

The cytotoxic effect of B(a)P exposure was examined in the haemocytes collected from *M. edulis* and was measured with the neutral red retention (NRR) assay. Since
haemoglobin is present in the erythrocytes of fish, this assay cannot be applied to this cell type due to the red colouration of the haemocytes masking the red hue of the neutral red supravital dye.

6.3.11.1 Preparation of slides

As with the micronucleus assay described in section 7.1.4.1, microscope slides were treated with 10% poly-L-lysine to increase the adhesion of the cells to the surface of the slides.

40 µl of the haemocyte-physiological saline (the recipe of which is presented in Appendix I) mixture held in siliconised microcentrifuge tubes, was then transferred to the centre of each slide. Cells were then allowed to attach to the microscope slides, whilst being incubated in a light-proof humidity chamber at room temperature (20 ± 2°C) for a period of 30 minutes. After this incubation period, the excess suspension was carefully drained from each slide before treatment with the neutral red dye.

6.3.11.2 Neutral red probe treatment

The neutral red probe was prepared as a stock solution in DMSO and as a working solution in physiological saline. The stock solution was prepared as 20 mg of neutral red (Sigma, Poole, UK) dissolved in 1 ml of DMSO (Sigma, Poole, UK). This was filtered through a 0.5 µl filter (Millipore, UK) which was attached to a hypodermic syringe (both of which were sterile packed) and stored in a amber glass vial held within a light-proof container (since neutral red is photo-sensitive) and refrigerated at 4°C (±1°C). Prior to the preparation of the working solution, the stock solution was removed from the refrigerator and allowed to reach melting point at room temperature. To prepare the working solution, 5 µl of the stock solution was added to and mixed with 995 µl of physiological saline and held in a smoked amber vial covered with aluminium foil.
After removal of the excess cell suspension from the microscope slides, 40 μl of the neutral red working solution was applied to the centre of the slide where the haemocytes had attached and allowed to incubate for a further 15 minutes (Lowe, 1988). After this period, 22mm x 22mm coverslips were applied, any excess solution carefully blotted from the edge of the coverslips with filter paper and the slides stored in a light-proof humidity chamber to ensure minimum light exposure and also to maintain a constant temperature.

6.3.11.3 Analysis of haemocytes

The slides were randomly arranged within the humidity chamber and then systematically examined with a light microscope (Leica, DMR) at 15 minute intervals and after an hour, were examined every 30 minutes (Lowe & Pipe, 1994). The condition of the cells and their lysosomal compartments were noted at each time increment and recorded in a table (an example of which is illustrated in table 7.1). Particular attention was paid to observing any evidence for the loss of dye from the lysosomal compartment into the cytosol.

The retention time of the neutral red probe by the lysosomes, was recorded by estimating the proportion of cells which exhibited leaking lysosomes and/or abnormal morphology. When 50% or more of the cells displayed leakage of the neutral red dye (recognised by the probe being distributed throughout the cytosol), the examination of the cells on that particular slides would be terminated; it was this stage that was recognised as the endpoint.

6.3.12 Statistical analyses

Data was treated with the one-way analysis of variance (ANOVA) multivariate technique, wherever the distribution of data was parametric. Non-parametric data was normalised by transformation (by log or square-root) and treated with ANOVA, assuming
the variance between the populations was not statistically significant (p<0.05). In these cases, the data was presented as plots of the mean (with standard deviation or 2 times the standard error where applicable). Where data could not be normalised by transformation, the non-parametric Kruskall-Wallis test was applied to compare the medians between populations and in these cases, the data was presented as box-and-whisker plots. Regression analyses were applied wherever dose-dependent responses were observed.

Statistical analyses were carried out using the statistical package Statgraphics Plus Version 4.0 and Microsoft Excel XP. Bar graphs were constructed using Microsoft Excel XP, box and whisker plots were constructed using Statgraphics Plus 4.0.

6.4 Results

6.4.1 Measurements of UV-B penetration through the shells of adult *M. edulis*

The penetration of ultraviolet radiation within the UV-A (315-400 nm) and UV-B (280-315 nm) wavelengths were measured through the shells of a selection of adult *M. edulis* of approximately similar size, the results of which have been presented in table 6.1. It was found that the levels of UV-A and of UV-B (DNA damaging UV-B) which were able to penetrate through the shells were negligible indicating the high level of protection provided by the shell to the soft tissue of bivalve molluscs.
Table 6.1. Measurements of the penetration of UV-B through the shells of adult *M. edulis*}

<table>
<thead>
<tr>
<th>Number</th>
<th>Length (mm)</th>
<th>Width (mm)</th>
<th>Breadth (mm)</th>
<th>Ambient UV-B (Wm$^{-2}$)</th>
<th>UV-B through shell (Wm$^{-2}$)</th>
<th>Ambient UV-A (Wm$^{-2}$)</th>
<th>UV-A through shell (Wm$^{-2}$)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>53.2</td>
<td>25.5</td>
<td>19.9</td>
<td>0.2612</td>
<td>0.00162</td>
<td>5.140</td>
<td>0.0204</td>
</tr>
<tr>
<td>2</td>
<td>50.1</td>
<td>25.9</td>
<td>18.2</td>
<td>0.2802</td>
<td>0.00201</td>
<td>6.003</td>
<td>0.0212</td>
</tr>
<tr>
<td>3</td>
<td>55.4</td>
<td>26.6</td>
<td>21.5</td>
<td>0.2667</td>
<td>0.00059</td>
<td>5.874</td>
<td>0.0129</td>
</tr>
<tr>
<td>4</td>
<td>52.0</td>
<td>22.5</td>
<td>22.3</td>
<td>0.2552</td>
<td>0.00086</td>
<td>5.140</td>
<td>0.0127</td>
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<tr>
<td>5</td>
<td>52.5</td>
<td>28.0</td>
<td>22.0</td>
<td>0.2552</td>
<td>0.00036</td>
<td>5.212</td>
<td>0.0108</td>
</tr>
<tr>
<td>6</td>
<td>48.6</td>
<td>23.4</td>
<td>19.7</td>
<td>0.2995</td>
<td>0.00038</td>
<td>5.176</td>
<td>0.0202</td>
</tr>
<tr>
<td>7</td>
<td>52.4</td>
<td>25.6</td>
<td>24.0</td>
<td>0.2668</td>
<td>0.00046</td>
<td>5.218</td>
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</tr>
<tr>
<td>8</td>
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<td>26.7</td>
<td>23.6</td>
<td>0.2677</td>
<td>0.00042</td>
<td>5.246</td>
<td>0.0124</td>
</tr>
<tr>
<td>9</td>
<td>51.5</td>
<td>24.5</td>
<td>20.4</td>
<td>0.2588</td>
<td>0.00039</td>
<td>5.238</td>
<td>0.0118</td>
</tr>
<tr>
<td>10</td>
<td>52.3</td>
<td>24.8</td>
<td>21.3</td>
<td>0.2554</td>
<td>0.00034</td>
<td>5.215</td>
<td>0.0126</td>
</tr>
<tr>
<td>Mean</td>
<td>52.23</td>
<td>25.35</td>
<td>21.29</td>
<td>0.2667</td>
<td>0.00074</td>
<td>5.338</td>
<td>0.01496</td>
</tr>
<tr>
<td>± SD</td>
<td>± 1.9</td>
<td>± 1.62</td>
<td>± 1.79</td>
<td>± 0.013</td>
<td>± 0.0005</td>
<td>± 0.287</td>
<td>± 0.004</td>
</tr>
</tbody>
</table>
6.4.2 Chemical analyses of water samples containing benzo(a)pyrene exposed to ultraviolet radiation

The results of this preliminary study to identify any potential photo-degradation of B(a)P are presented in figure 6.3. The levels of B(a)P were measured by fluorescent spectrophotometry as described in section 6.2.2. The emission peaks identified at 430 nm were smoothed and the mean from the replicates for the standards and the samples calculated. The standard curve has been presented in figure 6.3a which showed a strong dose-dependent increase in emission peak size ($R^2 = 0.99$) and the mean peaks from the irradiated and non irradiated samples are presented in figure 6.3b.

There was a significant decrease in the fluorescence emission peaks in the seawater samples containing B(a)P following UV irradiation, indicating that photo-alteration of the B(a)P molecules had occurred.

6.4.3 Mytilus edulis exposed to benzo(a)pyrene (B[a]P)

6.4.3.1 Micronucleus (MN) assay

Plate 6.2 illustrates the morphology of some normal haemocytes and some haemocytes which contained micronuclei (MN) which were observed in the samples collected. Figure 6.4 presents the data for MN induction and shows the mean frequency of haemocytes which contained one (or more) MN. When the data was tested with the F-test in one-way ANOVA, there was no significant difference between the treatment groups at the beginning of the experiment (prior to exposure). Following 3 days, there was a statistically significant increase in the frequency of micronucleated cells in animals exposed to 10µg/L B(a)P ($p<0.05$) compared with the control samples. A dose-dependent increase in MN frequency was detected, which was of high statistical significance ($R^2 = 0.95$). When the haemolymph samples were collected again after 6 days of exposure, there was a further increase in the frequency of micronucleated cells (compared with those detected after 3 days of exposure), with statistically significant increases ($p<0.05$) from the
Figure 6.3a. Standard curve measuring fluorescence emission peaks (430nm) for increasing concentrations of B(a)P at an excitation wavelength of 340 nm.

Figure 6.3b. The effect of UVR on the stability of B(a)P measured by fluorescent spectrophotometry. (Error bars = SEM; * denotes a statistically significant difference from the seawater blank (p<0.05); # denotes a statistically significant decrease in the emission peaks between the non irradiated and irradiated samples.
control sampled on the same day, in both concentrations (1 µg/L and 10 µg/L B(a)P). A dose-dependent increase in MN frequency was observed in samples collected on day 6, with a strong correlation ($R^2 = 0.92$). The data was then treated with multiple ANOVA to test the relative impact of the dose of the B(a)P and the time of exposure. Both of these factors had a highly significant effect on the induction of MN in *M. edulis* haemocytes ($p<0.00001$) at the 95% confidence interval.

The effects of simultaneous UVR and B(a)P exposure on the induction of MN in *M. edulis* are presented in figures 6.5a and 6.5b. It was found that after 3 days of exposure (figure 6.5a), there was a statistically significant increase ($p<0.05$) in the mean frequency of micronucleated cells in mussels exposed to both 1 µg/l and 10 µg/l B(a)P with simultaneous UVR compared with the seawater control. A dose-dependent increase in the MN was detected which was of a highly significant correlation ($R^2 = 0.93$). There was also a significant difference ($p<0.05$) between the samples exposed to B(a)P in the absence of UVR and those which were simultaneously exposed to UVR for 3 days in the samples exposed to 1 µg/l B(a)P suggesting that the PAH was being photoactivated into a more genotoxic product or products.

When the haemolymph samples were collected again after 6 days of exposure, there was a further increase in the frequency of micronucleated cells in mussels exposed to B(a)P with simultaneous UVR (figure 6.5b). This data reflects the trend observed in samples exposed to B(a)P in the absence of UVR. After 6 days of exposure, there was a significant difference ($p<0.05$) between B(a)P treatments without UVR and those exposed to both agents simultaneously in samples exposed to 10 µg/l B(a)P, however, this was a decrease in mean MN frequency (compared with samples exposed to B(a)P in the absence of UVR).

It was also noted that there was an increase in the frequency of MN in the cells extracted from the mussels on day 6, compared with day 3 in the seawater controls. This suggests that the repeated extraction of haemolymph, in addition to causing mechanical
Plate 6.2. (a) Normal *M. edulis* haemocyte; (b) a *M. edulis* haemocyte containing a single micronucleus; (c) a *M. edulis* haemocyte containing 3 micronuclei, above a normal haemocyte (micronuclei indicated with arrows). Photographs a and c taken at x400 magnification, photograph b taken at x1000 magnification with oil immersion.

Figure 6.4. The effect of prolonged exposure of adult *M. edulis* to B(a)P on the induction of micronucleated haemocytes. (Error bars = SEM; * denotes a statistically significant difference from the seawater control sampled at the same time period (p<0.05); ** denotes a strong statistically significant difference from the seawater control, sampled at the same time period (p<0.001)).
Figure 6.5. The effect of simultaneous UVR on the genotoxic potential of B(a)P measured with the MN (a) following 3 days of exposure; (b) following 6 days of exposure. (Error bars = 2SEM; * denotes a statistically significant difference from the control; # denotes a statistically significant difference between the photoactivated treatments and non-photoactivated treatments at the same B(a)P concentration (p<0.05).
damage, may have induced levels of endogenous DNA damage. It is possible that by extracting significant proportions of haemolymph, the rate of cellular proliferation is increased to replace these cells and therefore perhaps increased levels of MN may be induced.

6.4.3.2 Comet assay

The comet assay was carried out on haemolymph samples collected from mussels following 3 days of exposure. Due to the constraints imposed by space and restrictions on the number of samples that could be electrophoresed simultaneously in each experimental session, the comet assay was carried out on six animals per treatment from the seawater control, DMSO (solvent) control and the higher dose of 10 \( \mu g/L \) B(a)P only. The data examining the effects of B(a)P in terms of DNA damage measured with the comet assay are presented in figure 6.6. This data was normally distributed and when treated with the F-test in ANOVA, there was a strong statistically significant difference (\( p<0.0001 \)) between the level of DNA damage observed in cells from mussels exposed to 10 \( \mu g/L \) B(a)P compared with both the seawater and solvent controls. There was no significant difference between the seawater and solvent controls.

When statistical analyses was used to determine the effect of simultaneous UVR exposure, an increase in the level of DNA damage was observed in UVR treated samples compared with samples exposed only to B(a)P. However this was not statistically significantly different (\( p<0.05 \)) when tested with one-way ANOVA.
No UV: $R^2 = 0.7749$
Simultaneous UV: $R^2 = 0.7944$

Figure 6.6 The effect of simultaneous UVR exposure on the DNA damaging potential of B(a)P to adult *M. edulis* determined with the Comet assay measured by visual classification of DNA damage. (Error bars = 2SEM; ** denotes a statistically significant difference from the seawater control, $p<0.0001$).
6.4.3.3 Neutral red retention assay

Following 6 days of exposure to B(α)P, the neutral red assay gave an indication of the level of cytotoxicity caused by the PAH. Plate 6.3 illustrates the morphology of the haemocytes and the appearance of the lysosomes in healthy and stressed cells. Healthy cells (plate 6.3a) were identified to be those containing numerous, small spherical lysosomes and stressed cells were those which contained large fused lysosomes (plate 6.3b). Since the original data was non-parametric, it was normalised by log transformation, allowing the F-test of ANOVA to be applied. This test indicated that there was no statistically significant difference (p>0.05) between the seawater control (logged data produced a mean retention time of 3.9 minutes) and the samples exposed to 10μg/L B(α)P (logged data produced a mean retention time of 2.7 minutes). A means plot of the transformed data is presented in figure 6.7a. However, when the original non-parametric data were tested with the Kruskall-Wallis test, a statistically significant difference (p=0.003) was detected between the medians of animals exposed to the seawater control and those exposed to 10μg/L B(α)P (figure 6.7b).

When the B(α)P treatments were compared with the samples exposed to the B(α)P with simultaneous UVR, there was no significant change in the retention time, suggesting that UV photoactivation was not altering the cytotoxicity of the PAH contaminant. Nonetheless a decrease in retention time of the neutral red dye within the lysosomal compartment in B(α)P treatments compared with the control samples was observed. This indicated that there was an increase in the level of cytotoxicity in animals exposed to B(α)P.
Plate 6.3. (a) Healthy *M. edulis* haemocytes. Note the high frequency of small, spherical lysosomes stained by the neutral red probe; (b) Stressed *M. edulis* haemocytes. Note the difference in size and shape of the cells, the fused and enlarged lysosomes and the distribution of neutral red within the cytosol. Photographs were taken at x400 magnification.
Figure 6.7a. Log-transformed neutral red retention data. A decrease in the retention time indicates an increase in the cytotoxic response of the haemocytes from *M. edulis*.

Figure 6.7b. The retention time (non-transformed) of the lysosomal compartment of *M. edulis* haemocytes, following 6 days' exposure with B(a)P. (* Denotes a statistically significant difference from the control, p<0.05).
6.4.4 *Platichthys flesus* exposed to PAHs

6.4.4.1 Micronucleus assay

Plate 6.4 illustrates the appearance of some normal erythrocytes and an erythrocyte containing a single MN. The leucocytes and erythrocytes are easily distinguished from one another, the leucocytes having a much lower proportion of the cell component of cytosol with the nucleus taking up the majority of the cell. The erythrocytes are found in a much greater frequency than the leucocytes, since they carry the oxygen to body tissues and carbon dioxide away (Raven & Johnson, 1996).

Figure 6.8 presents the data for MN induction and shows the mean frequency of haemocytes which contained one or more MN. The data was normally distributed and when tested with the F-test in one-way ANOVA, it was found that there was a statistically significant difference between the populations which were exposed to the higher dose of PAH (500 mg/kg) and the seawater control (p<0.00005). There was a slight increase in the frequency of MN detected in samples treated to 100 mg/kg PAH, but this was not significantly different from the seawater control samples. The response was dose dependent, with an increasing frequency of MN induction with increasing dose. A regression analysis showed the level of significance of the correlation between PAH dose and MN induction ($R^2 = 0.8547$).
Plate 6.4. Blood cells from *P. flesus*. MN = micronucleated erythrocyte; ERY = normal erythrocytes; LEU = leucocyte. Photograph taken at x400 magnification.

Figure 6.8 The effect of PAH exposure on MN induction in *P. flesus* haemocytes. (Error bars = 2SEM; * denotes a statistically significant difference from the seawater control, p<0.05).
6.4.4.2 Comet assay

The comet assay was carried out on the blood cells of *P. flesus* following an 11 month period of exposure to PAH, administered via pelleted food. The level of damage in the cells was classed visually and presented as arbitrary units.

When the median level of DNA damage in the blood cells collected from fish in the exposure groups (100 and 500 mg/kg) is compared with the controls, there is a statistically significant difference between the exposed and control groups. The level of variance within the groups however violates the requirements for ANOVA tests when comparing the means. Since the data cannot be normalised, the Kruskall-Wallis test, a non-parametric method of analysis, has been applied. This test indicates that there is a statistically significant difference (p=0.0020) between the exposed and control groups, but no significant difference between the 100 and 500 mg/kg treatment groups of fish, when the medians are compared (presented in figure 6.9). A dose-dependent increase in the level of DNA damage was observed ($R^2 = 0.9823$), with the correlation being strongly significant.

6.4.4.3 Other endpoints

The data obtained by collaborative workers from CEFAS have also been included in this section for comparison with the genotoxic markers measured here. The induction of DNA adducts in liver tissue of the flounder were found to increase with dose (a summary of the data has been presented in figure 6.9), with a correlation detected between the frequency of DNA adducts per $10^8$ nucleotides and concentration of PAH exposure ($R^2 = 0.9221$). Due to the high variability of the data (the error bars on presented in figure 6.10 represent the standard error) there was no significant increase in the frequency of DNA adducts detected.

However, when the EROD activity was measured in the liver tissue samples, there was no dose-dependent relationship. There appeared to be a depression in the activity in
Figure 6.9 The effect of PAH exposure on the level of DNA damage in *P. flesus* blood cells. (Error bars = 2SEM; * denotes a statistically significant difference from the seawater control, *p*<0.002).

Figure 6.10 The effect of PAH exposure on the induction of DNA adducts in the liver of *P. flesus*. (Error bars = SE).

Figure 6.11 The effect of PAH exposure on the EROD activity detected in the liver of *P. flesus*. (Error bars = SE).
the samples exposed to 100 mg/kg, but an increase relative to this was then detected in samples exposed to the higher dose of 500 mg/kg PAH. A summary of the data has been presented in figure 6.11.

6.5 Discussion

It is evident from the data collated from these studies that PAHs, with particular reference to B(a)P, have a sublethal genotoxic effect upon the adult stages of the two species, *M. edulis* and *P. flesus* examined here. The sublethal and lethal effects of PAHs upon the larval stages of a variety of aquatic species have been widely reported (Neff, 1979) and have been described at length in chapter 5, however, it has also been reported that adult life stages are impacted by these contaminants.

The studies investigating the potential for UVR to photoactivate B(a)P indicated that UVR photodegrades B(a)P within three days of UVR exposure, evidenced by the fluorescent spectrophotometric analyses. Of the biological markers studied, the MN and comet assays indicated that there was an alteration in the genotoxic potential of B(a)P induced by UV photoactivation. However no changes in cytotoxic potential were detected with the NRR assay.

6.5.1 Comet assay

6.5.1.1 Bivalves

A study undertaken by Mitchelmore *et al.* (1998), showed a near linear dose-response in isolated digestive gland cells from *M. edulis* exposed to B(a)P at doses ranging between 10-100 μM. The concentrations of B(a)P in the studies included in this chapter were 1μg l⁻¹ and 10μg l⁻¹ B(a)P, nonetheless a dose-dependent increase in DNA damage comparable to these concentrations were observed in the haemocytes of *M. edulis* under the experimental conditions described in this chapter. Where haemocytes from oysters (*Crassostrea virginica*) were the target tissue in a study by Nacci *et al.* (1996), PAH
exposure via spiked sediment induced a significant increase in DNA strand breakage following 53 days exposure. Following 25 days of recovery in clean water, the levels of DNA damage between different treatments were similar, indicating DNA repair or cell turnover had occurred and also confirming the temporal nature of DNA strand breaks. A dose-dependent increase in DNA damage was also found in the gill tissue of other bivalve species such as *Tapes japonica* and *Patunopecten yessoensis* which were exposed to B(a)P (Sasaki *et al.*, 1997).

Steinert *et al.* (1998b) used the comet assay in addition to growth measurements to determine the photoinduced toxicity of PAH to adult mussels. In this study, it was found that slower growth and higher DNA damage in the haemocytes was induced in animals collected from sunlight exposed mussels from the PAH contaminated field site. However, 24 hours of depuration returned the levels of DNA damage to baseline levels indicating the animals' efficient capacity for DNA repair.

6.5.1.2 Fish

Fish cell lines derived from *Cyprinus carpio* exposed to different concentrations of organic sediment extracts from the North Sea were analysed for DNA strand breaks using the comet assay. Measurements from this study by Kammann *et al.* (2001) confirmed a dose-dependent genotoxic effect, this was in agreement with the observations made in the studies carried out on *P. flesus* in this chapter. A field study where chub (*Leuciscus cephalus*) were collected from locations in the River Rhone, found increased levels of DNA damage with the comet assay, in fish from sites contaminated with polychlorinated biphenyls (PCBs), heavy metals and PAHs, when compared with fish from the reference site (Devaux *et al.*, 1998). A consistent trend was observed by Pandrangi *et al.* (1995) where benthic feeding bullheads (*Ameiurus nebulosus*) and carp (*Cyprinus carpio*) were collected from different field sites of different contaminant levels. Nacci *et al.* (1996) carried out a study where flounder *Platichthys americanus*, were exposed in the laboratory
to B(α)P spiked food and sediment. Three tissues from the fish, blood, liver and gut, were analysed for DNA damage using the comet assay. It was observed that there was a high level of variation within treatments, however, this may have been due to the relatively low sample number within each treatment (between four and eight fish per treatment). In the experiments of this chapter, ten individuals from each treatment group were sampled in duplicate, providing a more robust set of data for statistical analyses. It was noted that the variation was nonetheless greatest in the fish exposed to the higher concentration of PAH and therefore it was necessary to apply the non-parametric Kruskall-Wallis test for statistical analysis of the data set.

The results observed in this chapter in addition to previous studies carried out on other fish species, indicate that the comet assay is a potentially useful assay for measuring DNA damage. These have included studies where fish have been exposed to a range of chemical contaminants, including organotin compounds (Pandrangi et al. 1995; Devaux et al., 1998; Gabbianelli et al., 2002).

6.5.2 Micronucleus assay

The micronucleus (MN) assay has been used as a biomarker in human studies as an indicator of PAH exposure (Karahalil et al., 1999). The frequency of MN in a population of cells at any particular time following clastogenic treatment is dependent on a number of factors including: the mitotic rate; the variation in the frequency of acentric chromosomal fragments in time; the proportion of acentric fragments that give rise to MN; the life span of the cells; and the selection pressure against cells with MN (Heddle et al., 1984). In the studies included in this chapter, there was evidence that increases in MN induction were caused by PAH exposure in marine mussels. In flounder the data indicates that the MN was not sufficiently sensitive to detect increases in genotoxicity in animals exposed in long-term studies to PAH concentrations less than 100 mg/kg, however a significant increase was detected in animals exposed to the higher dose of PAH (500 mg/kg). There
are contradicting reports of the sensitivity of this assay in teleosts, however different species have proven to have different levels of response. The application of this assay to fish species has been reviewed by Al-Sabti & Metcalfe (1995). There have been suggestions from previous workers that the MN assay is not a sufficiently sensitive method for detecting genotoxicity in fish species where little or no association was determined between the frequencies of micronuclei and levels of chemical contamination (Metcalfe, 1988; Carrasco et al., 1990; Vigano et al., 2002). These authors also indicated that even if the test had been found to sensitive, the variability of the frequencies would have presented difficulties in application of the test. Furthermore, Das & Nanda (1986) undertook a study where chronic exposures of the teleost Heteropneustes fossilis to paper mill effluent were carried out. Indications were that there was a time-response relationship with a gradual decrease in the incidence of MN with an increase in exposure period (between one and three month exposures). This response was suggested to be caused by the effluent inducing an inhibitory effect on cell division and subsequent hinderance to the passage of micronucleated cells into the peripheral circulation.

6.5.2.1 Bivalves

The MN assay has been used in a significant number of studies involving marine invertebrates in both laboratory and field conditions (Dixon et al., 2002). In the current investigations, the induction of MN in the haemocytes in M. edulis was significantly higher in B(a)P treated animals compared with the controls, following both 3 and 6 days of exposure. These results were in accordance with the increase in MN frequency in gill cells from M. galloprovincialis following exposure to B(a)P (Scarpato et al., 1990; Vernier et al., 1997) as well as in the haemocytes (Vernier et al., 1997) which were exposed to concentrations ranging between 0 and 1000 ppb and exposed for 48 hours. Furthermore, in another study by Burgeot et al. (1995), spat and adult oysters (Crassostrea gigas) were exposed to B(a)P at concentrations ranging between 0 and 500 μg l⁻¹, as well as other
chemical contaminants and contaminated effluent samples. A increase in MN frequency was observed for samples exposed to B(a)P of 5 µg/L and above following 48 hours of exposure in adults (with up to 45 MN per thousand cells observed). However, the frequency of MN in the gill cells from the spats were lower than that induced in the adult haemocytes (the frequency did not exceed 10 MN per thousand cells), suggesting that the earlier life stages had a higher level of sensitivity compared with the adults, thus resulting in an inhibition of cell division.

Where the MN assay was applied to determine whether photoactivation of B(a)P altered the genotoxic potential of the model PAH, increases in MN induction were observed at 3 days' exposure for both 1 µg/L and 10 µg/L B(a)P plus simultaneous UVR treatments, although the latter treatment was not statistically significantly different from the non irradiated 10 µg/L B(a)P treatment indicating that there was an increase in genotoxicity in photoactivated samples. Following 6 days' exposure, there was actually a decrease in the induction of MN in the higher does of B(a)P plus simultaneous UVR treatment. It is observed that this decrease in MN frequency is in accordance to the longer term exposures of fish, this may be due to an inhibition on the cell cycle kinetics of haemocytes resulting in lower frequency of MN (Burgeot et al., 1995). A similar response was reported by Scarpato et al. (1990), where at the highest doses of B(a)P a reduction in the level of MN was observed also explained by an inhibition of cellular division. However, there is little information regarding the replication kinetics of haemocytes (and gill cells) of bivalve molluscs and it is not clear where different subpopulations of cells originate and divide (Venier et al., 1997).

6.5.2.2 Fish

The use of the MN assay in different fish species has been reviewed by Al-Sabti & Metcalfe (1995). As reported by Carrasco et al. (1990), there was not a significant relationship between the frequency of variations of nuclear morphology and the levels of
chemical pollution measured in sediments that the white croaker (Genyonrmus lineatus) were exposed to. This may be due to the low mitotic index usually found in adult fish erythrocytes. The authors also postulated that the individuals measured, had not been actually exposed to the chemical contaminants since the animals were able to migrate. However, a number of other studies are inconsistent with the investigations carried out by Carrasco et al. (1990), where positive relationships between MN induction and levels of contamination were found (Longwell et al., 1983; Das & Nanda, 1986; Al-Sabti, 1986; Hose et al., 1987; Nikinmaa, 1992; Ayllon & Garcia-Vazquez, 2000). Of some of these studies, criticisms of the number of field stations being compared and the viability of the cells were made. It was further reported that there is a possibility that nuclear abnormalities are caused by factors other than genotoxin exposure, such as anemias induced by dietary vitamin B12 or folic acid deficiencies (Carrasco et al., 1990). The mean frequency of true MN-containing cells observed in the flounder exposed to 500mg/kg PAH, was only 1.8 (with a standard deviation of 0.83) and although statistically significantly greater than the control, this is not a vast increase from the base-line levels.

Where the MN assay has been demonstrated in the erythrocytes of amphibian Pleurodeles waltl, positive responses to PAH exposure have been reported. Larvae were exposed to four different PAHs (napthalene, anthracene, phenanthrene and B(a)P). Significant increases in MN induction were observed in samples exposed to napthalene and B(a)P, with a strong linear dose-relationship reported in samples exposed to B(a)P (Djomo et al., 1995).

6.5.3 Neutral Red Retention assay

For correct interpretation of results from the neutral red technique when assessing cytotoxic effects, it is necessary to consider any fluctuations which may affect the responses, such as seasonal variances and gametogenesis, age and sex of the animals (Regoli, 1992). Bayne et al. (1988) demonstrated that spawning disrupts lysosomal latency
in the digestive gland. This is caused by the processes of nutrient mobilisation, changes in energy budgets and general tissue reorganisation and repair that are associated with the final stages of gametogenesis and spawning. The disturbance of this compartment caused by the reproductive state has to be taken into consideration in any study of this nature. During the sampling and examination of haemocytes from *M. edulis* for the neutral red retention assay, the presence of any spermatocytes within the samples were always recorded and the results not included in the data collation. Work carried out by Hole *et al.* (1992) showed that age does not have any significant effect upon the stability of the lysosomal compartment, but the potential for recovery of lysosomal integrity is age-related. It was observed by Santarém *et al.* (1994) that the type and number of haemocytes in *Mytilus galloprovincialis*, were influenced by seasonal factors. The haemolymph represents the most important internal immune defence system against foreign material, hence any seasonal variations would have an effect upon the mussels' ability to sequester or metabolise contaminants.

It is known that the lysosomal compartment is particularly sensitive to contaminants such as hydrocarbons and organochlorides (Lowe, 1988; Lowe & Pipe, 1994; Moore *et al.*, 1984). Field and experimental exposure to oil-derived PAHs have been shown to induce profound changes in the rate of fusion events of the lysosomal-vacuolar systems and lysosomal membrane stability in molluscan digestive cells (Moore & Farrar, 1985; Moore *et al.*, 1987; Fernley *et al.*, 2000). In the studies carried out in this chapter, it is evident that B(a)P had a significant cytotoxic effect upon the haemocytes of *M. edulis* at a concentration of 10 µg/L, indicated by a significant decrease in retention time of the lysosomal compartment. At a concentration of 1 µg/L, there was a decrease in the median retention period of the lysosomes, though this was not significantly different from the controls. It has been hypothesised that PAHs exert their toxicity directly on the lysosomes of cells, a possible consequence of such lysosomal damage being immune
impaired potentially leading to reduced resistance to infectious disease (Grundy et al., 1996a).

However, when comparing the retention times of the samples exposed to $B(a)P$ and those exposed to $B(a)P$ with simultaneous UVR, there is no evidence for changes in the cytotoxicity measured by this assay. It is possible that the photoproducts of $B(a)P$ are less cytotoxic than the parent compound or that the products are more readily metabolised and eliminated by the mussels.

6.5.4 Whole organism responses

The response of invertebrates to PAH exposure may be due to the impacts upon the symbiotic relationships between two different organisms. An example of this was demonstrated in hydrocorals (*Millepora* sp.), which were found to have reduced rates of calcium deposition in the skeleton following phenanthrene exposure, possibly due to the inhibitory effects of the contaminant upon the photosynthetic ability of the associated algae (Neff, 1979).

In the present study, adult mussels were selected of approximately similar size, although in a study by Wang & Chow (2002) where the absorption and exposure pathways of $B(a)P$ in the green mussel *Perna viridis* were investigated, indications were that the body size of the mussels does not significantly affect the absorption efficiency of $B(a)P$. In freshwater mussels (*Driessena polymorpha*, *Anadonta cygnea*, *Unio pictorum*, and *Unio tumidus*) a significant decrease in the filtration rate was noted following exposure to $B(a)P$, possibly due to inhibited ciliary activity or even an inhibition on the metabolism of the whole animal (Neff, 1979). This suggests that the uptake of contaminants is related to the rate of filtration rather than the size of the animals. It would have been an interesting and pertinent factor to have measured the rate of filtration of the mussels during their exposure to $B(a)P$, as well as their recovery from exposure, had they been allowed to depurate after the period of exposure.
6.5.5 Other sublethal effects

The exposure of marine animals to certain organic and metal contaminants is thought to enhance the production of reactive oxygen species (ROS), with concomitant alterations of antioxidant defence mechanisms. A study by Orbea et al. (2002a), where *Mytilus galloprovincialis* were exposed to B(a)P (as well as other single contaminants) for 21 days, found that there was a significant increase in the induction of catalase and glutathione peroxidase. B(a)P also caused a slight increase in acyl-CoA oxidase activity and peroxisomal activity. A field study by Orbea et al. (2002b) however, suggested that seasonal factors might influence biomarker responses such as antioxidant enzyme activities to a greater extent than pollution variations between different sampling stations. It would have been useful to have measured such antioxidant and peroxisomal enzymes in both of the species in the current investigations to identify the potential impact upon other mechanisms other than DNA damaging effects. These endpoints could then have been compared with the biomarker responses already measured to identify which methods were the most sensitive.

B(a)P and other PAH contaminants have been reported by a number of authors to induce the formation of DNA adducts in marine organisms (Venier & Canova, 1996; Canova et al., 1998; Lyons et al., 1999; Malmström et al., 2000; Ching et al., 2001; Rose et al., 2001). In the liver samples from the *P.flesus* fish processed in the current studies, a dose-dependent increase for the induction of DNA adducts was detected. This observation corresponds to findings by the authors mentioned above. The detection of DNA adducts have therefore been suggested as a useful biomarker technique in teleosts. There was a suggestion however, that the detection of DNA adducts may not be a sufficiently sensitive method for detecting PAH exposure in marine invertebrates such as *Halichondria panacea* and *Mytilus edulis* (Harvey et al., 1999).

The EROD activity in the liver of the *P.flesus* samples in this chapter did not show any dose-related response in the current investigation. Quantitative analysis of CYP1A
mRNA expression in the same fish was also measured by colleagues at the CEFAS laboratories and this data mirrored the responses identified by EROD activity measurements (Lyons, personal communication). An investigation by van Schanke et al. (2001) reported that dab (*Limanda limanda*) exposed to a range of doses of B(α)P (0-10 mg/kg) did not exhibit any significant induction of microsomal EROD or cytosolic glutathione-S-transferase activity. Only a tentative correlation between EROD activity and PAH and PCB exposure in fish (*Serranus cabrilla*) were measured in samples collected from field stations along the French coast by Michel *et al.* (2001). Furthermore, previous authors have found that the sex of the chub (*Leuciscus cephalus*) influenced the level of EROD activity (Devaux *et al.*, 1998). This may have been a factor not previously considered during the exposure of *P. flesus*.

6.6 Conclusions

There is no doubt that PAHs in the marine environment pose a significant impact on the adult stages of marine organisms. The genotoxic responses however, can vary between species and even within species. In conclusion, it is evident that a suite of biomarkers are necessary to be able to determine relative toxic effects of PAH exposure since different endpoints respond with different levels of sensitivity. Furthermore, the period of exposure has a significant influence on the level of response observed and the potential for different cell types to recover and for the organism to depurate needs to be investigated. The experiments in the current chapter using the MN assay suggested that this assay is not particularly sensitive in the flounder *P. flesus* in long-term exposures possibly due to the contaminants inhibiting the cell cycle or leading to cell death and resulting in a misleading low incidence of MN. This stipulation is supported by the response of MN induction in the laboratory exposures of *M. edulis* to B(α)P. In these studies, a significant increase in MN induction after 3 days of exposure was observed, however, the rate of MN induction was reduced after 6 days of exposure. The neutral red cytotoxicity test measuring the integrity
of lysosomal membranes, appeared to a sensitive marker of B(α)P exposure in the laboratory, however, this method could not be applied to the fish haemocytes due to the colouration of the samples being the same as that of the neutral red probe. There is a potential for the use of alternative fluorescent dyes that could be used in the place of neutral red which have the same cationic properties, such as BODIPY-FL-verapamil as used by Moore et al. (1996) in molluscan haemocytes.

Indications are that the interactions of PAH contaminants and UVR may result in chemical products which have a higher toxic potential than the parent compounds. The results of the MN and comet assays applied to M. edulis haemocytes indicated that at the lower concentration of B(α)P (1 μg l⁻¹), photoactivation of the compound resulted in an increase in genotoxicity. The photo-oxidation of PAH contaminants may result in the formation of endoperoxides, diones, or quinones (Neff, 1979). The rate of B(α)P degradation is relatively protracted compared with other PAH compounds such as anthracene, phenanthrene and benz(α)anthracene, nonetheless B(α)P in the natural environment is degraded with rates of decomposition being related to water depth, levels of solar radiation, temperature and dissolved oxygen (Neff, 1979). Although the toxic potential of PAHs may be altered by photoactivation, the action of the light may result in shortened half-lives of the contaminants and a reduction in the persistence of these products compared to the parent compounds. The measurements of the penetration of UV through the shell of adult mussels indicated that negligible levels of photoactivation of PAH contaminants would be expected to occur within the soft tissues.

There is certainly scope for much further research into the sublethal effects of PAH exposure, the interactive effects of UVR and PAHs and the use of biological markers for the detection of such exposure-effects. Inter-species comparisons are necessary to determine differences in their levels of sensitivity and to assess which suites of assays would be most suitable for the different species. Furthermore, the repair of DNA and the
recovery of different species following depuration after they had sustained PAH exposure need to be characterised as often PAH contamination occurs as intermittent incidents.
Chapter 7

The Detection of Genotoxic Effects in Marine Bivalves: A Field Assessment
Hypothesis: A suite of biomarkers can be used to detect a pollution gradient in indigenous populations of invertebrates collected from field sites.

7.1 Introduction

Most ecotoxicological studies require validation and confirmation of the sensitivity and response of target organisms in controlled laboratory conditions before attempting to apply the techniques in field studies, where multiple environmental and or anthropogenic factors may interact to produce synergistic, additive or antagonistic effects. The limitation of univariate acute toxicity testing in laboratory conditions is that it is difficult to extrapolate the responses to real environment situations (Kimball & Levin, 1985). The complex nature of populations, communities and ecosystems and the biological and chemical mechanisms by which they interact makes it problematic to fully understand the significance of acute toxicity test results. Further complications arise when attempting to compare actual levels of chemical exposure in the field and the laboratory. In the environment, chemicals may volatize, adsorb on to surfaces, be passively or actively taken up by living or dead organic material, or they may form complexes with other substances (Chapman & Long, 1983; Monk, 1983). It is therefore relevant and important that biological monitoring is carried out utilising indigenous populations which have been exposed to contaminants in their natural environment. Numerous field studies have been carried out in the past to investigate the biological responses of pollution exposure, and attempts made to identify differences between sampling locations using biological markers (Brunetti et al., 1988; Cheung et al., 1998; Wedderburn et al., 1998, 2000; Hughes & Hebert, 1991; Ralph & Petras, 1997; Steinert et al., 1998; Quiniou et al., 1999; de Lafontaine et al., 2000; Frenzilli et al., 2001).

Following on from the laboratory studies which have been described in the previous chapters, an attempt was made to employ some of the validated biomarkers for the purpose of detecting a pollution gradient in samples collected from various sites along the Tamar estuary, Plymouth, UK. Five rivers run into the Tamar estuary, the Tamar, Tavy, Lynher, Plym and Tiddy, of these the Tamar estuary is the largest which then
empties into Plymouth Sound (Hiscock & Moore, 1986; Environment Agency, 1996b). The Tamar estuary is tidal for approximately 30 km landwards from the Sound, where it meets the sea (Lindsay & Bell, 1997). This estuary is relatively sparsely populated and non-industrialised, with villages such as Calstock having less than 2000 inhabitants (Lindsay & Bell, 1997). The catchment area of Plymouth has a population of approximately 329,250 residents, of which 243,350 live in the city of Plymouth itself (Environment Agency, 1996b). With this in mind, the sampling sites were selected near the mouth of the estuary on the west and east banks close to the city of Plymouth and to where the Royal Naval base of Devonport is situated.

A number of chemical studies have been carried out in the Tamar estuary area, investigating the concentrations of heavy metals and radionuclides present in the sediments (Lindsay & Bell, 1997) as well as the presence of polycyclic aromatic hydrocarbons in seawater (Law et al., 1997) and sediments (Woodhead et al., 1999). Indications from such reports are that the Tamar estuary contains elevated levels of metalliferous contaminants compared to many other estuaries around the British coast.

The field sampling for the current investigations was carried out within a period between September 2001 and October 2001. Tissue samples from indigenous populations of adult Mytilus edulis and Cerastoderma edule were used for genetic toxicological assays in an attempt to detect differences in biological response among the sites from where they were collected. These data were then compared with data collated from chemical analyses of sediment and body tissue samples from the corresponding sites.

7.2 Aim & Objectives

The main aim of this field study was to investigate whether the genotoxic and cytotoxic biomarkers which had been applied in laboratory conditions in the previous chapters, could be used to detect pollution responses in indigenous organisms collected from various field sites. Furthermore, to determine if any correlation existed between the
biomarker responses observed and levels of contamination present in the animals or their environment. This was determined by chemical analyses.

Genotoxicity was measured by assessing levels of DNA damage with the comet assay and micronucleus assay; and cytotoxicity was determined with the neutral red retention assay. Other biomarkers including the determination of metallothionein in whole tissue, heart rate measurements, and micronuclei induction were also carried out during the same study period, on different individuals of the target species by a co-worker from the University of Plymouth. This allowed for collation of further data and comparison between different biomarkers.

7.3 Materials & Methods

7.3.1 Sampling sites

Six sampling stations were selected from the Tamar estuary area based upon the ease of sampling for biological and sediment samples, in addition to their relative location along the estuary, as illustrated in figure 7.1. Site number 1 was situated at Bantham (SX 545 500), on the Avon estuary and was selected as the reference site since relatively little contamination occurs within the vicinity. An oyster farm is located a few kilometres from the sampling site where bivalve molluscs are cultured and farmed for human consumption. This site was therefore considered to be a relatively clean location. Site number 2, was located on the shore close to the Torpoint vehicle and passenger ferry terminal (west side of the estuary) (SX 442 551) and accessed via Marine Drive. With the exception of the reference site (Bantham), this site was the furthest south and the closest to the sea. Site number 3 was located approximately 2 kilometres north-west of Torpoint close to a jetty at Cove Head, Wilcove (SX 434 567). This was located approximately opposite to the Devonport Royal Dockyard where submarine re-fits are carried out and radioactive wastes are discharged, it was also observed that a few small craft were moored at this location. Site number 4 was situated at Jupiter Point, at a jetty leading from Anthony House (SX
Figure 7.1 Location of the sampling stations along the Tamar estuary, south Devon, UK.
416 568). This was located on the southern bank of the river Lynher and was noted that there were a number of dinghy boats moored which are used for training by the Navy. Site number 5 was situated under the Tamar Bridge, close to the marina at Saltash (SX 433 586). Site number 6 was situated at Neale Point close to Wearde Quay, where the river Tavy meets the river Tamar (SX 436 612).

7.3.2 Biological sampling

The presence of the species of interest at each potential sampling station were established in advance of the sample-collection. In addition, the ease of access to the stations and the distance from the laboratory was taken into account when selecting the sampling stations, to minimise the transportation time and thus the amount of stress placed upon the animals before tissue-sampling.

7.3.2.1 *Mytilus edulis*

12 organisms (6 for biomarker studies and 6 for metal analyses) were collected from each site. Excess animals were not collected to minimise the impact upon the local populations. *M. edulis* adults within the size range of approximately 4.2 – 6.2 cm were selected from each sampling site at low tide, from the rocky outcrops at sub-littoral level. The organisms were carefully removed from their substrates by cutting the byssus threads. The animals were then transported back to the laboratory in a cool box containing damp tissue paper, at ambient temperatures to minimise thermal stress.

7.3.2.2 *Cerastoderma edule*

In accordance to the sampling of *M. edulis*, 12 adult *C. edule* (6 for biomarker studies and 6 for metal analyses) were collected from each sampling site. These were collected at low tide from the silty sediments at sub-littoral level. The animals were then
transported back to the laboratory in a cool box containing damp tissue paper to maintain humidity.

7.3.3 Water sampling

On the same day that the biological and sediment samples were collected, hydrological parameters including pH, temperature, salinity and dissolved oxygen levels were measured in situ at high tide using a YSI multi-incorporated meter (model 550 DO). Approximately 10 litres of water was collected from each site in clean plastic containers at high tide, avoiding collection of the water surface microlayer. On return to the laboratory, 1 litre from each seawater sample was filtered through a 0.45 μm filter assembled in a Buchnar funnel filter unit. To determine the amount of suspended particulate matter in each sample the filters were weighed before filtering and then re-weighed after filtering once they had been dried in an oven at 40°C for 24 hours. Subsequently the amount of suspended particulate matter was calculated as units of milligrams per litre (mg l⁻¹) of seawater.

7.3.4 Sediment sampling

Approximately 5 kg of the sediment (wet weight) was collected from each sampling site using a small plastic trowel and transported back to the laboratory in labelled plastic sample bags. These samples were collected at low tide at the same time and close to where the biological samples (M.edulis and C.edule) were collected.

7.3.5 Chemical analyses for heavy metals

7.3.5.1 Sediments

The sediment samples were stored in plastic sample bags, at -80°C until the samples were ready for processing. The determination of metals in the sediments was carried out as per the methods described by Jha et al., 2000a. In brief, the sediment
samples were placed in an oven at 60°C and allowed to air dry for 7 days. The dried samples were then ground in a mortar and pestle and then sieved through a 180 μm nylon mesh. From each sample, approximately 0.25 g of the sieved sediment samples was placed into acid washed beakers, and 5 ml of concentrated nitric acid (Fisher Scientific, Loughborough, UK) added to each sample. The samples were covered with a sheet of paper to prevent the ingress of extraneous material, and left overnight to pre-digest in a fume cupboard. After pre-digestion, the beakers were placed onto a hotplate and boiled until all the biological material had dissolved. The samples were then allowed to cool, after which they were transferred quantitatively into pre-cleaned 25 ml capacity volumetric flasks. Each sample was spiked with an aliquot (0.25 ml) of a 10 μg ml⁻¹ indium solution and diluted to volume with milli-Q water. A blank containing only 5 ml of nitric acid, and a certified reference material (LGC 6137, Estuarine Sediment, Laboratory of the Government Chemist, Queens Road, Teddington, UK) were prepared simultaneously in an identical manner.

Although the nitric acid extraction is insufficient to dissolve the aluminosilicate material, it is capable of extracting 90% or more of heavy metals e.g. copper, cadmium, lead etc. Other analytes would be less efficiently extracted, such as arsenic and chromium, although efficiency is often greater than 50%.

7.3.5.2 Bivalve soft tissues

On return to the laboratory, the external shells of the mussel and cockle samples were cleaned of epiobionts and sediments. The whole animals were then placed into labelled plastic sample bags and stored at -80°C until ready for processing. Prior to treatment for heavy metal analyses, the samples were removed from the freezer, and freeze-dried for 24 hours. The soft tissues were then extracted from the shell (the shells were discarded), the soft tissue from the six animals of each site pooled and they were then pulverised using ceramic pestle and mortars.
Approximately 0.25 g of the pulverised tissue samples were placed into 10 ml acid washed beakers, and 5 ml of concentrated nitric acid (Fisher Scientific, Loughborough, UK) added to each sample. The samples were covered with a sheet of paper to prevent the ingress of extraneous material, and left overnight to pre-digest in a fume cupboard. After pre-digestion, the beakers were placed onto a hotplate and boiled until all the biological material had dissolved. The samples were then allowed to cool, after which they were transferred quantitatively into pre-cleaned 25 ml capacity volumetric flasks. Each sample was spiked with an aliquot (0.25 ml) of a 10 μg ml⁻¹ indium solution and diluted to volume with milli-Q water. A blank containing only 5 ml of nitric acid and a certified reference material (TORT-2, Lobster hepatopancreas, National Research Council, Canada) were prepared simultaneously in an identical manner.

7.3.5.2.1 Analysis of samples

All the dissolved sediment and biological samples were analysed using the semi-quantitative analysis software of an ICP-MS instrument (PlasmaQuad PQ2+ Turbo, VG Elemental, Winsford, Cheshire, UK). This software enables the calibration of the instrument using a mixed standard of six elements all at a concentration of 100 ng ml⁻¹. From this, a mass response curve was produced. This curve was then used to estimate the elemental composition of the samples. It is normally accurate to within a factor of two. The metals analysed for are presented in table 7.1.

7.3.5.2.2 Data output

The data collected from the ICP-MS output were expressed as ng g⁻¹ of solid sample. Check standards were run intermittently to ensure that instrumental drift did not pose a problem. The results of the certified material (run in parallel as a standard) were used to calculate more accurately the concentration of the analytes from the biological and sediment samples. Similar calculations were made for most of the elements detected with
Table 7.1 Metals analysed in sediments and biological material from the selected field sites.

<table>
<thead>
<tr>
<th>Element</th>
<th>Chemical Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium</td>
<td>Cr</td>
</tr>
<tr>
<td>Manganese</td>
<td>Mn</td>
</tr>
<tr>
<td>Iron</td>
<td>Fe</td>
</tr>
<tr>
<td>Cobalt</td>
<td>Co</td>
</tr>
<tr>
<td>Nickel</td>
<td>Ni</td>
</tr>
<tr>
<td>Copper</td>
<td>Cu</td>
</tr>
<tr>
<td>Zinc</td>
<td>Zn</td>
</tr>
<tr>
<td>Arsenic</td>
<td>As</td>
</tr>
<tr>
<td>Selenium</td>
<td>Se</td>
</tr>
<tr>
<td>Cadmium</td>
<td>Cd</td>
</tr>
<tr>
<td>Mercury</td>
<td>Hg</td>
</tr>
<tr>
<td>Lead</td>
<td>Pb</td>
</tr>
</tbody>
</table>
the exception of Al, Ca, Fe, Mg and Na in the sediment samples since in these analytes, the concentration for each of these elements was so high that the ICP-MS drastically under-estimates their concentration.

7.3.6 Selected biomarkers

The biomarkers selected for the field study were ones which had been successfully employed in laboratory exposures described in previous chapters and were most suited to field samples. The comet assay was carried out to determine levels of genetic damage (DNA strand breaks) and the neutral red retention assay was used to determine cytotoxicity. For each of these biomarkers, the haemocytes were used as the target cell type. In addition, other biomarker assays were carried out by a co-worker also undertaking research within the same laboratory. As indicated in figure 7.2, these included the determination of metallothionein content in whole tissue samples to provide an indication of metal exposure; measurements of micronuclei induction in haemocytes for the detection of genotoxicity; and heart rate measurements in the live animals for the detection of physiological effects. A summary of the treatment of the biological, sediment and water samples, and the biomarkers employed is presented in figure 7.2.

Considering the number of different biomarkers being carried out, in addition to the number of animals required for the chemical analyses of heavy metals in the tissues, the number of animals used for each assay was kept to a minimum. This ensured minimal impact upon the population dynamics of the indigenous animals whilst maintaining viable statistical analyses.

7.3.6.1 Sampling of haemolymph

From each individual of both species, 0.2 ml of haemolymph was extracted from the posterior adductor muscle into 0.2 ml physiological saline, as described in section
Figure 7.2 Summary of the endpoints measured in the biological, sediment and water samples (assays or procedures carried out by the author are indicated with a solid line).
2.3.1. Each sample was transferred into siliconised (2 ml) microcentrifuge tubes and held on ice to minimise cellular stress.

7.3.6.2 Eosin Y cell viability assay

The viability of the haemocytes in each sample was checked using the Eosin Y viability assay, in advance of the comet assay, as described in section 3.3.2.3.

7.3.6.3 Comet assay

The comet assay was carried out in the haemolymph samples collected from the individual animals, as described in section 3.3.2.4. The level of DNA damage in 100 cells per sample was measured using the Kinetic Imaging® image analysis software. The comet parameters, tail moment and tail length were used for data analyses.

7.3.6.4 Neutral red retention assay

The NRR assay was carried out on live cells collected from the animals simultaneously to the comet assay. The procedure was carried out as described in section 6.3.11.

7.3.7 Statistical analyses

Statistical analyses were carried out using the statistical package Statgraphics Plus Version 4.0 and Microsoft Excel XP. Bar graphs were constructed using Microsoft Excel XP, box and whisker plots were constructed using Statgraphics Plus 4.0 as per the previous chapters.
7.4 Results

7.4.1 Hydrological parameters

A variety of hydrological parameters were measured in the water within the vicinity of where the bivalve samples were collected from. Table 7.2 presents the data that were recorded.

7.4.1.1 pH

The pH of the water was slightly lower (more acidic) at the sites that were located further upstream, compared with that of the sites closer to the open sea. This may have been due to a combination of the relatively higher levels of contamination at the upstream sites and a lower amount of circulation and dilution at these sites.

7.4.1.2 Temperature

The water temperature recorded at each of the sites was quite similar with a maximum difference of 1.2°C between one site and another. At the time of sampling, the weather conditions were consistently fine, with no precipitation occurring between the sampling days. The variation of temperature was more likely to have been due to some areas having more foliage (and therefore more shade from the sun) marginally reducing the water temperature in the vicinity.

7.4.1.3 Salinity

The salinity measured at each site at high tide ranged between 33.3 and 35.1. The highest levels of salinity were measured at the sites 1 and 2 (Bantham and Torpoint) which were situated closest to the open sea.
Table 7.2 The results of the hydrological measurements.

<table>
<thead>
<tr>
<th>Site No.</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Salinity</th>
<th>Dissolved $O_2$ (mg l$^{-1}$)</th>
<th>SPM (mg l$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.02</td>
<td>16.4</td>
<td>35.1</td>
<td>6.93</td>
<td>31.3</td>
</tr>
<tr>
<td>2</td>
<td>7.88</td>
<td>17.0</td>
<td>34.8</td>
<td>5.37</td>
<td>35.2</td>
</tr>
<tr>
<td>3</td>
<td>7.83</td>
<td>16.2</td>
<td>33.5</td>
<td>6.46</td>
<td>29.8</td>
</tr>
<tr>
<td>4</td>
<td>7.81</td>
<td>17.0</td>
<td>33.3</td>
<td>6.4</td>
<td>14.9</td>
</tr>
<tr>
<td>5</td>
<td>7.8</td>
<td>17.9</td>
<td>34.1</td>
<td>7.83</td>
<td>23.8</td>
</tr>
<tr>
<td>6</td>
<td>7.76</td>
<td>17.4</td>
<td>33.4</td>
<td>6.20</td>
<td>21.5</td>
</tr>
</tbody>
</table>
7.4.1.4 Dissolved oxygen

There was no clear trend among the sites with regard to the distance from the open sea and the levels of dissolved oxygen. The highest level of dissolved oxygen was measured at site number 5 (under the Tamar Bridge), and the lowest at site number 2 (Torpoint).

7.4.1.5 Suspended particulate matter (SPM)

Once again for this parameter there was no clear correlation between the distance from the open sea and the levels of SPM. However, it was noted that the highest level of SPM (35.2 mg l⁻¹) was measured in water collected from site number 2 (Torpoint). This site also had the lowest dissolved oxygen levels, suggesting that the aerobic breakdown of organic matter present in the SPM was depressing the local oxygen levels.

7.4.2 Heavy metal analyses

7.4.2.1 Sediments

Table 7.3 presents the calculated total concentrations of the heavy metals measured in the sediments collected from the 6 sampling sites. It should be noted that measurements of the certified sediment material indicated that there was a significant underestimation of the measurements for iron and mercury. The data collected for these two elements are therefore deemed to be inaccurate and have not been included in the discussion of the results. Each of the heavy metals measured in the sediments, site number 1, Bantham, was consistently found to have the lowest concentrations. Chromium concentrations were found to be the highest at site number 4 (Jupiter Point), totalling 37.32 μg g⁻¹ sediment. At the same site, the highest concentrations of manganese and lead were also measured. The highest concentrations of zinc and cadmium were measured at site 5 located under the Tamar bridge (546.03 and 1.74 μg g⁻¹ sediment respectively). Of greatest biological significance, the highest concentrations of cobalt (14.73 μg g⁻¹), nickel (30.84 μg g⁻¹),
Table 7.3 The concentrations of total heavy metals in the sediment samples collected from the different sites along the Tamar estuary (µg g⁻¹).

<table>
<thead>
<tr>
<th>Site</th>
<th>Element</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cr</td>
<td>13.96</td>
<td>30.25</td>
<td>30.01</td>
<td>37.32</td>
<td>32.73</td>
<td>32.75</td>
</tr>
<tr>
<td>2</td>
<td>Mn</td>
<td>343.88</td>
<td>411.12</td>
<td>430.71</td>
<td>490.28</td>
<td>437.60</td>
<td>469.23</td>
</tr>
<tr>
<td>3</td>
<td>Fe</td>
<td>506778.63</td>
<td>17171.19</td>
<td>35130.77</td>
<td>4083.87</td>
<td>7205.91</td>
<td>6496.35</td>
</tr>
<tr>
<td>4</td>
<td>Co</td>
<td>5.98</td>
<td>11.04</td>
<td>10.41</td>
<td>13.19</td>
<td>12.03</td>
<td>14.73</td>
</tr>
<tr>
<td>5</td>
<td>Ni</td>
<td>18.12</td>
<td>27.66</td>
<td>26.07</td>
<td>31.79</td>
<td>28.01</td>
<td>30.84</td>
</tr>
<tr>
<td>6</td>
<td>Cu</td>
<td>6.33</td>
<td>106.91</td>
<td>139.67</td>
<td>168.84</td>
<td>143.48</td>
<td>191.98</td>
</tr>
<tr>
<td>7</td>
<td>Zn</td>
<td>51.70</td>
<td>266.10</td>
<td>150.02</td>
<td>182.88</td>
<td>546.03</td>
<td>194.82</td>
</tr>
<tr>
<td>8</td>
<td>As</td>
<td>7.72</td>
<td>42.75</td>
<td>57.12</td>
<td>71.81</td>
<td>65.12</td>
<td>79.37</td>
</tr>
<tr>
<td>9</td>
<td>Se</td>
<td>1.43</td>
<td>3.96</td>
<td>6.62</td>
<td>4.90</td>
<td>6.60</td>
<td>4.95</td>
</tr>
<tr>
<td>10</td>
<td>Cd</td>
<td>0.17</td>
<td>0.80</td>
<td>0.94</td>
<td>0.74</td>
<td>1.74</td>
<td>1.23</td>
</tr>
<tr>
<td>11</td>
<td>Hg</td>
<td>0.16</td>
<td>0.27</td>
<td>0.27</td>
<td>0.32</td>
<td>0.37</td>
<td>0.30</td>
</tr>
<tr>
<td>12</td>
<td>Pb</td>
<td>10.43</td>
<td>97.12</td>
<td>105.27</td>
<td>117.62</td>
<td>109.31</td>
<td>117.85</td>
</tr>
</tbody>
</table>

* Nb. Metals marked with an asterisk (*) are unreliable results due to the ICP-MS instrument underestimating concentration, thus limiting the detection level of the elements concerned.*
copper (191.98 μg g⁻¹), arsenic (79.37 μg g⁻¹), and lead (117.85 μg g⁻¹) were determined in sediments collected from site number 6.

7.4.2.2 Biological material

7.4.2.2.1 Mytilus edulis

The same heavy metals as that of the sediments were measured in the soft tissue pooled from six individuals from each site. The total body burden metal concentrations are presented in table 7.4. As was observed in the sediment samples, the lowest concentrations of each element (with the exception of copper and mercury) were measured in the tissues of mussels collected from Bantham, the reference site. Consistent with the measurements of the sediments, the highest chromium concentrations were found at site number 4 (Jupiter Point), totalling 4.66 μg g⁻¹ dry tissue. Furthermore, the highest concentrations of manganese, cobalt and cadmium were also measured in the mussel tissue from this site. Of the six sites, mussels from site number 5 contained the highest concentrations of the most significant number of metals, those being iron, nickel, zinc, selenium and lead.

7.4.2.2.2 Cerastoderma edule

As with the method for extracting and measuring metal content of the soft tissue from M. edulis, the same method was employed for C. edule. The total body burden metal concentrations are presented in table 7.5. As was observed in the sediment samples in addition to M. edulis soft tissue samples, the lowest concentrations of each element (with the exception of chromium, arsenic and mercury) were measured in the tissues of mussels collected from Bantham, the reference site. Consistent with the measurements of the sediments, the highest manganese concentrations were found at site number 4 (Jupiter Point), totalling 90.29 μg g⁻¹ dry tissue. Of the six sites, cockles collected from site number 4 contained the highest concentrations of the most significant number of metals, those being manganese, copper, arsenic and lead.
Table 7.4. The concentrations of total heavy metals in the soft tissue of adult *Mytilus edulis* collected from the different sites along the Tamar estuary (μg g⁻¹).

<table>
<thead>
<tr>
<th>Site</th>
<th>Element</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cr</td>
<td>0.97</td>
<td>1.78</td>
<td>4.66</td>
<td>1.41</td>
<td>2.84</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>Mn</td>
<td>6.28</td>
<td>22.39</td>
<td>96.67</td>
<td>44.14</td>
<td>36.01</td>
<td>29.39</td>
</tr>
<tr>
<td></td>
<td>Fe</td>
<td>166.12</td>
<td>340.02</td>
<td>453.59</td>
<td>360.34</td>
<td>715.33</td>
<td>466.43</td>
</tr>
<tr>
<td></td>
<td>Co</td>
<td>0.00</td>
<td>0.65</td>
<td>5.32</td>
<td>1.62</td>
<td>3.11</td>
<td>3.56</td>
</tr>
<tr>
<td></td>
<td>Ni</td>
<td>0.99</td>
<td>1.27</td>
<td>2.86</td>
<td>2.90</td>
<td>3.61</td>
<td>2.59</td>
</tr>
<tr>
<td></td>
<td>Cu</td>
<td>8.73</td>
<td>13.74</td>
<td>8.57</td>
<td>10.38</td>
<td>12.35</td>
<td>8.93</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>34.25</td>
<td>103.92</td>
<td>152.62</td>
<td>117.62</td>
<td>193.65</td>
<td>137.10</td>
</tr>
<tr>
<td></td>
<td>As</td>
<td>8.54</td>
<td>12.11</td>
<td>12.72</td>
<td>10.63</td>
<td>13.02</td>
<td>13.21</td>
</tr>
<tr>
<td></td>
<td>Se</td>
<td>2.69</td>
<td>3.28</td>
<td>9.35</td>
<td>5.47</td>
<td>10.74</td>
<td>8.50</td>
</tr>
<tr>
<td></td>
<td>Cd</td>
<td>0.35</td>
<td>0.80</td>
<td>2.10</td>
<td>0.99</td>
<td>1.50</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td>Hg</td>
<td>0.15</td>
<td>0.23</td>
<td>0.00</td>
<td>0.07</td>
<td>0.04</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Pb</td>
<td>1.31</td>
<td>11.52</td>
<td>21.31</td>
<td>9.22</td>
<td>22.97</td>
<td>13.19</td>
</tr>
</tbody>
</table>
Table 7.5. The concentrations of total heavy metals in the soft tissue of adult *Cerastoderma edule* collected from the different sites along the Tamar estuary (μg g\(^{-1}\)).

<table>
<thead>
<tr>
<th>Site</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr</td>
<td>1.61</td>
<td>3.63</td>
<td>1.36</td>
<td>3.41</td>
<td>3.05</td>
<td>1.44</td>
</tr>
<tr>
<td>Mn</td>
<td>15.67</td>
<td>32.51</td>
<td>57.11</td>
<td>90.29</td>
<td>69.29</td>
<td>48.41</td>
</tr>
<tr>
<td>Fe</td>
<td>930.57</td>
<td>1069.62</td>
<td>1153.21</td>
<td>1369.62</td>
<td>1516.60</td>
<td>1228.30</td>
</tr>
<tr>
<td>Co</td>
<td>6.21</td>
<td>7.99</td>
<td>10.77</td>
<td>11.95</td>
<td>15.62</td>
<td>15.47</td>
</tr>
<tr>
<td>Ni</td>
<td>22.86</td>
<td>37.79</td>
<td>45.33</td>
<td>30.84</td>
<td>42.86</td>
<td>55.87</td>
</tr>
<tr>
<td>Cu</td>
<td>6.03</td>
<td>9.78</td>
<td>12.29</td>
<td>24.40</td>
<td>14.53</td>
<td>11.73</td>
</tr>
<tr>
<td>Zn</td>
<td>44.28</td>
<td>59.67</td>
<td>86.51</td>
<td>79.89</td>
<td>59.00</td>
<td>68.82</td>
</tr>
<tr>
<td>As</td>
<td>14.46</td>
<td>15.42</td>
<td>15.78</td>
<td>17.28</td>
<td>17.22</td>
<td>13.95</td>
</tr>
<tr>
<td>Se</td>
<td>7.53</td>
<td>8.61</td>
<td>10.15</td>
<td>8.53</td>
<td>13.87</td>
<td>10.03</td>
</tr>
<tr>
<td>Cd</td>
<td>0.40</td>
<td>0.22</td>
<td>0.32</td>
<td>0.36</td>
<td>0.33</td>
<td>0.37</td>
</tr>
<tr>
<td>Hg</td>
<td>0.01</td>
<td>0.00</td>
<td>0.38</td>
<td>0.03</td>
<td>0.17</td>
<td>0.05</td>
</tr>
<tr>
<td>Pb</td>
<td>1.90</td>
<td>6.35</td>
<td>6.72</td>
<td>15.55</td>
<td>11.38</td>
<td>7.65</td>
</tr>
</tbody>
</table>
With some exceptions and with an overall view, it appears from the sediment, and both *M. edulis* and *C. edule* tissue samples, that the sites most consistently found to be significantly contaminated with heavy metals were sites 4 (Jupiter Point), 5 (Town Quay) and 6 (Neale Point).

### 7.4.3 Biological markers

#### 7.4.3.1 Comet assay

The comet assay was carried out on haemolymph samples collected from the mussels and cockles immediately after they had been cleaned of extraneous epibionts and sediments. Due to the constraints imposed by the quantity of animals that could be collected from each site and the number of samples which could be processed per experimental session, the comet assay was carried out on six animals per site for each species. There were insufficient *M. edulis* adults within the acceptable size range at Torpoint (site number 2) for the comet assay to be carried out.

#### 7.4.3.1.1 Mytilus edulis

The data showing the level of DNA damage measured by the tail length and tail moment of the cells with the comet assay are presented in figures 7.3a and 7.3b respectively. These data were non-parametric and could not be normalised by transformation, the data were therefore treated with the Kruskall-Wallis test. For the tail length (figure 7.3a), the results of the statistical analyses indicated that there was a statistically significant difference ($p<0.0001$) between the medians of the comet tail lengths from the various sites. By comparing the median notches from the box-and-whisker plots (figure 7.3a), it can be seen that the animals collected from site 3 (Cove Head) and site 6 (Neale Point) contained significantly more DNA damage than the organisms from the reference site, Bantham (indicated with asterisks). For the tail moment
Figure 7.3. The level of DNA damage indicated by (a) the tail length of cells; and (b) tail moment measured with the comet assay in haemocytes from *M. edulis*; * denotes a statistically significant difference from the reference site 1 (p<0.0001).
(figure 7.3b), the Kruskall-Wallis test indicated that there were statistically significant differences between the medians of the samples from the same sites (p<0.0001).

When these data were compared with the concentrations of heavy metals in the soft tissue of *M. edulis* collected from the same sites (table 7.4), there was a correlation between the two parameters. There were consistently high concentrations of a number of the elements including Cr, Mn, Fe, Co, Ni, Zn, Se, Cd, Pb in the soft tissues of the mussels collected from site 3 (Cove Head) and similarly there were significantly high levels of DNA damage measured with the comet assay. High concentrations of Fe, Co, As and Hg were also observed in the soft tissues of mussels collected from site 6 (Neale Point), the site from which haemolymph samples were collected from mussels and also exhibited high levels of DNA damage.

7.4.3.1.2 *Cerastoderma edule*

The data showing the level of DNA damage indicated by the tail length and the tail moment of the haemocytes, measured with the comet assay are presented in figures 7.4a and 7.4b respectively. The data were non-parametric, however, the tail length data could be normalised by log transformation and therefore could be treated with one-way ANOVA. An elevation in the mean tail length of the samples collected from *C. edule* from site number 4 (Jupiter Point) compared with the reference site (Bantham) was statistically significant (p=0.0002) in the log-tranformed data. However, when the Kruskall-Wallis test was applied to the non-transformed (non-parametric) data, there was not a statistically significant increase between the medians when comparing the various sites with the reference site (Bantham).

When considering the data for the tail moment, these were also non-parametric. However, it was not possible to normalise the data by transformation, therefore the Kruskall-Wallis test was applied to test the significance between the medians. With this test, it was found that the level of DNA damage was significantly higher in samples
Figure 7.4. The level of DNA damage indicated by (a) the tail length of cells; and (b) tail moment measured with the comet assay in haemocytes from *C. edule*; * denotes a statistically significant difference from the reference site 1 (p<0.005).
collected from sites 4 and 6 (Jupiter Point and Neale Point respectively) when compared with the reference site (Bantham). Significant differences from the reference site in non-transformed data are indicated with an asterisk in figure 7.3b.

When these data are compared with the concentrations of heavy metals in the sediments (table 7.3) and the soft tissue of *C. edule* collected from the same sites (table 7.5), there is a correlation between the chemical and the comet assay data. There were consistently high concentrations of a number of the elements including Cr, Mn, Cu, Zn, As, Pb in the sediments and soft tissues of the cockles collected from site 4 (Jupiter Point) and similarly there were significantly high levels of DNA damage measured with the comet assay. High concentrations of Fe, Co, Ni and Cd were also observed in the soft tissues of cockles collected from site 6 (Neale Point), the site from which haemolymph samples were collected from mussels and also exhibited high levels of DNA damage.

### 7.4.3.2 Neutral red retention assay

The neutral red retention (NRR) assay was carried out on haemolymph samples collected from mussels and cockles immediately on return to the laboratory after they had been cleaned of extraneous sediments and epibionts. Of the samples collected from each individual for the comet assay, a 40 µl volume was used for the NRR assay. The NRR assay was carried out on six animals from each site, with duplicate slides being prepared from each animal. As with the comet assay, there were insufficient *M. edulis* adults within the acceptable size range at Torpoint (site number 2) for the NRR assay to be carried out.

#### 7.4.3.2.1 *Mytilus edulis*

The data showing the mean retention time of neutral red is presented in figure 7.5. The data were normally distributed, therefore allowing one-way ANOVA tests to be applied. There was no statistically significant difference between the means of the samples
Figure 7.5 The neutral red retention time of the lysosomal compartment of *M*. *edulis* haemocytes collected from field sites along the Tamar estuary. (Error bars indicate 2SEM).

Figure 7.6 A box-and-whisker plot of the neutral red retention time of the lysosomal compartment of *C*. *edule* haemocytes collected from field sites along the Tamar estuary; * denotes a statistically significant difference from the reference site (p<0.001).
from each of the sites \(p=0.7606\). However, there was a reduction in the mean retention
time in samples collected from sites 3 and 4 indicating the these sites were more impacted
by xenobiotic influences (such as chemical contaminants) and these were causing a
detrimental effect upon the cellular physiology of the animals from those sites.

When comparing these results with those of the chemical data mentioned
previously, it was found that site number 4 (Jupiter Point) was most consistently
contaminated with heavy metals.

7.4.3.2.2 *Cerastoderma edule*

The data showing the mean retention time of neutral red is presented in figure 7.6.
The data were non-parametric and were therefore log transformed to allow the application
of one-way ANOVA for comparison of the means between samples. This test indicated
that there was a statistically significant difference between the means of the samples
collected from the reference site (Bantham) and that of the samples collected from cockles
from site number 5 (Town Quay) in the log transformed data \(p=0.0113\). However, the
retention time was actually an increase in relation to the reference site, indicating that the
animals were less stressed at site 5 than at the reference site.

When comparing these results with those of the chemical data extracted from the
body tissues of *C. edule* there is no significant correlation between the body burden
concentrations and the cytological effects indicated by the NRR assay.

7.4.3.3 Other biomarkers

Three further biomarkers were employed and executed by a co-worker
concomitantly with the assays described earlier in this chapter and mentioned in figure 7.2.
These included the biochemical biomarker analysis of metallothionein concentrations; the
cytogenetic micronucleus assay; and a physiological measurement of heart rate.
The full data are not presented here, however, a summary of the mean data is presented in table 7.6. Indications were that the highest levels of metallothionein (MT) concentrations could be found in the whole tissue of the mussels and cockles (a mean of 58 μg g⁻¹ and 26 μg g⁻¹ wet weight tissue respectively) collected from site number 1 (Bantham). Samples from site number 2 (Torpoint) contained the next highest concentrations of MT, with the remaining sites containing similar levels of MT (ranging between 23 and 30 μg g⁻¹ for mussel tissue, and 18 and 24 μg g⁻¹ for cockles). These results did not appear to have any significant relationship with the concentrations of metals measured in the sediments or soft tissues as would be expected.

A high mean frequency of micronuclei (MN) (5.6 MN per 1000 cells) was scored in the haemocytes from *M. edulis* collected from site number 5 (under the Tamar Bridge), however the same trend was not observed in *C. edule*. In *C. edule* relatively high frequencies of MN were observed in samples collected from site number 2 (Torpoint) (4.1 MN per 1000 cells). There was no obvious trend detected between the two species and no consistency of one species containing more MN than another, nor was there any significant correlation between the MN frequencies and the chemical analyses of sediment and soft tissue samples. It would be expected that animals collected from site number 1 (Bantham), the reference site would contain the lowest frequency of MN, however this not the case.

For the heart rate data, *M. edulis* consistently had a higher rate per minute, with the highest heart rate for both species observed in the samples collected from the reference site (Bantham). The heart rates of *M. edulis* and *C. edule* collected from the remaining sites (sites 2-6) were all suppressed in comparison to the reference site, however, there was no significant difference for the heart rates of the animals collected from these sites. There was no obvious relationship between the heart rates and the metal data other than that there was a consistently low concentration of metals in the sediments and a corresponding high heart rate.
Table 7.6 Summary of mean data collated from metallothionein, micronucleus and heart rate assays in *M. edulis* and *C. edule* collected from the various field sites along the Tamar estuary.

<table>
<thead>
<tr>
<th>Site</th>
<th>Metallothionein (µg g⁻¹)</th>
<th>Micronucleus (per 1000 cells)</th>
<th>Heart Rate (beats min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M. edulis</em></td>
<td><em>C. edule</em></td>
<td><em>M. edulis</em></td>
</tr>
<tr>
<td>1</td>
<td>58</td>
<td>26</td>
<td>2.8</td>
</tr>
<tr>
<td>2</td>
<td>43</td>
<td>18</td>
<td>1.7</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>18</td>
<td>2.0</td>
</tr>
<tr>
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<td>27</td>
<td>21</td>
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</tr>
<tr>
<td>5</td>
<td>30</td>
<td>24</td>
<td>5.6</td>
</tr>
<tr>
<td>6</td>
<td>29</td>
<td>24</td>
<td>3.2</td>
</tr>
</tbody>
</table>
7.5 Discussion

There is evidence from the data collected in these studies that some biological markers can be used to identify sites of high contaminant concentrations by measuring pollution responses in indigenous populations. There were some correlations between the genotoxic responses measured with the comet assay in both species and the metal concentrations. In addition, the NRR assay detected cytotoxic responses in *M. edulis* which correlated with the chemical data where metal concentrations were determined.

It is thought that metals cross cell membranes essentially by passive transport processes although endocytosis may also occur (Viarengo, 1985). Some trace metals are essential for the maintenance of cellular functions. For example, zinc ions are essential in the maintenance of the structure and function of cell membranes stabilising the plasma and internal membranes by binding to structural components or by preventing metal-catalysed lipid peroxidation (Viarengo, 1995). Copper is also an essential metal, holding a fundamental role in electron transport, respiration, growth and development (Linder, 1991). However, in excess, copper is able to bind to DNA bases and causes unwinding of the double-helix structure resulting in DNA damage such as single and double-strand breaks, modified bases, abasic sites, DNA-protein cross-links and bulky adducts (Eichhorn & Shin, 1968; Rodriguez *et al*., 1995; Lloyd & Phillips, 1999).

In the current studies only total heavy metals were measured in the sediment and soft tissue samples, whereas in some field studies by other workers, PAHs have also been measured. Previous authors (Roy *et al*., 2002) exposed the larvae of the Atlantic tomcod in laboratory conditions to dioxin-like compounds and PAHs and had found there were elevated levels of CYP1A1 in exposed samples compared with the vehicle-exposed controls. However, in fish exposed in the Hudson River, New York, there was no significant increase in the CYP1A1 expression indicating that in environmental conditions, the transcriptase-polymerase chain reaction was not sufficiently sensitive to detect low levels of PAH exposure from environmental samples. This emphasises the importance of
carrying out bioassays in environmentally exposed samples in addition to laboratory-based exposures.

In addition to the limited types of contaminants which were measured, the quantification of the metals was presented as total metals, as opposed to specifying the species of metals. Metal speciation in natural waters is of high importance and relevance since toxicity, bioavailability, environmental mobility and biogeochemical behaviour are all strongly dependent on the chemical species of metals (Fytianos, 2001).

7.5.1 Chemical analyses

A number of the metals analysed in the sediment and biological tissue samples in the present studies have been reported to cause genetic toxicity in mammalian and aquatic organisms. In this particular study, constraints implicated by resources did not permit the analyses of specific metal species in the sediment and tissue samples, nor were the samples analysed for PAH and other organic contaminants. Nonetheless, a correlation between sites of high levels of heavy metals and biological indicators of DNA damage and cellular toxicity were observed.

Some of the elements which are found in high concentrations in the South-west of England area are due to the geological background of rock strata. Mineralisation occurred when granite masses and other igneous intrusions came into contact with sedimentary rocks in the vicinity, altering the rocks on their margins and creating a metamorphic aureole with associated tin, copper, arsenic and tungsten being deposited. These mineral-rich rocks were exploited by mining and have caused mobilisation of metals into the natural environment (Environment Agency, 1996b). The rate of settlement and adsorption of suspended particulate matter have a significant effect on the distributions of contaminants with the kinetics of the rate of adsorption having a direct effect upon the quantities of contaminants taken up by filter feeders such as mussels (Liu et al., 1998). Hence the correlation between high concentrations of heavy metal contaminants measured
in sediment samples and subsequent high concentrations of the same heavy metals in the body burden measurements of *M. edulis* and *C. edule* soft tissue samples are as expected.

There has been a history of exploitation of metalliferous rocks in the Tamar area, with a significant amount of arsenic being mined and produced from the area approximately a century ago (Bryan & Langston, 1992; Lindsey & Bell, 1997). Also, a cement and lime manufacturer (Blue Circle Industries) is located on the River Plym (illustrated in figure 7.1) which is known to have released controlled amounts of arsenic into the tributary in 1999 as monitored by the Environment Agency.

### 7.5.2 Biomarkers

#### 7.5.2.1 Comet assay

Consistent with the findings in this chapter, field exposures of indigenous or deployed marine organisms produced higher levels of DNA damage measured with the comet assay and DNA alkaline unwinding assay in samples where there were high levels of organic and metal contaminants in a number of previous reports (Nacci & Jackim, 1989; Nacci *et al.*, 1992; Everaarts, 1995; Sasaki *et al.*, 1997; Steinert *et al.*, 1998; Frenzilli *et al.*, 1999; Frenzilli *et al.*, 2001). The DNA damage responses detected with the comet assay appeared to correlate with the relative heavy metal concentrations in sediments. Animals from sites where soft tissue concentrations were found to be high (compared with the other sites) also had relatively high comet tail lengths and/or high comet tail moment measurements. This biomarker correlated most significantly with the chemical analyses when compared with the other endpoints measured.

#### 7.5.2.2 Micronucleus assay

In a field study carried out by Burgeot *et al.* (1996), the MN assay was unable to differentiate between levels of genotoxicity at different sites, and did not always relate to long-term measurements of chemical pollutants. Their conclusion was that the assay
required improvement before application in field monitoring. Brunetti et al. (1988) measured MN in indigenous mussel populations in the Venetian lagoon. They found different levels of MN frequencies at different sampling stations, although a more direct observation of the population density provided information regarding the pollution levels. They hypothesised that the unexpected low frequencies of MN at highly contaminated sites, may have been due to a selective phenomenon, the specimens that were collected being the most resistant to polluting agents. As was observed in the current studies, there was not a significant correlation between MN induction in either *M. edulis* or *C. edule* and the heavy metal contaminants at the various sites in either the sediments or soft tissue samples. In a field study by Wrisberg et al. (1992), indigenous populations of mussels were sampled for MN assays. In two of the four populations, elevated MN frequency was observed indicating the presence of genotoxic pollution. The base-line MN frequencies were compared from samples at different seasons of the year. Brunetti et al. (1992a) noted that there were significant increases in MN frequencies in samples collected during the summer. They hypothesised that this may have been due to thermal stratification of the water column, which segregated pollutants in more superficial water layers, or there may have been a direct effect of temperature on the mitotic rate of the mussels. However, these authors carried out the analyses intermittently over a period of over a year, and although they noted the sex ratio of the animals sampled, they did not attempt to correlate the sexual maturity of the animals with the induction of MN. In the field study described in this chapter the animals from each species were collected and the samples processed within a month of one another (September-October, 2001), minimising any potential impact that seasonality may have had on the results and ensuring that all the animals were not spawning.

As well as cytogenetic toxicity with the MN assay, other methods can be applied to determine genotoxic effects. Theodorakis et al. (2000) undertook a study investigating the relationship between genotoxicity, mutagenicity and fish community structure in a
contaminated stream. Genotoxicity was evaluated by measuring chromosomal damage with flow cytometry and strand breakage with gel electrophoresis. Salmonella/microsome assays were used to determine mutagenicity and community level responses were determined with community diversity and percent pollution-tolerant species. These authors found that there were similar patterns of downstream effects to the data collated from the current work. Increasing distance from upstream sites had more significant biological responses and these correlated with chemical measurements.

7.5.2.3 Metallothionein assay

Metallothioneins (MT) are low-molecular-mass cysteine-rich proteins which are induced by and bind to certain heavy metals (Viarengo, 1985; Roesijadi, 1992; Pederson et al., 1994). In the current study, the results were not as expected considering that MT are thought to be induced by high concentrations of certain non-essential water-borne metals (Roesijadi, 1992). However, the significantly high concentrations of whole tissue MT in both the mussel and cockle tissues were not reflected by high metal concentrations of the sediment or soft tissue samples at the same field sites, but rather the inverse of this relationship (i.e. high MT concentrations corresponding with low metal concentrations in the sediments and tissues) was observed. A similar trend was reported by Roesijadi (1995) in oysters (Crassostrea virginica) collected from the estuarine reaches of the Patuxent River. It should be noted however, that the total heavy metal concentrations were determined in the sediment and body tissue samples, rather than the bioavailable concentrations. Chemical speciation, concentration gradients in the water column and biological responses would all contribute and affect the rate of bioaccumulation (Roesijadi, 1995).

As well as sequestering potentially toxic, non-essential metals, MT have a function for the regulation of essential metals such as copper and zinc. It is possible that perhaps essential metals were in depletion or low concentrations in the water at the reference site.
(Bantham) and therefore MT were being induced to aid the binding of some of the essential metals for the maintenance of the normal function of the animals. In other field studies by Roesijadi (1995; 1999), the levels of MT in oysters were not induced by metal-contaminated waters. It was observed that MT-bound fractions of zinc did not exhibit any correlation with total or cytosolic concentrations of this metal and that concentrations of the MT-bound fractions actually declined in oysters collected from the site with the highest tissue metal concentrations. Furthermore, indications are that the non-essential metal cadmium at low concentrations in the natural environment, may produce observations of elevated MT concentrations due to stabilisation of cadmium and its accretion while being synthesised at basal rates, rather than MT induction as such. Cadmium has on the other hand, been observed to induce MT levels in laboratory conditions. Furthermore, recent observations have indicated that MT can also act as free radical scavengers and are involved in cellular antioxidant defences and it is regulated and inducible by a variety of factors including steroid hormones, metal ions oxidative stress and ionizing radiation (Vukovic et al., 2000).

7.5.2.4 Neutral red retention assay

The NRR assay has been used in a number of field studies to assess cell injury (Lowe, 1988; Moore & Simpson, 1992; Cheung et al., 1996; Wedderburn et al., 1996). Although the acidic nature of the internal environment of the lysosomal compartment would not be expected to be suitable for metal accumulation, data demonstrates that lysosomes are nonetheless important sites of metal compartmentation in the cell (Viarengo, 1985). It has been found that copper and cadmium cause detrimental effects on the stability of the lysosomal membrane (Moore et al., 1984). However, not all heavy metal ions cause damaging effects on the lysosomal compartment. Some studies have indicated that zinc is able to stabilise the lysosomal membranes (Sternlieb & Goldfischer, 1975) and stimulatory effects on the lysosomal enzymes have been observed (Webb,
In addition to heavy metals, polycyclic aromatic hydrocarbon (PAH) contaminants have been reported to induce destabilisation of the lysosomal compartment and the NRR assay has been used to identify cytotoxic effects of such pollutants. In a study of mussels located close to the location of the spillage of oil from the Sea Empress tanker, the stability of the lysosomal compartment was found to be inversely correlated with PAH concentration in the mussel tissues (Fernley et al., 2000).

In the current study, the haemocytes of *M. edulis* were found to be less stable (resulting in a reduced NRR time) in mussels collected from sites 3 and 4 indicating that there was more cytotoxic effects at these sites. There is a correlation between the heavy metal concentrations in the sediments and the soft tissue samples measured at these sites and the NRR time.

### 7.5.2.5 Heart Rate Monitoring

Physiological and behavioural responses of organisms to pollution have been previously investigated in aquatic organisms. Among the various responses, changes in heart rate have been used as a response to physical stress (Bamber & Depledge, 1997b). In crustaceans such as crabs, heart rate is typically elevated in response to physical stress presumed to be critical to the survival of these animals to be able to escape from predators or physical perturbation. In laboratory studies, heart rate changes have been reported following exposure to various contaminants (Depledge, 1984; Aagard et al., 1991; Depledge & Lundebye, 1996). However, in studies where samples have been collected from indigenous populations, results have not been conclusive and indications have been that the method of heart rate monitoring would require further investigation (Bamber & Depledge, 1997b). The data collated from the current field study indicated that animals from sites where more xenobiotic contaminants were present had a lower heart rate than the animals from the reference site, however, this endpoint was not apparently sufficiently
sensitive to differentiate between contaminant effects between the populations from remaining sites.

7.6 Conclusions

In conclusion, the biomarker responses previously identified and implemented in laboratory studies are applicable to indigenous populations of *M. edulis* and *C. edule* collected from various field sites. The genotoxic responses measured with the comet assay and sediment contaminant concentrations were found to be highest at sites furthest away from the sea and where water exchange would be the lowest due to the confounding physio-chemical nature of the Tamar estuary. In general, there was an increase in the genotoxic biomarker responses with distance from the sea (i.e. sites further upstream). These biomarker responses appeared to correlate with high concentrations of heavy metals within the sediment indicating that there was a causal relationship between contamination and biological effects. However, it would be necessary to measure metal concentrations in the water collected from these sites and to determine the species of the metals, in addition to PAHs and other organic contaminants (such as polychlorinated biphenyls) and other agents such as ionising radiation, to fully understand the causes of the biological responses observed.

There have been suggestions that ecotoxicological studies are difficult in natural environments due to the genetic variability within natural populations as well as individuals. This inherent variability makes the use of "control" or "reference" populations very difficult (Hasspieler *et al.*, 1995). The authors suggested that alternatives to the use of *in vivo* organisms should be addressed and instead use human and fish cell lines for the screening and testing of water quality, however, such studies do not take into consideration the inter-species interactions, nor the response of natural populations to pollution events or long-term exposures of indigenous populations. It would have been a more robust study had time allowed sampling from at least one further reference site, since
"control" sites in field studies are only relative to other sites and never absolutely pristine. Furthermore, a study where seasonal factors were taken into consideration would have been beneficial and informative.

To have been able to identify individuals and species with high levels of resistance to contaminant effects would assist in answering questions related to either physiological acclimatization or evolutionary adaptation (Nevo et al., 1986; Tatara et al., 2001). Further studies would be required to identify such factors which could explain unexpected results such as those measured by the metallothionein assay.
Chapter 8

DNA Repair Mechanisms in the Haemocytes of *Mytilus edulis*
Hypothesis: DNA repair processes occur in *Mytilus edulis* haemocytes *in vitro*.

8.1 Introduction

As demonstrated in earlier chapters, the DNA of living cells is highly reactive to a great variety of chemical and physical agents. For convenience, two major classes of DNA damage have been identified; those cause by (i) spontaneous, and (ii) environmental factors (Friedberg, 1985). However, it is difficult to differentiate between the biochemical changes which result in DNA damage as those that occur "spontaneously" from those which may be brought about by interaction with xenobiotic agents (Friedberg, 1985). Spontaneous damage to DNA may arise from a variety of mechanisms, including the mispairing of bases during DNA synthesis and replication; the replacement of nucleotides during repair synthesis of DNA (non-semireplication of DNA) and DNA rearrangement; and in the culturing of cells, clastogenic agents within culturing media have been found to induce chromosomal breakages (Friedberg, 1985). Environmental damage to DNA may be induced following exposure to chemical agents which may include alkylating agents, DNA-DNA cross-linking agents, DNA-protein cross-linking agents, or chemicals that may be metabolised into electrophilic reactants. Also, physical agents such as ultraviolet radiation (UVR), or ionising radiation are considered to be environmental factors which can target DNA material (Friedberg, 1985).

Much of the work presented previously has focused upon DNA damage and its detection, and the endpoints have given measure of net levels rather than gross levels of DNA damage. In contrast to other macromolecules, one of the most incredible characteristics of DNA is that it has the capacity to repair damaged sections and remove inflicted lesions (Griffiths & Carr, 1998). The spectrum of damage that cellular DNA can suffer is extensive and as mentioned earlier, in addition to DNA damage being caused by environmental factors, a significant level of DNA damage arises from the activities of reactants generated naturally within cells themselves (Burdon, 1999). DNA breaks may be produced by a variety of chemicals and reactive oxygen species (ROS), the latter may be
induced following endogenous metabolism or may be produced in excess following redox cycling or other free radical interactions associated with organic contaminants, metabolites and metals. Strand breaks may also result from alkali-labile sites or during the excision repair of moieties such as DNA adducts (Mitchelmore et al., 1998b).

The defence mechanisms of invertebrates differ from that of vertebrates mainly in that invertebrates do not have a basis for the development of lymphoid tissue giving rise to a sufficient number of cell lineages and there is no cellular cooperation during defence reactions (Galloway & Depledge, 2001). Most invertebrates including the bivalve molluscs, have freely circulating haemocytes which share their immune function with the mid-gut and sinus linings. One of the main mechanisms of cellular defence in invertebrates is phagocytosis performed by the haemocytes (Noël et al., 1993; Barraco et al., 1999; Fournier et al., 2000). In bivalves such as Mytilus edulis and Crassostrea virginica, phagocytosis occurs following ingestion and bacteriocidal factors are then discharged. These may include the activation of lysosomal degradative enzymes and reactive oxygen intermediates such as superoxide, hydrogen peroxide, or singlet oxygen may be produced (Cheng, 1983; Adema et al., 1991; Pipe & Coles, 1995; Pipe et al., 1995; Torreilles et al., 1997; Anderson, 1999; Burdon, 1999; Fournier et al., 2000). These reactive oxygen intermediates have the potential to cause damage to DNA material by forming DNA adducts (such as 8-hydroxy-2'-deoxyguanine), or forming apurinic/apyrimidinic sites in DNA via the oxidation of sugar moieties, as well as causing structural alterations in the phosphodiester backbone resulting in the formation of single- and double-strand breaks (Newcomb & Loeb, 1989). In mammalian systems, oxidative DNA damage has been considered to be a causative factor in human cancer induced by ionising radiation, transition metals, chronic inflammation, and age-related decline in function, as well as a factor in hereditary syndromes with a proclivity to malignancy and a number of other diseases (Newcomb & Loeb, 1998).
Oxidative stress can potentially be experienced by all aerobic life and hydroxyl free radicals are capable of reacting with critical macromolecules such as DNA and proteins (Burdon, 1999; Cheung et al., 2001). Oxidative damage occurs when antioxidant defences are overcome by pro-oxidant forces and it has been shown that xenobiotic contaminants, including PAHs and metals, can induce oxidative stress in aquatic organisms (Winston, 1991; Dyrynda et al., 1998). The different stages which occur during phagocytosis are demonstrated in figure 8.1. In the first stage, (a) the free radical superoxide is produced from the reduction of molecular oxygen through the action of a plasma membrane NADPH-oxidase complex. This is followed by step (b) where hydrogen peroxide is formed by the spontaneous dismutation of superoxide radicals. The hydrogen peroxide is able to kill some bacteria which have been uptaken into the plasma membrane vesicle. Once the membrane vacuole has been formed, fusion with other granules in the phagocytic cell cytoplasm releases the enzyme myeloperoxidase (c) which is able to use hydrogen peroxide as a substrate and chloride is oxidised to hypochlorous acid. Hypochlorous acid is highly reactive and is able to oxidise biomolecules and will induce the killing of engulfed organisms (Burdon, 1999). It was shown by Livingstone et al. (1988) that in mussels, Mytilus edulis, NADPH-dependent metabolism of B(a)P results in the production of hydroxyl and superoxide anion radicals.

The extent to which oxyradical generation results in biological damage is dependent on the effectiveness of antioxidant defences (Michiels & Remacle, 1988). Antioxidant enzymes play a crucial role in the maintenance of cellular homeostasis with their induction reflecting specific responses to pollutants. Oxidant-related effects may manifest if these protective enzymes are inhibited (Stegeman et al., 1992) and DNA damage, enzymatic inactivation and lipid peroxidation may occur if the antioxidant enzymatic systems are impaired (Cheung et al., 2001). Hence, hydrogen peroxide has been commonly used as a positive control in numerous comet assay studies (Singh et al., 1988; Kruszewski et al., 1994).
Figure 8.1 A summary of the mechanisms involved during phagocytosis and the potential for oxidative damage (based on Burdon, 1999).

NADPH-oxidase

\[ 2O_2 + \text{NADPH} \rightarrow \text{NADP}^+ + \text{H}^+ + 2O_2^- \]

superoxide

spontaneous mutation

\[ 2O_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

hydrogen peroxide

myeloperoxidase

\[ \text{H}_2\text{O}_2 + \text{Cl}^- \rightarrow \text{OH}^- + \text{HOCl} \]

hypochlorous acid
As well as oxidative damage, the integrity of DNA can be compromised by other mechanisms. A wide variety of chemicals are classed as alkylating compounds many of which are proven or suspected carcinogens (Lawley, 1989). These chemicals have an affinity for nucleophilic centres in organic macromolecules. Alkylating compounds can be classed as either monofunctional or bifunctional, whereby the former have a single reactive group and therefore covalently bond with single nucleophilic centres in the DNA. Bifunctional alkylating agents have two reactive groups and each molecule of the compound is able to react with two nucleophilic sites in the DNA (Friedberg, 1985; Friedberg et al., 1995).

Friedberg et al. (1995) state that all four bases of DNA (i.e. adenine, thymine, guanine and cytosine) have numerous potential reaction sites, although their potential for reactivity are quite variable. In general, alkylating agents react most readily with ring nitrogens of the bases compared with the relatively less reactive ring oxygens and alkylations at phosphodiester linkages at the N\(^7\) position of guanine and the N\(^3\) position of adenine are the most reactive and most prevalent. Modification of the bases by alkylation weakens the N-glycosylic bond, therefore this often leads to depurination or depyrimidinidation and the appearance of alkali-labile abasic sites.

In recent years, the field of DNA repair has attained increasing interest and recognition associated with its fundamental importance in genomic maintenance, and the fact that decreases in repair efficiency could lead to many pathological conditions including cancer (Hanawalt, 1998). Much of the insight regarding 'DNA repair' mechanisms has been elucidated by the identification of a number of mutants in bacteria and yeast cells, in addition to the generation of mutant mammalian cell lines following treatment with chemicals and radiation and DNA repair deficient syndromes (e.g. Xeroderma pigmentosum, ataxia telangiectasia etc.) (Karmarkar et al., 1998). Furthermore, approaches to study DNA repair processes have involved the inhibition of the action of enzymes by applying so-called DNA repair 'inhibitors' (Natarajan et al.,
1982; Collins et al., 1984). Compared to bacterial, yeast and mammalian systems, the lack of the identification of mutants has constrained the understanding of DNA repair processes in aquatic organisms. A few preliminary studies have been made regarding aquatic organisms' ability to repair DNA damage. Zahn et al. (1981) indicated that one type of B(a)P-induced DNA damage was repairable in sponges whereas another type of lesion induced by the same agent was persistent and could not be repaired in their laboratory conditions. In lobsters, James et al. (1992) showed that DNA repair processes were able to remove adducts induced by B(a)P metabolites.

A simple classification of the different types of DNA repair was proposed by Friedberg et al. (1980) and has been presented in table 8.1. Photoreactivating enzymes (PRE) such as photolyase have been identified in aquatic animals such as amphibians whereby the presence of cyclobutane pyrimidine dimers and 6-4 photoproducts have been found to be reduced in species which exhibited higher levels of the enzyme (Blaustein et al., 1994; Kim et al., 1996). The processes involved in this pathway have been described in the first chapter (section 1.5.1.3) and has been demonstrated using cell-free extracts as well as in vivo (Friedberg et al., 1980). The pathways of interest in the studies of the present chapter related to the base excision repair processes since these tend to be followed when oxidative DNA damage occurs.

In brief, the base excision repair processes involve a number of enzyme-mediated events including:

(i) cleavage of a phosphodiester bond adjacent to the damaged base;
(ii) removal of the damaged base;
(iii) replacement with an undamaged base;
(iv) ligation of the DNA strand.

If for any reason, any one of the latter three steps is interrupted, a strand break may remain. Such misrepair of the damaged DNA resulting in a single strand break (SSB) may be formed from numerous forms of base damage including covalent adduct formation or
oxidative damage (Friedberg et al., 1995; Hasspieler et al., 1995). A wide variety of genotoxic agents may induce the formation of SSBs such as PAHs, aromatic amines or compounds which stimulate the production of reactive oxygen species (Coles et al., 1994; Hasspieler et al., 1995). Such SSBs may in turn result in the formation of double strand breaks (DSBs) which if left unrepaired may manifest as chromosomal aberrations and can lead to cellular transformation or in extreme cases of mutation, cell death.

Damage induced by alkylation is fundamentally repaired by two processes; that of direct transfer of the modifying alkyl group to the repair protein or the removal of the modified base by a glycosylase, resulting in an apurinic or apyrimidinic site which is subsequently repaired by an excision-repair process (Beranek, 1990; Friedberg et al., 1995). Monofunctional alkylating agents may cause the production of O-alkylated and or N-alkylated products. Of the O-alkylated products, O\(^6\)-alkylguanine and O\(^4\)-alkylthymine are potentially mutagenic lesions since they can misrepair with thymine during semi-conservative DNA synthesis (Friedberg et al., 1995; Margison et al., 2002). Such lesions are repaired by direct reversal of the base damage. In ultraviolet radiation induced pyrimidine dimers, enzymatic photoreactivation catalyses the monomerisation of the dimers. In the case of O-alkylated products, direct reversal of base damage is catalysed by proteins such as O\(^6\)-alkylguanine DNA transferase (O\(^6\)-AT) which induce demethylation where the methyl group is removed, this process has been illustrated in figure 8.2 (Friedberg et al., 1980; Friedberg 1995; Burdon, 1999; Norbury & Hickson, 2001). These proteins accept the alkyl group and by so-doing destroy their own action and have therefore been classed as a specific protein reagent rather than an enzyme (Elliot & Elliot, 2002). Hence the amount of alkylation damage that this type of repair system can manage is limited by the level of the availability of unreacted alkyltransferase molecules (Burdon, 1999). The repair induced by such proteins occur by an error-free mechanism since only the modified alkyl group is removed from the DNA (Beranek, 1990). In the current studies methyl methane sulfonate (MMS) was used as a representative alkylating agent. This is
Table 8.1 Classification of DNA repair (based upon Friedberg et al., 1980).

<table>
<thead>
<tr>
<th>A. Reversal of Base Damage</th>
<th>1. <strong>Enzymatic photoreactivation</strong> (enzyme-catalysed monomerisation of pyrimidine dimers in DNA).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2. <strong>Base modification</strong> (enzyme-catalysed removal of specific chemical groups, thereby restoring modified bases to their normal chemistry).</td>
</tr>
<tr>
<td>B. Removal of Base Damage</td>
<td>1. <strong>Nucelotide excision</strong> (enzyme-catalysed excision of bases from DNA as mono- or oligo-nucleotides).</td>
</tr>
<tr>
<td></td>
<td>2. <strong>Base excision</strong> (enzyme-catalysed insertion of bases from DNA as the free base).</td>
</tr>
<tr>
<td>C. Base Restoration</td>
<td>1. <strong>Repair synthesis</strong> (enzyme-catalysed insertion of one or more nucleotides at sites of pre-existing base damage in DNA).</td>
</tr>
<tr>
<td></td>
<td>2. <strong>Base insertion</strong> (enzyme-catalysed covalent linkage of a base to the deoxyribose-phosphate backbone of DNA at sites of base loss).</td>
</tr>
</tbody>
</table>

**Figure 8.2** The direct reversal of damage to a DNA guanine residue by an alkyltransferase (based upon Burdon, 1999).
known to be a radiomimetic chemical and has been shown to induce both single and double strand breaks (Park et al., 1991; Nickoloff & Hoekstra, 1998).

A number of studies investigating the DNA repair mechanisms in a wide range of cell types have been reported. In the current studies, the haemocytes were selected as the target cell type since they are important for the immunodefence mechanisms against xenobiotics including chemical contaminants and pathogens (Coles et al., 1994; Pipe & Coles, 1995; Torreilles et al., 1997; Galloway & Depledge, 2001).

8.2 Aims & objectives

The aims and objectives of the studies described in this chapter were to evaluate the DNA repair capacity of the haemocytes from *M. edulis*. To examine the hypothesis that these cells are able to repair DNA damage, haemocytes from *M. edulis* were exposed *in vitro* to hydrogen peroxide (an oxidising agent) or methyl methane sulfonate (a monofunctional alkylating agent) in the presence or absence of the DNA polymerase inhibiting chemical, cytosine β-D-arabinofuranoside (AraC). The levels of DNA damage were then determined using the comet assay and comparisons made between samples incubated with AraC and those without AraC. This technique then allowed the determination of the gross (unrepaired) and net (repaired) levels of DNA damage experienced by the haemocytes.

8.3 Material & methods

All chemicals and reagents were supplied by Sigma-Aldrich (Poole, UK) unless otherwise stated.

8.3.1 Sampling of haemolymph

Adult *M. edulis* were collected from their natural beds at Sharrow Point, Whitsand Bay and allowed to acclimatise in laboratory conditions for a minimum of 24 hours as
described in section 2.1.1.1. prior to collection of haemolymph samples. From each mussel, 0.3 ml haemolymph was extracted from the posterior adductor muscle into 0.3 ml physiological saline as described in section 2.3.1. The haemolymph samples were then pooled by transferring the samples from the animals into a siliconised (10 ml) centrifuge tube which was kept on ice. 20 individuals were used for each test chemical used (i.e. 20 animals’ haemolymph pooled for \( \text{H}_2\text{O}_2 \) treatments and 20 animals’ haemolymph pooled for MMS treatments).

The pooled samples were each gently mixed by inversion, then 500 \( \mu \)l of the haemolymph suspension was transferred into 20 siliconised (1 ml) microcentrifuge tubes. These were then centrifuged at room temperature for 2.5 minutes at 1500 rpm.

### 8.3.2 Exposure of haemocytes to \( \text{H}_2\text{O}_2 \) and MMS

A series of solutions of hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) and methyl methane sulfonate (MMS) were prepared in 10.0 ml centrifuge tubes. The final concentrations of \( \text{H}_2\text{O}_2 \) were 0, 100, 500, 1000 and 5000 \( \mu \)M, each diluted with filtered seawater (filtered through a 10 \( \mu \)m carbon filter) in a similar method as that of the studies described in section 3.4. The final concentrations of MMS were 0, 0.1, 1, 10 and 100 \( \mu \)M, again diluted with 10 \( \mu \)m carbon-filtered seawater in a range of concentrations comparable to that of Jha et al. (1996). A 2 mM stock solution of AraC was first prepared separately by dissolving the chemical in 10 \( \mu \)m filtered seawater (0.04864 g in 100 ml) and this was later used for incubation with a parallel series of \( \text{H}_2\text{O}_2 \) and MMS solutions the final concentration of AraC was 1 mM as per Mitchelmore et al. (1998b). The treatments that were prepared are summarised in table 8.2.

Following centrifugation, the supernatant was carefully removed from each haemolymph sample by pipetting, then 300 \( \mu \)l of the test solutions were added to the haemocyte pellet. Each treatment was prepared in duplicate. To ensure equal exposure of
the cells, each sample was gently mixed by vortexing for 10 seconds. The samples were then maintained in the dark in incubation cabinets held at 15 ± 2°C for one hour.

8.3.3 Eosin Y cell viability assay

In advance of undertaking the comet assay, 20 µl sub-samples were pipetted onto microscope slides and the viability of the cells assessed using the Eosin Y assay. This was carried out as described in section 3.2.2.3. The viability of the cells in these samples ranged from 89.9% to 94.5%.

8.3.4 Comet assay

All reagents and solutions were prepared two days in advance of running the comet assay, as defined in Appendix I.

8.3.4.1 Slide preparation

Microscope slides were prepared for the comet assay immediately before running the comet assay, whilst the cells were being incubated with the test chemicals. Plain microscope slides were prepared as described in section 3.4.2.4.

Once the samples had been incubated for one hour, the samples were centrifuged again for 2 minutes at room temperature, at a speed of 1500 rpm. The cells were resuspended in 0.5% low melting point agarose (dissolved in Kenny's solution) and transferred to the base layer of normal melting point agarose in duplicate as described in sections 3.4.2.4 and 6.3.10.1.

8.3.4.2 Cell lysis

The lysis solution was prepared as described in sections 3.3.5.4 and 6.3.10.2, with Triton X-100 and dimethylsulfoxide (DMSO) being added immediately before treating the slides at a concentration of 1 and 10% respectively in a total volume of 250 ml. The
Table 8.2. A summary of the concentrations of the test chemicals prepared for each exposure scenario.

<table>
<thead>
<tr>
<th>$\text{H}_2\text{O}_2$</th>
<th>$\text{H}_2\text{O}_2 + \text{AraC}$</th>
<th>MMS</th>
<th>MMS + AraC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM</td>
<td>0 µM + 1 mM</td>
<td>0 µM</td>
<td>0 µM + 1 mM</td>
</tr>
<tr>
<td>100 µM</td>
<td>100 µM + 1 mM</td>
<td>0.1 µM</td>
<td>0.1 µM + 1 mM</td>
</tr>
<tr>
<td>500 µM</td>
<td>500 µM + 1 mM</td>
<td>1.0 µM</td>
<td>1.0 µM + 1 mM</td>
</tr>
<tr>
<td>1000 µM</td>
<td>1000 µM + 1 mM</td>
<td>10.0 µM</td>
<td>10.0 µM + 1 mM</td>
</tr>
<tr>
<td>5000 µM</td>
<td>5000 µM + 1 mM</td>
<td>100.0 µM</td>
<td>100.0 µM + 1 mM</td>
</tr>
</tbody>
</table>
coverslips were removed from each microgel and discarded before being placed into a nalgene® staining trough and the lysing solution was carefully poured into the trough to cover the slides without disturbing the microgels. This was then incubated at 4 °C in the dark for one hour as per previous experiments.

8.3.4.3 Alkaline DNA unwinding and electrophoresis

The procedures carried out for the unwinding of the DNA and electrophoresis were as described in section 6.3.10.3. The electrophoresis was undertaken with a continuous recirculating water flow around the electrophoresis chamber to ensure that a stable temperature of 20°C (± 2°C) was maintained throughout the electrophoresis of the samples. The samples were incubated for 20 minutes at room ambient temperature (20 ± 2°C) to allow the DNA to unwind before the electrophoresis commenced and the power supply was switched on and a voltage of 15V and a current of 320 mA was delivered for a period of 25 minutes as optimised in chapter 3.

8.3.4.4 Neutralisation and staining of slides

Following the period of electrophoresis, the power supply was switched off, the slides removed from the electrophoresis buffer and the microgels were neutralised as described in section 6.3.10.4. The microgels were then stained with 40 μl 0.02 mg/ml ethidium bromide solution, coverslipped and stored in airtight containers at 4°C, as per the previous experiments.

8.3.4.5 Scoring of comets

Slides were selected at random and scored blind to avoid biased measurements. 50 cells per replicate microgel (100 cells per slide) were selected at random whilst being simultaneously visualised via the image processing monitor and the level of DNA damage of each cell was measured using the Komet version 5.0 software (Kinetic Imaging, UK).
All measurements including % tail DNA, tail length and tail moment (among a series of other measurements) were automatically recorded for each cell selected using the software and stored as excel files.

8.4 Results

As mentioned in chapter 3, the most widely used measurements from the comet assay which have been reported in the literature are tail length, tail moment and head-to-tail ratio, however, the latter has not been well-defined by previous authors. Therefore the tail length and tail moment measurements have been employed in the current studies in the same manner as that described in section 3.4.2.4.

8.4.1 H₂O₂ with or without AraC

The amount of net and gross DNA damage (without and with AraC respectively) measured as the tail length and tail moment with the comet assay are presented in figures 8.3a and 8.3b. There was a significant dose-dependent response in both the treatments exposed solely to H₂O₂ and those also exposed simultaneously to 1mM AraC (R² = 0.8273 and 0.854 respectively) measured with tail length (figure 8.3a). Similarly, dose-dependent responses were observed when the DNA damage was measured with the tail moment (figure 8.3b). When the data was treated with one-way ANOVA it was found that there was a statistically significant increase in the level of damage measured by both the tail length and the tail moment, with the exception of the tail length measured in samples exposed to 500 μM H₂O₂.

8.4.2 MMS with or without AraC

The amount of DNA damage measured with the comet assay as the tail length and tail moment, with and without the presence of AraC are presented in figures 8.4a and 8.4b. There was a no significant dose-dependent response in the tail length measurements...
(figure 8.3a) in either of the treatments, that being only MMS or MMS with 1 mM AraC ($R^2 = 0.0293$ and 0.6375 respectively). Similarly, there was no dose-dependent trend measured with the tail moment (figure 8.3b) (MMS, $R^2 = 0.0348$; MMS with AraC $R^2 = 0.5504$).

When the data was treated with one-way ANOVA it was found that there was no statistically significant difference between the amount of damage measured in any of the samples incubated with and without AraC. There was an increase in the amount of gross DNA damage measured by tail moment in the control samples (as reflected in the control samples of the H2O2 experiment) although this is not statistically significant. This indicates that there is a certain amount of base-line damage being induced by background metabolic activity within the cells and this level of damage has no influence either with the addition of MMS on its own or in combination with AraC.
Figure 8.3a Tail length measured with the comet assay in *M. edulis* haemocytes exposed *in vitro* to hydrogen peroxide +/- AraC. Error bars = 2SEM; * denotes a statistically significant difference from the control; # denotes a statistically significant difference between the treatments with and without AraC (p<0.05).

Figure 8.3b Tail moment measured with the comet assay in *M. edulis* haemocytes exposed *in vitro* to hydrogen peroxide +/- AraC. Error bars = 2SEM; * denotes a statistically significant difference from the control; # denotes a statistically significant difference between the treatments with and without AraC (p<0.05).
Figure 8.4a Tail length measured with the comet assay in *M. edulis* haemocytes exposed *in vitro* to methyl methane sulfonate +/- AraC. Error bars = 2SEM. No significant dose-dependent response was observed.

Figure 8.4b Tail moment measured with the comet assay in *M. edulis* haemocytes exposed *in vitro* to methyl methane sulfonate +/- AraC. Error bars = 2SEM. No significant dose-dependent response was observed.
8.5 Discussion

It has been shown that the comet assay can be a powerful tool for detecting DNA strand breaks and alkali-labile sites in eukaryotic cells as reviewed by Fairbairn et al. (1995) and Rojas et al. (1999). With modification, this assay has also been used to detect DNA repair deficiencies (Alapetite et al., 1996; Rojas et al., 1999) as well as to determine the kinetics and capacity for DNA to repair itself (Gedik et al., 1992; Collins et al., 1997b; Mohankumar et al., 1998; Mediola-Cruz & Morales-Ramirez, 1999). By blocking the DNA polymerase which is involved in the repair processes, the action of AraC inhibits repair which follows the nucleotide excision repair pathway (Mitchelmore et al., 1998b; Andreoli et al., 1999), this chemical also inhibits other DNA polymerase dependent processes such as DNA replication. For these reasons, AraC was used in the present studies to test the hypothesis that DNA repair mechanisms are present in the haemocytes of *M. edulis*.

The gross level of DNA damage caused by H$_2$O$_2$ was significantly increased by AraC presumably through its ability to inhibit the long-patch DNA repair mechanisms following nucleotide excision (Collins et al., 1984; Mitchelmore et al., 1998b; Kammann et al., 2000). It was found by Mirzayans et al. (1992) that AraC can inhibit α- and β-polymerases and DNA ligase. In mussel digestive gland cells, there has been evidence that the β-form is the predominant polymerase, however, there is no report to indicate that AraC might have any effect on the repair capacity of *M. edulis* haemocytes (Accornando et al., 1985, 1989). In contrast, there was no significant change in the level of DNA damage induced in samples treated with AraC and MMS.

In agreement with previous experiments using marine mussels (Mitchelmore et al., 1998b; Wilson et al., 1998), H$_2$O$_2$ produced extensive DNA damage indicated by increases in tail length and tail moment in the cells analysed immediately following *in vitro* exposure. Furthermore, samples treated with H$_2$O$_2$ and AraC showed still significantly greater levels of DNA damage and strand breaks, compared with samples
exposed to \( \text{H}_2\text{O}_2 \) alone, indicating the level of DNA repair inhibited by AraC and supporting the hypothesis that the haemocytes of *M. edulis* have DNA repair capabilities. The genotoxic effect of agents such as \( \text{H}_2\text{O}_2 \) or other genotoxins which exert their genotoxicity indirectly for example *via* redox cycling and ROS production, is dependent upon the effectiveness of the antioxidant defences (Mitchelmore *et al.*, 1998b). Studies by Livingstone *et al.* (1994) have indicated that antioxidant defences are relatively high in *M. edulis*. In addition, in studies by Wilson *et al.* (1998), incubation with the antioxidant supplements of a vitamin E compound (\( \alpha \)-tocopherol acetate) was found to have a marked reduction on the background level of damage expressed by negative controls. The differences observed in the seawater control samples, which were incubated with or without AraC indicates that possibly AraC was generating additional levels of damage in its own right. This may have been caused directly by the formation of reactive oxygen species or indirectly by interfering with metabolic processes (de Andrade *et al.*, 2001).

The differences observed in the responses following *in vitro* \( \text{H}_2\text{O}_2 \) and MMS exposure of molluscan haemocytes highlight the differences in the type of DNA lesions that are induced by each of these chemicals. As mentioned earlier, alkylating agents such as MMS react with DNA to produce O-alkylated and N-alkylated products (Friedberg, 1985) and this reaction involves some biochemical reactions. The lack of a dose-response relationship following exposure to MMS either in the presence or absence of AraC, indicate that under *in vitro* conditions, the haemocytes have only limited or a low capacity to form alkylated DNA products. This is in contrast to the response observed in the samples exposed to \( \text{H}_2\text{O}_2 \). This chemical can produce reactive oxygen species resulting in the formation of DNA breaks due to direct action on the biomolecule. Furthermore, previous authors have indicated that mice exposed *in vivo* to the alkylating agent ethyl methanesulfonate (EMS) showed an increase in DNA damage measured with chromosomal aberrations (Mahmood & Vasudev, 1993).
Alkylating DNA damage is repaired by basically two processes; the direct transfer of the modifying alkyl group to the repair protein or the removal of the modified base by a glycosylase, leaving an apurinic or apyrimidinic site which is then repaired by an excision-repair process (Beranek, 1990). Furthermore, it has been reported that nucleotide excision repair, which is normally involved in the repair of adducts which distort the DNA helix, may play a "backup" role in the repair of lesions normally repaired by alkyl transferases or glycosylases (Beranek, 1990). With the method of direct removal of the alkyl group, it may be possible that only a limited amount of strand breaks were induced and therefore the comet assay was not able to detect this type of DNA damage. The lack of significant differences between MMS treated cells and those treated to MMS and AraC could also be due to the action of alkylation induced by MMS interacting with the AraC (Collins, personal communication). In reports by Schendel et al. (1978), another monofunctional alkylating agent N-methyl-N'-nitro-N-nitroguanidine (MNNG) was found not to yield mutants in a dose-dependent linear relationship and that when *Escherichia coli* were exposed to a moderate concentration of mutagen for varying periods of time a non-linear response was observed. A lack of a dose-related linear relationship was also observed in the current work when increasing concentrations of MMS were used. Furthermore, in a study carried out by Kammann et al. (2000), leukocytes extracted from carp (*Cyprius carpio*) were exposed *in vitro* to organic sediment extracts from the North Sea. AraC was used to increase the sensitivity of the comet assay, however, there was no increase in the level of DNA damage in samples exposed to one of the extracts with AraC. This was explained to be due to the contaminants in this particular extract inducing short-patch and other DNA lesions which are not influenced by the action of AraC. In contrast, sediment extracts with AraC from other sites induced significant enhancement of the levels of DNA damage measured with the comet assay. This was suggested to be due to significantly higher amounts of long-patch or other AraC-sensitive DNA lesions. The authors concluded that the effect of AraC was dependent upon the composition of the genotoxic...
substances present in the sediment extracts. In the present studies, the exact type of DNA lesion was not determined, although the literature indicates that following exposure to alkylating agents and H2O2, alkyl groups and SSB and DSB are formed respectively.

The significant differences in DNA damage measured by tail length and tail moment between the controls incubated with and without AraC, in the H2O2 experiments indicate that there was an inherent level of DNA damage being induced by normal metabolic processes within the cells which was being efficiently repaired.

8.6 Conclusions

The results of the studies in this chapter compared the different levels of DNA damage induced by oxidative and alkylating agents measured with the comet assay. The data provided evidence to indicate that the haemocytes of *M. edulis* under *in vitro* conditions have DNA repair capacities, with the significant differences being observed between the samples exposed to the oxidising agent H2O2 with and without simultaneous incubation with the DNA polymerase inhibiting chemical AraC, although the mechanisms of the repair were not studied in detail.

As mentioned earlier, the repair of oxidative damage follows the base excision repair processes of which the comet assay is able to detect DNA strand breaks when the damaged bases are removed before ligation occurs. Also, misrepair of this process may result in strand breaks. In contrast, repair of alkyl groups by transferase proteins (induced by monofunctional alkylating agents) occur by an error-free mechanism since only the modified alkyl group is removed from the DNA and this type of repair is a direct reversal of the bonding of the alkyl group (Beranek, 1990; Friedberg, 1985). The repair of such lesions may not necessarily involve excision repair mechanisms. Therefore, the addition of AraC in such exposures to alkylating agents may not necessarily enhance the response measured with the comet assay. Furthermore, the lack of a dose-response relationship in
the samples exposed to MMS, suggest that *in vitro* the haemocytes had a low capacity to produce alkylated products.

Although it has been shown here that DNA repair processes occur in the haemocytes of *M. edulis*, it is necessary to carry out further studies to fully understand the mechanisms of repair in *M. edulis* both *in vivo* as well as *in vitro*. Also, the kinetics of the repair processes need to be studied to identify the time taken to repair the induced damage and whether there are critical periods of exposure within the cell cycle which may have a greater impact on the cell’s integrity. In addition, it would be useful to investigate whether different doses of DNA damaging agents or repeated exposures of cells to these contaminants might result in an enhancement or decrease in sensitivity to DNA damaging agents as a result of adaptive responses.
Chapter 9

General Discussion
9.1 Genetic toxicology

The objective of the genetic toxicologist is to attempt to detect at sublethal concentrations, the potential for agents to interact with and alter genetic material. (Shugart, 1995). In mammalian systems, genotoxic agents that cause DNA damage and initiate neoplastic transformations (carcinogens) are of the greatest relevance and interest; in the field of eco-genotoxicology, the objective is to evaluate the direct and indirect impact of contaminants on the genetic material of natural biota (Dixon et al., 2002). In all biological systems, should the cellular responses to DNA damage involve the misrepair of lesions or if DNA repair fails to occur and the predominant response is an error-prone mechanism, mutations may arise in the descendants of the affected cells (Friedberg, 1985).

All organisms and genes have an inherent background rate of mutations. Somatic mutations may accumulate during the lifetime of an organism, therefore an older individual may be more vulnerable to disease due to a greater body burden of background and induced mutations, than a comparatively younger individual (UNEP, 1992). In addition nutritional content may have an influence on the levels of damage induced. Deficiencies in the levels of some vitamins, which act as antioxidating agents, may increase an organism's susceptibility to chromosomal mutations. As well as the concentration, the duration of exposure to a mutagenic substance may affect the resulting genetic risk, depending on the form of the dose-response curve and the specificity of the agent for particular stages of germ-cell development. Acute exposure to chemicals that induce mutation in late stages of germ-cell growth will result in a transitory genetic risk, confined to conceptions resulting from the gametes exposed during the sensitive stage. Acute exposure to chemicals that induce mutation in the early stages of germ-cell growth will result in a permanent genetic risk. (UNEP, 1992).

The effects of genotoxicity on the fate of natural populations are of particular concern since the alterations to the structure and function of DNA could potentially result in changes in genetic variability or alterations to the distribution of allele frequencies.
These alterations could potentially lead to population instability and may increase the risk of population disappearance in fluctuating environments (Nadig et al., 1998; Ross et al., 2002). The theory that genetic diversity can be altered by environmental contaminants has been proven in populations of gastropod (*Littorina brevicula*) in areas where heavy metal pollution were found to be high, and consequently genetic diversity was shown to be significantly reduced when measured with mtDNA markers (Kim et al., 2003).

Detrimental effects may include increases in mortality, impairment of reproductive productivity and potentially the introduction of novel selective pressure in a heterogeneous population (Nevo et al., 1986; Hose & Guillette, 1995). In extreme cases, in particular where there might be small populations, there have been suggestions that genetic deterioration could potentially result in extinctions (Lynch et al., 1995). However somatic changes, should they lead to a loss of individuals, might not be so critical in populations with a large reproductive surplus (Würgler & Kramers, 1992). In the present work, investigations have focused upon marine invertebrates. It has been shown by previous workers that many benthic invertebrate species produce a large reproductive surplus and that high juvenile mortality rates occur (Gosselin & Qian, 1997). It might therefore be assumed that low levels genetic mutations induced by genotoxic agents would have relatively insignificant implications on the population or community levels of biological organisation.

9.2 Toxic effects of ultraviolet radiation

The detrimental effect of ultraviolet radiation (UVR) on DNA has been well documented and this agent has been found to induce the formation of pyrimidine dimers and 6-4 photoproducts. The hypothesis put forward in chapter 4 was that the DNA damage induced by UVR in the early life stages of marine invertebrates could be detected using cytogenetic methods. The data collated in these studies indicated that the physical agent
ultraviolet radiation (UVR) is indeed able to induce sublethal perturbations in the genetic material of marine invertebrates. These lesions were detected by measuring the induction of chromosomal aberrations (CAbs) and sister chromatid exchanges (SCEs). Of particular interest were the observed differences in sensitivity between different life stages. The effects of UVR are more environmentally relevant in the early life stages of the two species studied, *M. edulis* and *P. dumerilii* since they are pelagic during this part of the life cycle, whereas the adult life stages are benthic. In addition, *M. edulis* is a sub-tidal species and even when exposed at low tide, they have a thick, dark blue coloured shell which protects the soft tissues through which negligible amounts of biologically damaging UV-B wavelengths are able to penetrate. In contrast, the adult polychaete worms are sub-littoral and reside within mucous tubes secreted by the animals.

From other authors' work and the studies described in chapters 4 and 5, the early life stages appear to be more sensitive to UVR and PAHs than adult life stages. Morphological deformities and genetic lesions were readily induced following exposure at significantly lower concentrations in early life stages than in adults. This agrees with the observations made by Bingham & Reyns (1999) who noted that the larval stages of the solitary ascidian *Corella inflate* were more sensitive than the juvenile and adults to UVR exposure. Furthermore, the rate of development appeared to be suppressed by exposure to these genotoxic agents, which would prolong the time spent in the early larval stages and render the individuals more susceptible to predators in the natural environment.

The early life stages are particularly susceptible to physical or chemical agents since their cell cycle during the cleavage period is short and often lacks mitotic checkpoints to ensure accurate transmission of DNA to the daughter cells (Epel et al., 1999). Such unrepaired DNA in cell division could impact embryogenesis and the transmission of the genome through the germ line. However, the levels of UVR which actually induced significant genotoxic and developmental effects on the embryo-larval stages of *M. edulis* and *P. dumerilii* were not within the realms of environmentally realistic
conditions, typically reductions of ozone have been approximately 12% (WMO, 1995). This suggests that these organisms have strategies for limiting the damage induced by UVR at environmentally realistic doses. A number of species have been found to produce sunscreens such as mycosporines (tunicates) and some species have pigments to aid absorption of damaging UVR (daphnia). The presence of such protective mechanisms in the species studied here need to identified. Furthermore, the nature of the UV damage induced in the embryos was not pin-pointed. Damage to protein in addition to DNA itself may have been the target for UVR, furthermore the capacity for UV-induced DNA repair was not assessed in these studies. Previous authors have postulated that a variety of mechanisms are employed by some aquatic organisms to limit oxidative damage (Epel et al., 1999). The risk that genotoxins pose on individuals and potentially populations, need to take into account a number of factors which may affect the level of the risk. These include the ability and mechanisms that an organism has for repairing damaged DNA. Photoreactivating enzymes (PRE) involved in UVR-induced DNA damage has been documented in a number of aquatic species (Blaustein et al., 1994; Kim et al., 1996), however, this has not yet been identified or quantified in *M. edulis* or *P. dumerilii*.

There was a difference in the level of sensitivity between the two species studied with *M. edulis* appearing to be more sensitive than *P. dumerilii* for genotoxic endpoints. When the induction of CAbs was considered, UVR levels equivalent to 18% and 32% ozone depletion respectively induced significant increases of CAbs in each of the species respectively. When the induction of SCEs was investigated, the levels of UVR which induced significantly increased frequencies were 10% and 18% ozone depletion for *M. edulis* and *P. dumerilii* respectively. In contrast to the genotoxic endpoints measured, when the phenotypic effects of UVR exposure were evaluated, it was observed that *P. dumerilii* was more sensitive to UVR than *M. edulis*. There were significant increases in the proportion of embryo-larvae which showed abnormal morphological development for *P. dumerilii* exposed to UVR equivalent to 10% ozone depletion, whereas for *M. edulis*
doses of UVR equivalent to 18% ozone depletion were required to induce significant increases in abnormal larvae. Since the survival of embryo-larvae was not quantified due to the high levels of reproductive surplus and naturally high death rates among early life stages of invertebrate species, it is possible that in *M. edulis*, larvae suffering from particular abnormalities may have died. Furthermore, at a similar age the biological complexity of the two species is significantly different. *P. dumerilii* has a more complex morphology (development of eyes and parapodia) and a greater differentiation of cells for different body parts than *M. edulis*. Therefore, other effects such as cell death or cellular toxicity may have a greater impact on the whole organism than normal development of *P. dumerilii* than *M. edulis*. It would be useful to identify the rates of cell death, for example, by measurement of apoptosis of the cells to identify any relationship between cell death and abnormal development.

As mentioned earlier, the levels of ozone depletion, although having been as much as 50% with extreme meteorological conditions, are normally around 12% (WMO, 1995). The Montreal Protocol, which limits the emission of ozone-depleting chemicals was finalised and approved in 1987. It was then enforced in January 1989 and has since significantly reduced the rate of ozone depletion. Although the hole in the ozone layer over Antarctica is predicted to heal over the next 50 years or so (Henderson, 2000), it is thought that there may be other as-yet unidentified ozone-destroying chemicals in widespread use (Pearce, 2001). Indications have been that only a relatively small proportion (of the total populations) of the early life stages of marine organisms are present in the first meter of coastal water columns. It is these individuals that are likely to be most susceptible to UV-B radiation (Browman, 2003). Since the species of interest reside in the coastal areas, where generally the amount of dissolved organic matter is higher and therefore the attenuation of UVR is greater, compared with open ocean environments where the water may be more transparent, the direct effects of UVR are likely to be minimal in such coastal zones (Bornman & van der Leun, 1998).
9.3 Toxic effects of PAH exposure

As a result of the reduction of discharges of raw sewage into watercourses, there have undoubtedly been major improvements in the quality of water in estuarine and river courses over the past century (Matthiessen & Law, 2002). Regardless, contaminants still continue to enter the aquatic environment and cause pollution effects in biological material. Chemical contamination in the environment can cause population alteration by the effects of somatic and heritable mutations, as well as non-genetic modes of toxicity such as alterations to immune systems (Bickam et al., 2000). Many chemical agents are not directly mutagenic themselves, but require conversion to a chemical form that can react with DNA and cause mutation. This is metabolic activation and is accomplished by enzymes which vary in type and amount among species and different tissues in an organism (UNEP, 1992). Many PAHs fall into this category and require metabolic activation before they assume genotoxic potential (Neff, 1979).

The studies which were conducted in chapters 5 and 6 investigated the effects of single or mixtures of PAHs on the early and adult life stages of marine organisms. The hypothesis that benzo(a)pyrene (B[a]P), a representative of the PAH group, or a mixture of PAHs could induce genotoxic responses was evaluated. Cytogenetic (CAbs and SCEs) and cytotoxic (proliferation rate index) endpoints were measured in the early life stages of *Mytilus edulis* and *Platynereis dumerilii*. Each of these biological responses indicated that a mixture of PAHs induced genotoxic and cytotoxic effects. A significant increase in the induction of SCEs was observed at a lower concentration of PAH than that for CAbs indicating that the induction of SCEs is a more sensitive endpoint. When comparing the two species, indications from the cytogenetic endpoints were that *M. edulis* was more sensitive to PAH exposure than *P. dumerilii*, however, from the observations made from the normal development of the embryo-larval stages, the reverse of this was discovered.

Molecular and cellular endpoints (comet assay and micronucleus induction) were measured in the adult stages of the mussel *Mytilus edulis* and the benthic fish *Platichthys*
For each of these species, significant increases in genetic damage measured with the comet assay were observed in samples exposed to the PAH treatments. The comet assay was shown to be a sensitive and useful endpoint for measuring DNA damage in the haemocytes of the target organisms. This was observed both in the acute exposures administered directly via the exposure water in the studies investigating the responses in mussels, and in chronic exposures undertaken via the food for the experiments evaluating the responses in fish. Unfortunately, different researchers use a variety of different parameters for measuring DNA damage with the comet assay therefore making it difficult to compare results between different studies. It has been suggested that the simplest method for measuring the level of DNA damage would be to calculate the percentage of damaged cells (otherwise known as the percentage of comets) (Cotelle & Férand, 1999). However, to be able to determine this parameter, different categories of damaged cells would need to be arbitrarily defined which would be a disadvantage (Tice, 1995). In spite of the lack of standardisation between different authors’ methods of presenting their results, the comet assay is able to offer many advantages. From the present studies and previous authors’ work, various reports have demonstrated its sensitivity for detecting DNA damage and it is a rapid method. Since individual cells are used, the quantification of different responses from single-strand breaks to apoptosis can be made. Also, there is no need to use cells which are mitotically dividing (unlike the micronucleus assay) (Cotelle & Férand, 1999).

When genotoxicity was measured with the micronucleus (MN) assay, acute exposures of mussels (3 days) showed significant increases in the frequencies of MN, however, longer exposures indicated a lesser increase in frequency. Similarly the blood samples collected from the long-term exposures of flounder (P. flesus) did not reflect the expected increase in MN frequencies. The insensitive response of the MN assay has been discussed earlier in fish species and it is thought that the low mitotic index found in fish erythrocytes has a significant impact on the frequencies of MN observed in the cells. In
the laboratory exposures, the MN assay appeared to be useful for detecting genotoxicity when the acute (up to 3 days) exposure scenarios were employed. However, in longer-term exposures, this assay shows that it is not a sensitive method and requires further investigations since comparatively low incidences of MN occur in both bivalve and fish species when exposed to longer periods of time.

The induction of DNA adducts in *P.flesus* was found to be related to the dose of PAH in the food administered to the fish in the laboratory exposures as supported by previous authors (Venier & Canova, 1996; Canova *et al.*, 1998; Lyons *et al.*, 1999; Ching *et al.*, 2001). However, in some field studies, measurements of DNA adducts have not necessarily correlated with PAH exposure (Akcha *et al.*, 2003) therefore this suggests that the assay has a limited applicability in environmental monitoring.

Although there is much concern over the detrimental effects of contaminants being introduced into the aquatic systems, there are indications that the major human impact on marine ecosystems in areas such as the North Sea is caused by the effects of fishing activities. In relation to this, it has been argued that the impacts caused by the exposure to contaminants are now relatively small in comparison (Matthiessen & Law, 2002).

### 9.4 Interactions between UVR and PAHs

The results of the studies involving exposure of the target organisms to UVR on its own, suggest that in environmentally realistic situations, only extreme levels of ozone depletion would result in significant detrimental effect. However, indications from the present and previous studies are that simultaneous UVR and chemical contaminant exposure in the natural environment is of far greater relevance. The research which has been documented recently has highlighted the potential environmental impacts of photo-induced PAH toxicity, with evidence for increases in toxicity being demonstrated in invertebrate and vertebrate model organisms from both marine and freshwater ecosystems (Afsten *et al.*, 1996; Pelletier *et al.*, 1997; Swartz *et al.*, 1997; Hatch & Burton, 1999).
The studies described in chapter 5 involved measurements of the induction of chromosomal aberrations (CAbs) in the early life stages of *M. edulis* and *P. dumerilii* under laboratory conditions. Both of these species indicated that photoactivation of PAHs had occurred and an increase in the genotoxic potential of these chemicals resulted, measured by an increase in the induction of CAbs in the metaphase chromosomes compared with samples exposed to PAH which had not been photoactivated before exposure to the test organisms. The induction of SCEs on the other hand was not affected by photoactivation of PAHs suggesting that different mechanisms occur between CAb and SCE formation. In addition, proliferation rate index (PRI) was significantly reduced in the samples exposed to photoactivated PAHs compared with the non-photoactivated, further supporting the observations of previous work where toxicity of such contaminants was increased.

The hypothesis that interactions between PAH contaminants and UVR caused changes in toxicity in the adult stages of *M. edulis* was tested in chapter 6. Both the micronucleus and comet assays indicated that was an increase in genotoxicity caused by UVR interaction with B(a)P, however, cytotoxicity measured with the neutral red retention assay was not affected. Fluorescent spectrophotometric analyses provided evidence that the chemical structure of B(a)P was altered by UVR interaction, however, the persistence of the resulting daughter compounds needs to be investigated.

9.5 Application of biomarkers in field studies

The ecotoxicological evaluation of mutagenic risks has a somewhat different emphasis to human risk assessment since the concern for ecosystem and ecological compartments may be directed toward the maintenance of the integrity of populations, whereas in humans it would rather be directed towards that of individuals (Mohn *et al.*, 1993). Many laboratory-based toxicity bioassays have been used to test the toxic potential of various contaminants, however these sorts of tests do not provide an adequate basis for
extrapolating to effects on populations, communities, or ecosystems, or for predicting transformation processes of xenobiotic chemicals in the environment (Levin et al., 1989).

Studies of bioaccumulation of contaminants in organisms have provided useful and pertinent information regarding the biological availability of various contaminants to animals in situ. However, bioaccumulation measures a phenomenon not an effect. The use of various biomarkers for the detection of pollution effects in environmental conditions have been employed in numerous studies in addition to bioaccumulation testing to provide information upon predictive and worst-case conditions (Chapman, 1993).

There are limitations to the use of methods such as the measurement of the induction of CAbs or SCEs in organisms exposed to mutagens in the marine environment. For instance, such studies have been restricted primarily to sedentary species since mobile organisms may have suffered genetic lesions following exposure from locations other than where they had been sampled, therefore providing little relevant information from the position of collection (Dixon, 1983). Also, the preparation of metaphase chromosomes requires the harvesting of mitotically dividing cells, the cell turnover of which is often slow in field samples (Dixon et al., 2002). Furthermore, the use of cells from early larval stages is restricted since simultaneous exposed to BrdU is necessary for the differential staining of sister chromatids.

In chapter 7, a field study was undertaken to evaluate the potential for applying the comet assay, the micronucleus assay and the neutral red retention (NRR) assay to samples exposed in situ from environmental contaminants. The levels of variability in the effects observed for a number of the endpoints emphasises the need for multi-assay approaches for toxicity studies for monitoring pollution effects. This is supported by high levels of variability in studies carried out by other authors such as Coles et al. (1995). Nevertheless, a significant relationship was observed between the levels of DNA damage measured with the comet assay and levels of cytotoxicity measured with the NRR assay, and chemical analyses of the heavy metals found in the sediments and soft tissues of the organisms. As
observed in the present study, the comet assay showed itself to be a useful and sensitive biomarker in field investigations carried out by Akcha et al. (2003) where the comet assay was able to distinguish samples of dab (*Limanda limanda*) collected from environmental sites with high contaminant levels from those collected from sites with lower pollution levels. A number of other reports have also indicated the assay to be applicable to field studies (Nacci & Jackim, 1989; Everaats, 1995; Steinert et al., 1997, 1998; Frenzilli et al., 1999, 2001). The results of the MN assay suggested that this is not a particularly sensitive method of detecting genotoxicity in samples exposed in the field. A similar observation was made by Burgeot et al. (1996) and Brunetti et al. (1988).

It would be necessary to identify other potential chemical and/or physical contaminants to further evaluate the sensitivity of the endpoints measured. Differences in the levels of DNA damage measured in *Mytilus edulis* and *Cerastoderma edule* highlight the differences in potential bioavailability of contaminants to target organisms as recognised by other workers (Kaag et al., 1998). The feeding habits and morphology of *M. edulis* requires that it resides in positions where hard strata is present to allow it to attach with the byssus threads and filter feed from the surrounding water. In contrast, *C. edule* resides in fine- to medium-grained sediments into which it is able to burrow, deposit feeding as well as filter feeding. The latter species is therefore in direct contact with the sediments and any contaminants which may be present within it, whereas *M. edulis* is exposed to contaminants that may be adsorbed onto the surface of suspended organic matter which it then filters from the water. This also emphasises the need to employ a multi-species approach when using test organisms to monitor environmental quality using biomarker techniques.

9.6 DNA repair mechanisms in *M. edulis* haemocytes

The kinetics of the formation and removal (repair) of DNA damage in addition to the probability that any persisting damage may result in mutations need to be established
before conclusions can be made about their significance in terms of mutagenesis (Mohn & De Raat, 1993). Many demonstrations of the mechanisms of DNA repair have involved the use of yeast mutants, cultured cells and bacterial systems (Griffiths & Carr, 1998; Siede, 1998). Relatively few studies have been conducted investigating the repair capabilities of aquatic organisms, though some studies have considered the repair activities in frogs (Carroll, 1998) and nematodes (Hartman & Nelson, 1998).

In the studies undertaken in chapter 8, the hypothesis that the haemocytes of *M. edulis* have DNA repair capabilities was evaluated. As mentioned previously, the spectrum of damage that cellular DNA can suffer is extensive and in addition to DNA damage caused by environmental factors, a significant level of DNA damage arises from the activities of reactants generated naturally within cells themselves (Burdon, 1999). The *in vitro* effects of exposure of the haemocytes from *M. edulis* to an oxidising agent (H$_2$O$_2$) and an alkylating agent, methyl methane sulfonate (MMS) was therefore evaluated. By incubating the cells simultaneously with cytosine β-D-arabinofuranoside (AraC) in conjunction with the agents, the repair of DNA involving DNA synthesis was inhibited.

It was evident from this study that when the haemocytes from *M. edulis* were treated with H$_2$O$_2$ there was a detrimental effect to the DNA caused by oxidative damage and repair mechanisms involving long-patch repair following nucleotide excision were active. This was demonstrated by the increased levels of DNA damage measured in the samples exposed with simultaneous AraC incubation, compared with the samples exposed to H$_2$O$_2$ in the absence of AraC. However, the mechanisms of damage induced by the alkylating agent MMS were not made clear by these studies although these appeared to be quite different from that of oxidative agents. There was no significant increase in the level of DNA damage measured in the samples exposed to the concentrations of MMS in these studies indicating that these in these conditions the cells were not able to induce the formation of alkylating products as would be expected. It would be necessary to further investigate the effects of MMS *in vivo* to the haemocytes of *M. edulis*, and to repeat the
study *in vitro* at greater concentrations. Furthermore, the affect of a chemical which inhibits the reversal of alkylating damage should be investigated in the place of AraC which only inhibits DNA repair mechanisms which involve the synthesis of DNA.

9.7 **Implications of genotoxic effects**

With the advance of techniques for the detection of genotoxicity in the marine environment, there have been increasing numbers of studies which measure the impact in large-scale field assessments (Hose, 1994). The application of genotoxicological biomarkers to indigenous organisms exposed to contaminants in the environment have advantages over *in vitro* assays in that they provide the most realistic information on levels of genetic damage induced by genotoxic agents in the field. However, there may be limitations in the amount of information which may be deduced from the biomarkers being measured. Many of the endpoints measuring genotoxicity give an indication of the level of genetic damage endured by the organisms of interest, but may not necessarily provide information regarding the specific identification of the DNA-damaging agent. An exception to this includes the measurements of DNA adducts, the identification of which can be used to determine the exposure of cells to specific PAHs. Nonetheless, non-specific genotoxicological assays do provide a method for the screening of environmentally exposed organisms for DNA-damaging agents and can present useful information regarding sites of high impact, for which further investigation can then be focused. The initial identification of sites of high contaminant impact by biomarkers can assist for prioritising sampling sites and act as a precursor to subsequent chemical analyses methods.

It is pertinent to consider the impacts upon the ecosystem structure that alterations in species and genetic variability due to adaptation to environmental or contaminant conditions, may have. The inheritance of contaminant tolerance in aquatic organisms has been suggested to be through single major genes (Barata *et al.*, 1998), it would therefore be relevant to identify such genes.
9.8 Future directions

The implications of the work included in this thesis and that of other researchers indicates that biomarkers are a useful tool for monitoring the relative health of indigenous populations of invertebrates exposed to environmental contaminants in the field. In addition to the research that remains to be undertaken, already discussed, there is also a need to pinpoint cause-effect relationships between specific genotoxic or toxic agents and consequent biological effects. For the detection of genotoxin-specific products, it would be relevant and necessary to produce and characterise polyclonal and monoclonal antibodies specific to particular genotoxic agents of interest.

Organisms chronically exposed in environmental conditions may have adapted to the background levels of contamination and therefore be more resistant to elevated concentrations of specific classes of contaminants. However, such genetic adaptations could render the individuals more susceptible, or alternatively, more resistant to other classes of contaminants which may sporadically occur. It would be therefore be of great importance to identify any changes in sensitivity of genetically resistant populations to alternative agents.

The use of the comet assay to determine the DNA repair capabilities in the target organism *M. edulis* described in chapter 8 was an indirect method of measurement. There is no doubt that the mechanisms of DNA repair in these organisms need to be elucidated and direct methods of measurement are required. It would be possible to develop antibodies to qualify and quantify enzymes involved in the DNA repair processes and therefore assist in the understanding of the different mechanisms involved in invertebrate species.

9.9 Conclusions

In conclusion, the methods employed in this thesis have proven to be valuable biomarkers of genotoxin exposure in laboratory conditions and some of the methods have
subsequently been found to have useful applications in field samples. The genotoxic agents selected, UVR and PAHs, were able to induce genotoxic and cytotoxic responses in the target organisms in both the early and adult life stages. Of the various biomarkers employed, the comet assay indicated broad applicability and potential utility in laboratory as well as field studies investigating environmental genotoxic exposures and effects. Furthermore, this assay has shown to be applicable to a number of different species as well as providing useful information regarding DNA repair capabilities of various cell types and it has also been widely used in a number of other ecotoxicological studies (Mitchelmore & Chipman, 1998a, 1998b; Steinert et al., 1998a, 1998b).

The cytogenetic assays which were employed were shown to be useful methods of identifying genotoxic effects caused by ultraviolet radiation and chemical agents in the early life stages of marine invertebrates, in laboratory conditions. The use of early life stages are appropriate since they are often the most sensitive life stage and it is during this period that they are most susceptible to ultraviolet radiation in the upper waters. Furthermore, their small size allows experiments to be conducted where space is limited, making considerable savings on reagents, space and apparatus (Dixon & Pascoe, 1994). In the present studies, links between the toxic effects observed at the early life stages and the survival and reproductive capacity of juvenile and adult life stages was not established. Such links between long-term exposures and potential effects at higher levels of biological organisation remain difficult to quantify due to the restrictions imposed by such long-term studies. There is certainly a need to correlate species-population studies with sub-lethal effects identified with biomarker responses.

There are still gaps in the information where studies need to be designed to investigate relationships between DNA damage and recovery, and between genotoxic effects and biological and ecological effects at the cellular, individual and higher levels of biological organisation. It has been a goal of many ecotoxicologists to accurately predict the consequences of exposure of organisms to contaminants in the environment and this
has often been based upon information gathered from laboratory-based studies (Monk, 1983; Evenden & Depledge, 1997). The results presented in this research project further emphasise the need for multi-assay and multi-species approaches in toxicity studies for monitoring pollution effects.
Appendix I

Formulations of reagents & solutions
Preparation of Marine Bivalve Physiological Saline

Physiological saline was prepared for the extraction of haemolymph from *Mytilus edulis* and *Cerastoderma edule*. It was used in all assays where haemolymph was extracted including the neutral red retention assay, the micronucleus assay, and the comet assay. The reagents included in the physiological saline are based upon the recipe of Peek & Gabbot (1989).

**Ingredients per 1000ml**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final Conc.</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepes</td>
<td>20 mM</td>
<td>4.77 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>436 mM</td>
<td>25.48 g</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>53 mM</td>
<td>13.06 g</td>
</tr>
<tr>
<td>KCl</td>
<td>10 mM</td>
<td>0.75 g</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10 mM</td>
<td>1.47 g</td>
</tr>
</tbody>
</table>

q.s. to 1000 ml distilled water

The pH was adjusted to 7.36, and stored in the refrigerator at 4°C in the dark. The saline was used at room temperature to prevent temperature shock to the cells.

Preparation of Buffered Salt Solution

Buffered salt solution was prepared for the dilution of haemocytes from *Pleuronectes flesus*. It was used to dilute cells in preparation for the comet assay, and for the cell viability test, Trypan Blue. The reagents included in this salt solution are based upon the recipe of Nacci *et al.* (1996).

**Ingredients per 1000ml**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>23.5 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.7 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Na₂HCO₃</td>
<td>0.2 g</td>
</tr>
<tr>
<td>CaCl₂₂H₂O</td>
<td>1.1 g</td>
</tr>
</tbody>
</table>

The pH was adjusted to 7.5, stored in the refrigerator at 4°C in the dark, but used at room temperature.
Reagents prepared for the 'Comet' Assay

Low melting point agarose and normal melting point agarose were made up in Kenny’s salt solution and Tris-Acetate-EDTA (TAE) solution respectively. Following mixing and melting in the microwave, 2.0 ml aliquots of each agarose solution were measured into 2.0 ml microcentrifuge tubes, allowed to cool and stored in air-tight containers in the refrigerator at 4°C in the dark, for up to 3 months. Individual aliquots of agarose were melted in water baths as they were required.

Preparation of Kenny’s salt solution for dissolving the Low Melting Point Agarose:
Ingredients per 1000ml

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final Conc.</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.4 M</td>
<td>23.37 g</td>
</tr>
<tr>
<td>KCl</td>
<td>9 mM</td>
<td>0.6709 g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.7 mM</td>
<td>0.12194 g</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>2 mM</td>
<td>0.16802 g</td>
</tr>
</tbody>
</table>

q.s. to 1000ml distilled water.

Preparation of Tris-Acetate-EDTA (TAE) solution for dissolving the Normal Melting Point (NMP) Agarose:
Ingredients per 1000ml

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final Conc.</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Acetate</td>
<td>40 mM</td>
<td>7.248 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>2 ml 0.5M solution</td>
</tr>
</tbody>
</table>

q.s. to 1000 ml distilled water.

Low Melting Point Agarose:
0.5% (5 mg/ml Kenny’s Salt Solution).

Normal Melting Point Agarose:
1.5% (15 mg/ml TAE Solution).

Preparation of Lysing Solution:
Ingredients per 1000ml

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final Conc.</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>2.5M</td>
<td>146.4g</td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
<td>100mM</td>
<td>37.2g</td>
</tr>
<tr>
<td>Tris Base</td>
<td>10mM</td>
<td>1.2g</td>
</tr>
<tr>
<td>NaOH</td>
<td></td>
<td>8.0g</td>
</tr>
</tbody>
</table>

Pure water added to give final volume of 890ml

This stock lysing solution was filter sterilised through a 0.45 µm filter, and stored at room temperature in the dark, for up to 1 week. Immediately prior to use, the following ingredients were added:
Triton X-100 1% (e.g. 0.5ml per 50ml)
DMSO 10% (e.g. 5ml per 45ml)
Preparation of Stock Electrophoresis buffer Solution:

**Preparation of 1N NaOH**

40g NaOH / 1000ml distilled water.
This was filter sterilised through a 0.45 μm filter, and stored at room temperature in the dark for up to one week.

**Preparation of 200mM EDTA** - Review date: 3 months

0.5M Na₂EDTA solution was diluted with distilled water 2.5 times (e.g. 1 ml EDTA plus 1.5 ml distilled water).
This was stored at room temperature in the dark for up to 3 months.

Immediately before use, electrophoresis buffer was made up by:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final Conc.</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>300 mM</td>
<td>300 ml</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM EDTA</td>
<td>5 ml</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td></td>
<td>695 ml</td>
</tr>
</tbody>
</table>

**Preparation of Neutralisation Buffer:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final Conc.</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>0.4 M</td>
<td>9.7 g</td>
</tr>
</tbody>
</table>

The pH was then adjusted to 7.5 using concentrated HCl acid. This was stored at room temperature in the dark for up to 3 months.

**Preparation of Stock Ethidium Bromide Solution:**

A stock solution of 2.0mg/ml ethidium bromide in distilled water (a 5 fold dilution of the original 10 mg/ml solution from Sigma), was made up. This was then diluted a further 100 times to produce the working solution of 0.02 mg/ml ethidium bromide in distilled water.
Preparation of the Stock Neutral Red Solution:
A stock solution of 20 mg/ml of neutral red (Sigma, Dorset, UK) in dimethyl sulfoxide (DMSO) was prepared in a smoked amber vial. This was filtered through a 0.5 μm Millipore filter which was attached to a hypodermic syringe, and stored in a smoked amber vial in the refrigerator (4°C) within a light-proof container (as neutral red is photosensitive).

Preparation of the Working Neutral Red Solution:
Prior to the preparation of the working solution during execution of the assay, the stock solution would be removed from the refrigerator and allowed to reach melting point at room temperature. To prepare the working solution, 5 μl of the stock solution would be added and mixed with 995 μl of physiological saline.
Appendix II

Extra Data
Table II.4.1a
Details of the spawning and fertilisation of *Mytilus edulis* gametes, in experiments carried out for Chapter 4.

<table>
<thead>
<tr>
<th>Endpoints measured</th>
<th>Number of Females used</th>
<th>Number of Males used</th>
<th>Total number of embryos produced</th>
<th>Fertilisation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAbs, development, growth</td>
<td>5</td>
<td>2</td>
<td>712000</td>
<td>93.2%</td>
</tr>
<tr>
<td>CAbs, development, growth</td>
<td>4</td>
<td>3</td>
<td>358000</td>
<td>96.0%</td>
</tr>
<tr>
<td>PRI, SCE</td>
<td>2</td>
<td>2</td>
<td>160000</td>
<td>99.45%</td>
</tr>
</tbody>
</table>

Table II.4.1b
Details of the spawning and fertilisation of *Platynereis dumerilii* gametes, in experiments carried out for Chapter 4.

<table>
<thead>
<tr>
<th>Endpoints measured</th>
<th>Number of Females used</th>
<th>Number of Males used</th>
<th>Total number of embryos produced</th>
<th>Fertilisation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAbs, development, growth</td>
<td>2</td>
<td>1</td>
<td>48000</td>
<td>91.95%</td>
</tr>
<tr>
<td>CAbs, development, growth</td>
<td>3</td>
<td>3</td>
<td>14000</td>
<td>98.6%</td>
</tr>
<tr>
<td>PRI, SCE</td>
<td>4</td>
<td>4</td>
<td>18000</td>
<td>96.1%</td>
</tr>
</tbody>
</table>

Table II.5.1a
Details of the spawning and fertilisation of *Mytilus edulis* gametes, in experiments carried out for Chapter 5.

<table>
<thead>
<tr>
<th>Endpoints measured</th>
<th>Number of Females used</th>
<th>Number of Males used</th>
<th>Total number of embryos produced</th>
<th>Fertilisation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAbs, development, growth</td>
<td>4</td>
<td>3</td>
<td>285000</td>
<td>89.5%</td>
</tr>
<tr>
<td>CAbs, development, growth</td>
<td>2</td>
<td>1</td>
<td>520000</td>
<td>88.9%</td>
</tr>
<tr>
<td>PRI, SCE</td>
<td>4</td>
<td>2</td>
<td>500000</td>
<td>92.5%</td>
</tr>
</tbody>
</table>
Table II.5.1b
Details of the spawning and fertilisation of *Platynereis dumerilii* gametes, in experiments carried out for Chapter 5.

<table>
<thead>
<tr>
<th>Endpoints measured</th>
<th>Number of Females used</th>
<th>Number of Males used</th>
<th>Total number of embryos produced</th>
<th>Fertilisation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAbs, development, growth</td>
<td>4</td>
<td>2</td>
<td>500000</td>
<td>92.5%</td>
</tr>
<tr>
<td>PRI, SCE</td>
<td>4</td>
<td>2</td>
<td>50000</td>
<td>92.5%</td>
</tr>
</tbody>
</table>
Appendix III

Scientific contribution
ABSTRACTS FROM CONFERENCES

- April 1997: 9th International symposium on Pollutant Responses in Marine Organisms (PRIMO 9), Bergen, Norway. Poster presentation entitled 'Molluscan lysosomal responses as a diagnostic tool for the detection of a pollution gradient in Tolo Harbour, Hong Kong.'


- April 1998: 8th Annual meeting of the Society of Environmental Toxicology and Chemistry (SETAC-Europe), Bordeaux, France. Poster presentation entitled 'Genotoxic and developmental effects of arsenic-containing samples on marine invertebrates.'

- September 1998: 28th European Environmental Mutagen Society (EEMS), Salzburg, Austria. Poster presentation entitled 'Developmental and genotoxic impacts of ultraviolet-B radiation on the embryo-larval stages of the marine polychaete, Platynereis dumerili.'


- July 2000: The United Kingdom Environmental Mutagen Society (UKEMS), Swansea, England. Poster presentation entitled 'Potentiating effects of UV radiation on PAH-induced genotoxicity in Mytilus edulis.'

- July 2001: 11th International symposium on Pollutant Responses in Marine Organisms (PRIMO 11), Plymouth, England. Oral presentation entitled 'Photo-enhanced toxicity of benzo(a)pyrene in the haemocytes of Mytilus edulis.'

- August 2001: Society of Environmental Toxicology and Chemistry (SETAC-UK), Norwich, England. Oral presentation entitled 'Combined toxic effects of benzo(a)pyrene and ultraviolet radiation in Mytilus edulis.'

This study examines the sub-cellular perturbations, in the lysosomal compartment of molluscan haemocytes. The integrity of lysosomes taken from *Perna viridis* was determined using the neutral red technique. This technique was used to identify a well defined contamination gradient between indigenous populations from five stations along Tolo Harbour, Hong Kong. Condition indices (shell length: dry tissue weight) and tissue metal concentrations were also measured in an attempt to identify a relationship between contamination level and adverse physiological effects in the mussels.

Mussels collected from stations located on offshore islands had greater lysosomal stability and condition indices, than those collected from inner harbour sites. Mean values for both lysosomal retention time and condition indicated a pollution gradient along the harbour. However, there was little correlation between metal concentrations and metal concentrations and lysosomal retention time or condition indices. This suggests that the metals analysed were not present in concentrations detrimental to the mussels' physiology. Other contaminants such as organics may have been responsible for the mussels' varying condition.

It is concluded that the supravital dye, neutral red, is effective in identifying perturbations in the lysosomal compartment of molluscan haemocytes extracted from mussels *in situ*. The results of this study further validated the technique to be one that can be used as an early indicator of chemical, physical or biological stress in molluscs.
BIOMARKERS OF BIOCHEMICAL AND CELLULAR STRESS IN *Carcinus maenas*: AN IN SITU FIELD STUDY

J. Wedderburn, V. Cheung, S. Bamber, M. Bloxham, M.H. Depledge.
Plymouth Environmental Research Centre, University of Plymouth,
Plymouth, PL4 8AA, UK.

Biological pollution indicators, known as biomarkers, are useful diagnostic tools for identifying the presence of physical, chemical, and environmental stresses. Although various biomarker techniques have been developed in the laboratory their application in the field has been limited. The object of the study was to use a suite of techniques, metallothionein induction, lysosomal integrity, and osmoregulatory ability, to examine effects at biochemical, cellular and physiological levels. Five estuarine sites were studied, two pristine sites, two relatively contaminated urban sites, and one intermediate. The shore crab, *Carcinus maenas* was collected using baited nets and haemolymph samples taken for the lysosomal and osmoregulatory techniques. A further set of animals were sacrificed and tissue preserved for metallothionein analysis. Measurement of trace metal content of sediment samples was also undertaken at the sites. Lysosomal integrity showed significant differences between the reference sites and the urban sites (n=18, p=0.005 and p=0.0003). Changes in metallothionein concentrations and osmoregulation ability also occurred. The potential use of the biomarker approach is that it could rapidly provide suitable data for environmental impact assessment and is easily applicable in the field.
Chromosomal aberrations (CAbs) and sister chromatid exchanges (SCEs) were measured in the embryo-larvae of the marine bivalve, *Mytilus edulis* following exposure to two reference mutagens: A direct acting mutagen, methyl methane Sulfonate (MMS) at concentrations ranging between $1.0 \times 10^{-7}$ M and $1.0 \times 10^{-3}$ M; and a mutagen requiring metabolic activation, benzo(a)pyrene (B(a)P) at concentrations ranging between $1.0 \times 10^{-8}$ M and $1.0 \times 10^{-5}$ M. These endpoints were also measured in embryos exposed to the water-soluble fraction of an environmental sediment sample, known to contain elevated levels of arsenic and other associated metals. The concentrations of arsenic in the sediments ranged from 0.1 mg/l to 0.32 mg/l.

Following 12 hours of development in synthetic sea water, the embryo-larvae of *Mytilus edulis* were exposed simultaneously to 5-bromodeoxyuridine (BrdU) (to enable differential staining of the sister chromatids) and to each reference mutagen dissolved in sea water. For the analysis of CAbs and SCEs, embryos were exposed for 6 and 8 hours respectively, corresponding to periods of one and half and two cell cycles respectively. The samples were then fixed, and prepared for the staining of metaphase spreads.

In each of the exposure regimes, an inverse dose-relationship was observed in cytotoxic measurements as indicated by proliferation rate index. A dose-dependent increase for the induction of CAbs was observed as a measure of genotoxicity. Compared to the sea water controls in which only 3% were aberrant, 17% of cells exposed to $1.0 \times 10^{-3}$ M of MMS, 9% of cells exposed to $1.0 \times 10^{-5}$ M B(a)P, and 11% of cells exposed to the water-soluble fraction from sediments containing 0.32 mg/l were aberrant. There was also a significant increase in the induction of SCEs at these concentrations. SCEs ranged from 0 to 3 in cells from sea water controls; up to 31 SCEs per metaphase were observed in cells exposed to MMS, 18 in cells exposed to B(a)P and 11 in cells exposed to the sediment elutriates. The results of this study further validate the use of cytogenetic endpoints for the detection of genotoxins using the embryo-larval stages of marine mussels.
Detection of potential genotoxins for environmental monitoring and effluent discharge compliance traditionally rely upon analytical techniques. In order to evaluate the integral effects of the contaminants on the environment, recently, besides these chemo-physical analytical methods, the importance of biological methods is being recognised (e.g. direct toxicity test).

In the present study, we have evaluated the genotoxic potential of arsenic-containing marine sediment samples on the embryo-larvae of marine invertebrates (polychaete worm, *Platynereis dumerilii*, marine mussels, *Mytilus edulis*) and have compared the relative sensitivity of chemical, microbial and biological methods for the detection of arsenicals. Following the chemical characterisation of the sediment samples suspected to have high levels of arsenic by analytical techniques such as GCMS, ICP-AES and ICP-MS, it was revealed that the samples contained high levels of arsenic and associated metals, confirming that (geno)toxic agents in the environmental samples could in all probability occur as complex mixtures. Examination of the water soluble fraction indicated that low levels of arsenic and copper were leached from the sediments under laboratory conditions, which supported the view that presence of metals in sediment samples do not necessarily reflect their biological availability. Embryo-larvae of the test organisms exposed to this water soluble fraction (containing arsenic and copper) induced cytotoxic, genotoxic (i.e. SCEs and CAbs) and developmental effects in a dose-dependent manner. A parallel exposure series using sodium arsenite of concentrations within the range of arsenic levels present in the sediments also produced similar results, while the bacterial test, Microtox®, produced relatively non-toxic responses. Our studies indicate that low levels of arsenic are capable of inducing genetic damage in aquatic invertebrates, and properly validated biological methods are at least as sensitive as traditional chemical methods for identifying genotoxins from environmental samples.
GENOTOXIC AND DEVELOPMENTAL EFFECTS OF ARSENIC-CONTAINING SAMPLES ON MARINE INVERTEBRATES

V.V. Cheung, M. Foulkes, S.J. Hill, M.H. Depledge, A.N. Jha

Plymouth Environmental Research Centre, University of Plymouth, Plymouth, PL4 8AA, UK.

In recent years, the importance of biological methods, as well as chemical analyses, have been recognised in environmental monitoring and effluent discharge compliance. Anthropogenic activities result in the discharge of arsenicals (arsenic compounds), a known human carcinogen, into the aquatic environment, placing them on the priority list for PARCOM and NSCD purposes. There have however been few, if any, studies on the genotoxic effects of arsenicals on aquatic organisms, which may have implications for their short- and long-term survival.

In this study, we evaluated the genotoxic potential of arsenic-containing marine sediment samples on the embryo-larvae of marine invertebrates. The sensitivity of chemical, microbial and biological methods for the detection of arsenicals have been compared. Following the chemical characterisation of the sediment samples by analytical techniques such as GCMS, ICP-AES and ICP-MS, it was revealed that the samples contained high levels of arsenic and associated metals. Examination of the water soluble fraction indicated that low levels of arsenic and copper were leached from the sediments under laboratory conditions. Embryo-larvae of the test organism were exposed to this water-soluble fraction, with a positive genotoxic control (methylmethane Sulfonate). A parallel exposure series using NaAsO₂ with concentrations within the range of arsenic levels present in the sediments, was also carried out. Biological test indicated that these low levels of contaminants were able to induce cytotoxic, genotoxic (i.e. SCEs and CAbs) and developmental effects. Microtox® tests on the sediment leachates produced relatively non-toxic responses. The findings of this study indicate that low levels of arsenic are capable of inducing genetic damage in aquatic invertebrates, and biological methods are at least as sensitive as traditional chemical methods for identifying environmental genotoxins.
DEVELOPMENTAL AND GENOTOXIC IMPACTS OF ULTRAVIOLET-B RADIATION ON THE EMBRYO-LARVAL STAGES OF THE MARINE POLYCHAETE, PLATYNEREIS DUMERILII

V.V. Cheung*, I. McFadzen^, M.H. Depledge^, A.N. Jha^  
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Following depletion of stratospheric ozone, there has been an increase in the amount of biologically harmful ultraviolet-B (UV-B) radiation (280-320nm) reaching the earth’s surface. This flux of UV-B will potentially have adverse consequences on human and ecosystem health. In aquatic environments UV-B has its greatest impact at or immediately below the earth’s surface. This is where most primary productivity occurs, and the gametes and early life stages of many important groups including invertebrates, dwell.

In the present study, we have investigated the impact of UV-B radiation on the various life stages of Platynereis dumerilii. Exposure of the free swimming trochophore stages to UV-B irradiance levels of 48.43 Wm\(^{-2}\) for 7 hours (corresponding to 17434.8 Jm\(^{-2}\)min\(^{-1}\)) resulted in significantly high incidences of gross malformations in morphology at later developmental stages (60% of the exposed population showed abnormalities compared with 13% in the control population). These abnormalities ranged from relatively minor features such as a reduction in the number of chaetae to sever deformities such as the lack of development of parapodia and eyes. These studies suggest that this level of exposure is detrimental to the survival of the organism. Investigations are in progress to detect genetic damage following UV-irradiation and to find any potential link of this damage to developmental and survival effects in marine invertebrates.
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POTENTIATING EFFECTS OF UV RADIATION ON PAH-INDUCED GENOTOXICITY IN *MYTILUS EDULIS*

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Ultraviolet radiation (UVR) is known to alter the toxicity of certain chemical compounds, in particular polycyclic aromatic hydrocarbons (PAHs). In the current study, miconuclei (MN) induction was assessed in haemocytes from adult *M. edulis* exposed to (a) B(a)P in the absence of UVR; (b) B(a)P which had received previous UV photoactivation; or (c) B(a)P with simultaneous UV irradiation. Samples were collected on days three and six of continuous exposure.

Spontaneous levels of micronuclei induction were within the range of 0-6 per 1000 cells (mean frequency of $1.7 \pm 1.6$). Following three days of exposure, the mean frequency of micronuclei was (a) $7.00 \pm 6.05$ and $10.5 \pm 4.04$ in mussels exposed to $1 \mu g/L$ B(a)P and $10 \mu g/L$ B(a)P respectively; (b) haemolymph sampled from mussels exposed to previously photoactivated B(a)P showed a micronuclei frequency of $13.17 \pm 4.07$ and $15.83 \pm 4.9$ ($1 \mu g/L$ B(a)P and $10 \mu g/L$ B(a)P respectively); and (c) mussels exposed to simultaneous UV irradiation, exhibited a mean micronuclei frequency of $16.7 \pm 4.5$, in animals which had been exposed to a concentration of $10 \mu g/L$ B(a)P.

These highly significant ($p<0.0001$) increases in genotoxicity clearly indicate that there is an interaction of UVR with B(a)P in the marine environment. Observations from this study indicate that the acute phototoxic effects of B(a)P and other PAHs should be taken into consideration when assessing the impact of physical and chemical agents on aquatic biota.
PHOTO-ENHANCED TOXICITY OF BENZO(A)PYRENE IN THE 
HAEMOCYTES OF MYTILUS EDULIS

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The depletion of stratospheric ozone has led to an increase in the amount of ultraviolet radiation (UVR) potentially causing detrimental affects to marine organisms. Additionally it is known that the interaction of UVR with polycyclic aromatic hydrocarbons results in an alteration of toxicity. In the present study, the haemocytes of Mytilus edulis were examined for genotoxic and cytotoxic effects induced by environmentally realistic levels of benzo(a)pyrene (B(a)P) (1 mg/L and 10 μg/L). Exposure was carried out with or without simultaneous UVR, of levels comparable to mid-summer irradiation. Genotoxic effects were assessed using the micronucleus and Comet assays, and cytotoxicity with the lysosomal neutral red assay. Exposure of mussels to B(a)P with simultaneous UVR resulted in an increase in the frequency of micronuclei, and an increase in DNA strand breaks measured with the frequency of micronuclei, and an increase in DNA strand breaks measured with the Comet assay, compared with mussels exposed solely to B(a)P. There was a statistically significant relationship between the frequency of micronucleated cells and DNA strand breaks. No trend was observed in the retention time of neutral red in the lysosomes when comparing mussels exposed to B(a)P, and B(a)P with simultaneous UVR. In conclusion, UVR photoactivated B(a)P caused increases in DNA damage but alterations in cytotoxicity could not be detected with the neutral red assay.
EVALUATION OF BIOMARKER RESPONSES IN EUROPEAN FLOUNDER (PLATICHTHYS FLESUS) FOLLOWING EXPOSURE TO PAH SPIKED FEED

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The validation of biomarker measurements is an essential prerequisite to their use in the field. In this study selected biomarkers were applied to flounder (Platichthys flesus) chronically exposed to polycyclic aromatic hydrocarbons (PAH) via an oral route. Fish were maintained in a flow through system for 4 months and fed an equal mix of four PAHs, pyrene, Phenanthrene, benzo[a]pyrene and benz[a]anthracene. Nominal concentrations of PAH were 100 and 500 mg kg⁻¹ of feed (nutra marine, Trouw aquaculture) along with a control of unspiked feed. Following 4 months of exposure fish were sacrificed and a suite of commonly used cellular and molecular biomarkers were applied. EROD activity was used to estimate hepatic cytochrome P450 induction and bile metabolites were analysed by synchronous fluorescence spectrometry. Hepatic DNA adducts were quantified by the ³²P-postlabelling assay, while DNA strand breaks and cytogenetic damage in peripheral blood erythrocytes were analysed using the comet and micronucleus assay respectively. Analysis of parent PAH compounds in the muscle was carried out by HPLC. The biomarkers employed demonstrated a clear dose response relationship between fish fed a control feed and those exposed to PAH. The exception to this was the induction of EROD activity, which did not show any difference between control fish and those to feed spiked with 100 or 500 mg kg⁻¹ PAH.
VALIDATION OF THE COMMON COCKLE *CERASTODERMA EDULE*, AS A POTENTIAL BIOMONITORING SPECIES FOR DETECTING GENOTOXICITY IN THE MARINE ENVIRONMENT USING THE COMET ASSAY

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The marine bivalve *Mytilus* sp. has been widely used as a sentinel species for biomonitoring. To be able to grow and proliferate, this species requires rocky substrata for attachment. In contrast, the common cockle *Cerastoderma edule*, is widely distributed in many shore areas including fine sediment environments into which it is able to burrow. It is therefore in close proximity to contaminants which may be present in the sediments. In this study, its potential for use in genotoxicity studies is evaluated.

Haemocytes were collected from the adductor muscle from 20 animals, pooled, and exposed *in vitro* to a range of concentrations (100-1000 \(\mu\)M) of \(\text{H}_2\text{O}_2\). The level of DNA damage induced was measured with the Comet assay. Following the *in vitro* exposure to \(\text{H}_2\text{O}_2\), a dose-dependent increase in the level of DNA damage was detected in the cells (\(R^2=0.8962\%\)), with statistically significant increases in the amount of DNA damage in the cells treated to 100 \(\mu\)M, 500 \(\mu\)M, and 1000 \(\mu\)M \(\text{H}_2\text{O}_2\) when compared with the seawater control (\(p<0.0001\)), as measured with ‘tail moment’ and ‘tail length’. However, a dose-dependent increase was not detected with ‘tail DNA’ measurements.

Following this study, haemolymph samples were collected from indigenous populations (*in vivo*) along the Tamar estuary (SW Devon) in an attempt to determine differences in levels of DNA damage using the Comet assay. A pollution gradient was detected with increasing levels of DNA damage being found in samples from animals collected from sites located upstream, away from the mouth of the estuary. A statistically significant difference was observed in samples from sites highest upstream compared with the control site (\(p<0.05\)). The level of DNA damage correlated with *in vivo* cytotoxicity measurements, in addition to heavy metal concentrations quantified in the sediments and tissues of *C. edule* collected from the same sites. *Cerastoderma edule* appears to be a potentially useful biomonitoring species of genotoxic contaminant effects.
PUBLICATIONS IN PEER-REVIEWED INTERNATIONAL JOURNALS


Atienzar FA, Cheung VV, Jha AN, Depledge MH. 2001. Fitness parameters and DNA effects are sensitive indicators of copper-induced toxicity in Daphnia magna. Toxicological Sciences, 59: 241-250.
Molluscan Lysosomal Responses as a Diagnostic Tool for the Detection of a Pollution Gradient in Tolo Harbour, Hong Kong

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ABSTRACT

Sub-cellular perturbations in the lysosomal compartment of molluscan haemocytes were examined in mussels (Perna viridis), collected along a pollution gradient. The neutral red technique was validated using a well defined contamination gradient among indigenous populations from five stations along Tolo Harbour, Hong Kong. Condition indices (shell length:dry tissue wt) and tissue metal concentrations were also measured in an attempt to identify a relationship between contamination level and adverse physiological effects. Correlations were found between lysosomal retention time and condition along the pollution gradient. There were significant differences between mussels collected from stations on offshore islands and those collected from inner harbour sites (p<0.05). There was, however, little correlation between metal concentrations and retention time or condition (p>0.05). © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Increased contamination of water, especially in the coastal zone, has been attributed to urbanisation and industrialisation along the shoreline. It has been estimated that approximately 70% of the human population resides within 60 km of the coastal region, and a significant proportion of the world's largest cities are linked either directly or indirectly, to the marine environment (Forbes and Forbes, 1994). Semi-enclosed areas of the shoreline are increasingly showing signs of contamination, and the biological impact of human activities can be observed as changes to inshore ecosystems (Peakall, 1992). Tolo Harbour is a near land-locked embayment and was selected as the study site due to its high contaminant input and its poor flushing rate (Cheung and Wong, 1992).

The basis of all xenobiotic-induced injury involves perturbation of biochemical and molecular processes within the cell, which then give rise to effects at higher levels of organisation. Ultimately this may lead to extensive changes in distribution or age structure, and alterations in ecosystem processes (Abel and Axiak, 1991). Biomarker responses
at the subcellular level can therefore potentially be used as early warning signals of damage at higher levels of organisation.

One of the more widely studied subcellular biomarkers is lysosomal stability. It has been applied to both molluscan digestive cell lysosomes and fish hepatocyte lysosomes in laboratory exposures to contaminants including heavy metals, and various organics (Moore, 1985; Kohler, 1989; Lowe and Pipe, 1994). Destabilisation of the lysosomal membrane has been quantitatively related to the extent of the stress response (Moore, 1985). The aim of this study was to attempt to detect a pollution gradient in a field study, using lysosomal integrity and condition index in *Perna viridis* as indicators of the degree of stress experienced by the animals. Metal concentrations in the dry tissue of the mussel samples were also quantified, in an attempt to identify a relationship between relative metal concentrations and bivalve health. The metals selected for analysis were representative of a suite of metal contaminants which can be associated with the discharges from domestic and industrial activity from Shatin and Tai Po new towns. Some of the industries in the area include food processing, metal finishing, laundering, photofinishing and vehicle maintenance (Cheung and Wong, 1992).

**MATERIALS AND METHODS**

*Perna viridis* were collected from five sites in and around Tolo Harbour (Fig. 1). At each collection site, hydrological parameters were measured including pH, salinity, temperature, dissolved oxygen and particulate matter. On return to the laboratory, epibionts were removed from the mussels. Test animals were kept in plastic tanks containing filtered, continuously circulating, aerated sea water at 25°C. Following a 24 h period of acclimatisation, 48 animals from each site were treated with the neutral red probe for the

![Fig. 1. Map of Hong Kong showing the positions of the five sampling sites in and around Tolo Harbour.](image)
measurement of lysosomal stability. They were then dissected for calculation of condition index, and 12 random samples from each site were measured to determine trace metal concentrations.

Neutral red retention assay

The protocol used for the neutral red assay in mussel haemolymph was described by Lowe et al., 1995. When 50% or more of the cells exhibited leaking lysosomes (recognised by the neutral red probe being distributed throughout the cytosol), the time was noted and the examination of the cells was concluded.

Condition index calculation

Various biological parameters including shell length, shell weight, and soft tissue wt (wet and dry) were measured for the calculation of condition index. Condition index was calculated as a dry tissue wt:shell length ratio (Luoma et al., 1995).

Heavy metal analysis

The metals copper, manganese, zinc and iron were measured in the soft tissue using a Spectra AA-400 Plus Atomic Absorption Spectrometer (Varian), following acid and microwave digestion (Howard and Statham, 1993).

RESULTS AND DISCUSSION

Figure 2 (A) and (B) show the neutral red retention times and the condition index, respectively, for mussel populations collected from each of the five sampling sites in and around Tolo Harbour. Crosses indicate the mean values, notches are adjacent to median values, with 95% confidence intervals. Following transformation of data (to normalise data distribution), one-way ANOVA tests showed a pollution gradient detected by the two biomarker techniques, with significant differences between the mean retention times and condition indices in mussels from the inner harbour sites and those from the control offshore harbour sites (F-test, p-value < 0.05). The higher condition index observed in the mussels collected from Yim Tin Tsai may be explained by a relatively high amount of suspended particulates at this site, since condition is a measure of nutritive status.

Metal concentrations in the tissue are summarised in Table 1. A negative correlation was found for populations at each site between copper concentrations in the soft tissue, and lysosomal membrane stability. No correlation was observed between the other three metals and lysosomal responses within populations, indicating that other xenobiotics in the study area could be responsible for the perturbations.

CONCLUSIONS

The neutral red assay is effective in identifying perturbations in the lysosomal compartment of haemocytes extracted from indigenous field samples of Perna viridis. Condition
Fig. 2. (a) Notched box and whisker plot for log lysosomal retention (RT) times. A pollution gradient can be detected by the differences in lysosomal responses between mussels from different sites (n=48). (b) Notched box and whisker plot for log condition index (CI). A pollution gradient can be detected by the differences in condition between mussels from different sites. Note the high condition of mussels collected from Yim Tin Tsai (n=48).

**TABLE 1**
Mean Metal Concentrations (µg g⁻¹ Dry Weight) in *Perna viridis* Collected from Each Site (Mean ± SD; n = 12)

<table>
<thead>
<tr>
<th>Site</th>
<th>Cu</th>
<th>Mn</th>
<th>Zn</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap Mun</td>
<td>6.37 ± 1.33</td>
<td>36.52 ± 15.96</td>
<td>95.83 ± 38.42</td>
<td>58.64 ± 9.95</td>
</tr>
<tr>
<td>Kat-O</td>
<td>5.35 ± 1.06</td>
<td>1.98 ± 0.79</td>
<td>35.30 ± 23.09</td>
<td>37.30 ± 15.37</td>
</tr>
<tr>
<td>Three Fathoms Cove</td>
<td>7.01 ± 1.52</td>
<td>38.44 ± 12.23</td>
<td>35.67 ± 9.66</td>
<td>46.17 ± 11.79</td>
</tr>
<tr>
<td>Ma Lui Shui</td>
<td>6.79 ± 1.58</td>
<td>15.31 ± 5.61</td>
<td>98.42 ± 19.76</td>
<td>39.85 ± 2.44</td>
</tr>
<tr>
<td>Yim Tin Tsai</td>
<td>7.78 ± 1.57</td>
<td>11.25 ± 3.50</td>
<td>49.99 ± 4.07</td>
<td>47.01 ± 33.33</td>
</tr>
</tbody>
</table>
index is an indicator of nutritive status, lysosomal stability, however, is a more sensitive indicator of bioavailable xenobiotics. This explains the disparity between the condition index and lysosomal response in the mussels from Yim Tin Tsai.

Although the responses observed in field samples cannot be used to pinpoint specific contaminants, such biomarkers are beneficial for providing biological information concerning the health status of organisms at a study site. Metal analyses in this study showed little or no correlation with the biomarkers suggesting that further work should be undertaken to identify the xenobiotics accountable for the biomarker responses observed. When using biomarkers, an interrelationship between field and laboratory studies is advantageous, and if used in collaboration with chemical monitoring, can provide an appropriate data base for hazard assessment.

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REFERENCES

Biomarkers of Biochemical and Cellular Stress in *Carcinus maenas*: an in situ Field Study

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**ABSTRACT**

Biomarkers are useful diagnostic tools for identifying exposure to physical, chemical, and environmental stresses. In this study a suite of techniques—measurements of metallothionein, lysosomal integrity, and osmoregulatory ability—have been used in an attempt to provide an in situ assessment of environmental quality. Five estuarine sites were studied, two clean sites, two urban sites, and one intermediate. Shore crabs, *Carcinus maenas*, were collected and haemolymph taken for the lysosomal and osmoregulatory assays. Animals were killed and tissue preserved for metallothionein analysis. Lysosomal integrity was significantly different in crabs from clean sites and the urban sites (n = 40, p < 0.05). The potential value of the biomarker approach is that it could provide data for impact assessment and is applicable in the field. © 1998 Elsevier Science Ltd. All rights reserved

**INTRODUCTION**

Biomarkers of pollution exposure, and effect, have been suggested for establishing the status of marine ecosystems. There is a need for sensitive, cost-effective biomarkers for ecological risk assessment (Depledge, 1994).

This study's objective was to discern the environmental quality of different field locations. Relative comparison of biomarker responses would be made. Emphasis was on evaluating in situ application of the biomarkers. Three biomarkers were chosen for study: haemolymph lysosomal stability, metallothionein (MT) concentration, and osmoregulatory ability. The shore crab, *Carcinus maenas*, was utilised as it is distributed throughout most estuaries.

Lysosomes are conserved cellular organelles. There is evidence for lysosomal damage by xenobiotics in aquatic organisms (Moore, 1990; Lowe et al., 1992). The neutral red dye assay utilises the fact that lysosomes in healthy cells retain the dye longer than perturbed lysosomes (Lowe et al., 1992). Retention time gives a health index in terms of contaminant
exposure. Lysosomal responses have been measured in several studies (Lowe et al., 1992; Krishnakumar et al., 1994). MTs are proteins which bind trace metals in the cells of a wide range of organisms. Field studies have shown that MT concentrations may be a useful indicator of trace metal exposure (Hogstrand and Haux, 1990). Measurement of crustacean osmolality has been investigated as an indicator of exposure to chemical stress (Bamber and Depledge, 1997). Crabs hyperregulate in response to external salinity. Exposure of crabs to low salinity, and measurement of osmolality, shows effects on physiology associated with osmoregulation. The effect of water quality on osmoregulation can thus be established.

MATERIALS AND METHODS

Field protocol

Five sites around the southwest United Kingdom were studied. Plym Estuary and Polphlett Creek receive effluent from industrial and urban sources. Restronguet is rich in trace metals from mining activity. The Erne and Avon are impacted by smaller numbers of domestic sources.

Sixteen intermoult *Carcinus maenas* males of carapace width 50–60 mm were obtained from nets. Lysosomal and osmoregulation tests were conducted in situ on eight crabs. Eight further crabs were killed and the midgut gland frozen for MT analysis. A van was equipped for in situ work and tissue storage.

Neutral red lysosomal assay

The technique described here is adapted from Lowe et al. (1995). Haemolymph (100 μl) was extracted from the arthrodial membrane using a Drummond pipette and mixed with 100 μl of anticoagulant (Smith and Ratcliffe, 1978). Mixture (40 μl) was then placed on a slide. Physiological saline (Smith and Ratcliffe, 1978) was added and cells incubated for 15 min at 10°C. Excess was removed and 40 μl of the dye working solution was added. Cells were scored according to Lowe et al. (1992).

Metallothionein analysis.

Quantification of MT was achieved using the indirect method described by Pedersen et al. (1996). Absorbency was read in a UNICAM UV/Vis spectrophotometer. MT concentration was measured using glutathione as reference standard.

Measurement of osmoregulatory ability

Measurement of osmoregulatory ability was performed as described in Bamber and Depledge (1997). However, after haemolymph samples were taken crabs were transferred to tanks containing distilled water. Samples were taken from each crab 3 h later and frozen in liquid nitrogen. Osmolality was measured by osmometer (Wescor 5500) requiring 10 μl of sample per evaluation. Site differences were examined by Kruskal–Wallis one-way analysis of variance and multiple range tests on means (Fisher’s LSD procedure).
RESULTS AND DISCUSSION

Lysosomal retention values were of 0–100 min which is typical for *C. maenas* haemolymph (Wedderburn, unpublished). Significant differences existed between mean retention times of Pophlett Creek (41 min, *n* = 16, *p* < 0.05) and the Erme (81 min) and Avon (77 min). Significant differences existed (*p* < 0.05) between the Plym (56 min) and the Erme (81 min) (Fig. 1).

![Means Plot (95% C.I) of Neutral Red Retention](image1)

**Fig. 1.** *Carcinus maenas*. Neutral red retention (minutes) by sampling sites. Significant differences exist between the Pom (Pomphlett creek) and the Avon and Erme sites, and the Plym and Erme sites (*p* < 0.05, 95% confidence interval, Kruskal–Wallis). Res = Restronguet Creek.

![Means Plot (95% C.I) of Osmolality Post/Pre Stress](image2)

**Fig. 2.** *Carcinus maenas*. Haemolymph osmolality values, pre-post-exposure to distilled water. ‘I’ indicates values obtained after exposure. A significant difference exists between Avon and Erme to other sites (*n* = 40, *p* ≤ 0.05, 95% confidence interval, Kruskal–Wallis).
There are no differences in MT concentration. MT values for the Pompflelt site are highest (301 µg/g wet wt tissue), and the Erme estuary has the lowest (228 µg/g wet wt tissue). The Avon and Restronguet sites had values of 230 and 238 µg/g wet weight tissue. Significant differences in osmolality values \( p < 0.01 \) exist between the Avon and Erme pre-stress values and the remaining sites. There is no difference between the osmolality end points \( p > 0.05 \). (Fig. 2).

The neutral red assay showed that animals from the Pompflelt and Plym sites had reduced retention times in haemocytic lysosomes. Retention has been linked with pollutant exposure (Lowe et al., 1995).

MT data presented here are consistent with the findings of other authors, using the same sampling sites and technique. Pedersen et al. (1998) found no significant difference in the levels of MT in \( C. maenas \) midgut between the Restronguet site, contaminated with trace metals, and the cleaner Avon site.

The osmolality data do not allow conclusions to be drawn concerning osmoregulation. The results are confounded by differences in site osmolality. This could be a result of hydrographic differences.

The lysosomal technique has performed well with \( C. maenas \). It is curious that the trace metals at the Restronguet site are not associated with increased MT, or reduced lysosomal stability. The technique's sensitivity for detecting MT needs further investigation. The lack of lysosomal responses in animals from the Restronguet site could be due to adaptation to the high background levels of trace metals. The in situ use of osmoregulation ability requires adaptation to demonstrate the potential indicated in other studies (Bamber and Depledge, 1997). Lysosomal assays are sufficiently sensitive to discern site-related differences in gross environmental quality (Krishnakumar et al., 1994; Lowe et al., 1995). The results found here are encouraging for the technique.

Rapid site characterisation means biological monitoring programs can be focused and more cost-effective, whilst providing useful information.

REFERENCES

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*

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Abstract

In genetic ecotoxicology or eco-genotoxicology, there is lack of well-validated systems which could demonstrate the utility of multiple endpoints in environmental quality assessment. For an evaluation of genotoxic potential of heterogeneous marine sediment samples collected from a small fishing harbour in the UK, an in vivo test system using embryo-larval stages of the common mussel, *Mytilus edulis* was validated against direct and indirect acting reference mutagens. The system appeared to be sensitive and reproducible for cytogenetic endpoints analysed (sister chromatid exchanges (SCEs) and chromosomal aberrations (CAbs)). Following validation and chemical characterisation of the environmental samples, multiple endpoints were measured. Determination of the maximum tolerated dose (MTD) was carried out as a measure to determine cytotoxic effects as a confounding factor for genotoxicity, based on developmental and cytotoxic (in terms of proliferative mit index or PRI) effects. Evaluation of the genotoxic potential of the samples gave a positive response for all the endpoints tested, linking different levels of biological organisation (i.e., chromosomal, cellular and organismal) for the observed effects. The study also emphasises the need for the assessment of the short and long-term impacts of dredge disposal on marine biota by including laboratory-based bioassays and incorporating an integrated approach which could yield as much useful information as possible in overall hazard and risk assessment for aquatic genotoxicity. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Genotoxicity; Cytotoxicity; Developmental toxicity; Sediment chemistry; Marine mussel; Multiple endpoints; Integrated approach

1. Introduction

In recent years, much attention has been given towards the detection of genotoxic agents in the aquatic environment. While presence of such contaminants are suggested to have adverse effects on human health via the food chain, it is being suggested that such exposure might have significant implications for the short (e.g. neoplastic diseases) and long-term survival (i.e. extinction) of the natural populations [1,2]. In contrast to the human health.
arena, where the health status of individuals is of paramount importance, in an ecological or ecogenotoxicological context, the main objective is to evaluate the population or community level responses following exposure to a contaminant [1,2]. At higher orders of biological organisation, however, the problem of identifying a particular biological response to a genotoxic or other environmental stressor becomes too complex which hinders our understanding of population or community level consequences of genotoxic exposures in natural biota. However, one approach to this problem has been to break the problem down into smaller organisational levels (molecular to individual levels), each level controlling the structure and function of subsequent levels. Mechanistic questions pertaining to how an organism relates to its environment can then be considered. Key questions therefore are: (1) which mechanisms link levels of biological organisation in a stressed ecosystem; (2) how can these mechanisms be expressed at higher levels of organisation in terms of Darwinian fitness (i.e., survival, reproductive output and time to maturity between breedings) of the species.

Another limitation to evaluating the integral effects of contaminants in an ecosystem is our traditional reliance on analytical techniques for their detection, in particular, for environmental monitoring and effluent discharge compliance. Simple detection of environmental contaminants by these techniques is not sufficient unless their biological effects are properly evaluated. Furthermore, genotoxic agents in the environment, in all probability, occur as complex mixtures, and the risk of such mixtures cannot be adequately anticipated on the basis of the effect and behaviour of individual components. In addition, in a complex mixture, the presence and potential interaction of unknown substances or substances in concentrations beyond the detection limits of the techniques used could lead to a discrepancy between the actual and the predicted risk of an effluent or contaminant using a substance-specific assessment. Thus in view of these limitations to chemical monitoring, the importance of biological monitoring is now being greatly realised (e.g., adoption of direct toxicity test or whole effluent environmental risk). As biological systems are the target for the action of toxicants, they can provide important information which is not readily available from chemical analyses of the environmental samples.

It is well-established that the genotoxic potency of chemicals is closely tied to their toxicity for many phylogenetic endpoints [3]. The toxicity of contaminants is therefore one of the major confounding factors in interpreting the results for genotoxicity assays. This important factor, which deserves due attention, has not been widely adopted when evaluating the genotoxicity in aquatic organisms. In mammalian genotoxicity assays, for a test material to be described with confidence as negative, it is considered to be necessary to evaluate it at a sufficiently high dose level. In general, mammalian regulatory guidelines recommend the use of a maximum tolerated dose (MTD) or a dose level producing some indication of cytotoxicity either on the target cell (organ) for the assay or at the whole animal level (death being the final arbiter). Although there is some discrepancy and ambiguity in defining MTD in mammalian regulatory guidelines [4], in aquatic genotoxicology, specially with the use of invertebrate species, it is relatively easier to determine apparent toxic effects by evaluating various behavioural, physiological, developmental and morphological endpoints following exposure to a contaminant [5]. This approach can facilitate the determination of MTD in the exposed organisms.

Despite growing concern over the presence of genotoxins in the aquatic environment, there is a lack of adequately validated test methods which could be used effectively to evaluate genotoxicity in aquatic organisms under environmentally relevant conditions. It will be worth mentioning here that while in vitro or bacterial test systems could be used as screening tools to define the intrinsic genotoxicity of a substance, definitive ecotoxicological risk assessments should consider the expressed genotoxic activity in ecologically relevant organisms. These will take into account environmentally realistic routes of exposure, the effects of metabolism and DNA repair efficiency. The development of such in vivo test systems is also essential in providing a scientific basis for comparing the relative risks of man-made vs. natural genotoxins.

While environmental scientists are attracted to studying heavily contaminated samples or sites to evaluate potential damage in the exposed biota, in
order to elucidate and solve many of the challenges in eco-genotoxicology (e.g. identification of physical, chemical and biological factors influencing the induction of genetic damage under natural conditions; identification of sensitive species and life stages; individual variability and genetic susceptibility, etc.), there is also a need for the monitoring sites or samples which are considered to be pristine, undisturbed or have moderate levels of contamination compared with heavily contaminated sites or samples. This is considered to be necessary in order to generate background and historical control data while evaluating the impact of anthropogenic contaminants on natural biota [6]. Monitoring of such sites is also necessary for many estuaries in industrialised areas that require periodic maintenance dredging of navigable waterways to sustain recreational, commercial and military shipping [7-9]. While such dredging operations might be helpful in maintaining the health of the biota in the coastal waters, if in excess, it could create an ecological imbalance for the deep sea ecosystems.

It is clear that in order to assess the integral effects of genotoxins on the ecosystems, we need to adopt a holistic approach to address many fundamental questions. These include: (a) how chemical analyses complement genotoxicological endpoints under in vivo conditions, and (b) how these endpoints compare and complement other parameters at various levels of biological organisation. Bearing these objectives in mind, in this study, an attempt to integrate different components of eco-genotoxicology described earlier has been made. In particular, an attempt has been made to (i) validate and use an in vivo test system using an ecologically relevant marine invertebrate (ii) take into account the developmental and cellular toxicity of an environmental sample (to determine MTD), prior to evaluating its genotoxic potential (iii) link genotoxic effects with cellular and individual level effects (iv) characterise the sediment sample from a small fishing harbour to determine its chemical composition and (v) postulate the possible source of contamination at the collection site, which had not been investigated before for the presence of any contaminants and, (vi) hypothesise its likely effects on the natural biota.

In this study, we used the embryo–larval stage of the marine mussel, *Mytilus edulis* (Mollusca: Bivalvia). This is a member of an ecologically important group of marine invertebrates, and has been used widely for monitoring the bioaccumulation, ecotoxico logical and genotoxicological effects of marine contaminants [10-12]. It will be worth mentioning that embryo–larval stages of aquatic organisms, in contrast to sexually mature adult animals are considered to be critical and several orders of magnitude more sensitive in terms of toxicological injury. Their small size also facilitates the distribution of contaminants throughout the body.

2. Materials and methods

2.1. Validation of the in vivo test system for genotoxicity assay

Adult specimens of *M. edulis* were collected from the lower intertidal zone at Whitsand Bay, Cornwall, UK, transported to the laboratory in a cool box, maintained in clean, aerated seawater (SW) at 15(±2)°C and fed with liquid aquifry twice a day prior to their use. To obtain the gametes and subsequently the embryo–larval stages, individual animals were transferred into glass beakers with clean SW (salinity 35‰) at room temperature (20 ± 2°C) and were given thermal shock as per the protocol described by Harrison and Jones [10]. Prior to their use, the gametes were checked qualitatively and quantitatively. In order to achieve optimum fertilisation, the sperm suspension was added to the egg suspension in the ratio of 1:1000 (egg:sperm), as described by Sprung and Bayne [13]. Once the maximum fertilisation was achieved (typically > 95%), the excess sperms were sieved-off, the embryos were transferred into clean SW kept at 15(±1)°C (maximum concentration: 30 embryos/ml) and were allowed to grow. From the embryo stage (up to 12 h post-fertilisation at 15°C) to the beginning of the first larval stage (up to 24 h at 15°C), free-swimming embryo–larval specimens of aquatic invertebrates are easy to manipulate experimentally, providing large numbers of actively dividing cells which can be arrested at metaphase following exposure to colchicine. Preliminary studies to establish the average generation time (AGT) of the embryo–larval cells based on the protocol proposed by Ivett and
Tice [14] suggested that the AGT for these cells (15 ± 1°C) is approximately 3.8 h (unpublished data). Following estimation of the AGT, investigations were undertaken to evaluate the genotoxic effects of two reference mutagens, which included methyl methane sulphonate (MMS) a direct acting compound and benzo[a]pyrene (BaP), a compound which requires metabolic activation. Genotoxic observations were primarily based on the analysis of chromosomal aberrations (CAbs) since this is considered to be the most biologically significant endpoint [15,16]. Sister chromatid exchanges (SCEs) were also analysed in order to provide information on the comparative sensitivity of the system with historical reports for this and other marine invertebrate species. Cytotoxicity assessment was based upon the use of sister chromatid differential (SCD) staining and calculation of the proliferative rate index (or PRI) [17,18]. As mentioned in our earlier studies, the SCD staining and analyses of SCEs required presence of 5-bromodeoxyuridine (BrdU: 1 × 10⁻³ M). The growing embryo-larvae were shielded from light following BrdU exposure to prevent photolysis. The selection criteria for the concentrations of reference mutagens for validation of the system, exposure conditions, preparation of metaphase spreads, SCD staining and, methods adopted to analyse cytogenetic endpoints have been described in details in previous publications using embryo-larval stage of polychaete worm Platynereis dumerilii [19,20].

2.2. Study site, sample collection and storage

The sediment samples were collected from the inner harbour of the small fishing port of Brixham, situated in the Torbay area of south Devon, UK (Ordnance Survey Landranger series reference 202; grid reference 937 568) (Fig. 1). The basic character of Brixham harbour can be said to be mainly the product of a 19th century fishing industry together with the activities associated with a modern commercial port and tourist resort. The geology of the area is predominantly limestone, with red sandstone in various associations. A geochemical survey of metals in the Brixham rocks notes the presence of certain heavy metal species [21].

In south Devon, large replacement bodies of iron oxides are developed in the middle Devonian limestones. In the Brixham district, this occurs in the form of red or brown hematite pockets in the limestones [22]. As a result, both mining and quarrying has been practised in the locality [23]. Rich iron oxide deposits in the limestones were exploited in the mid-19th century and goods produced from this mineral were exported all over the world. As only the inner harbour drains out at low tide, sampling of the black sediments was restricted to this area. The anoxic appearance of the sediment was evidenced also by a sulphurous odour. Six sites were selected for the collection of the samples in an area approximately 200 m × 100 m (Fig. 1b). A plastic trowel was used to sub-sample down to a depth of 10 cm and all materials were collected in separate, pre-acid washed sealed polyethylene containers. Wet samples were returned to the laboratory and either filtered to remove the bulk of associated liquid prior to drying e.g. Buchner filtration or freeze drying or sealed wet and held in cold storage below 5°C.

2.2.1. Physico-chemical measurements

The physico-chemical characteristics of the sediments were measured on site using calibrated sensors. The measurements taken were pH and oxygen content at 5 cm depth and redox potential at 1, 5 and 10 cm depth. All particle size analyses were conducted later using a Malvern Mastersizer X Analyser.
A.N. Jha et al./Mutation Research 464 (2000) 213–228

2.2.2. Chemical characterisation of sediments

Following collection, pre-treatment and storage, the sediment samples were analysed to determine their (a) organic content and (b) extractable metals.

2.2.2.1. Organic content of sediments. The total organic-matter was determined by an ashing technique. Accurately weighed masses of sediment (ca. 60 g) were added to pre-weighed crucibles and placed in an oven at 110°C for 24 h. The weight after loss of volatiles at this temperature was recorded. The crucibles were then admitted to a muffle furnace at 550°C for 6 h, cooled and re-weighed.

Organic matter was extracted from sediments using an automated Soxhlet technique. An accurately weighed mass of sample was transferred to a pre-cleaned Soxhlet thimble and extracted for 12 h with 500 ml of dichloromethane (DCM). The solvent was removed carefully and the total organic extract (TOE) determined by weight. The TOE was then redisolved in a minimum volume of DCM and added to a silica gel open column. Aliphatic and aromatic fractions were obtained by sequential elution with solvents of increasing polarity, i.e., hexane and DCM. High resolution gas chromatography (HRGC) was performed using a Carlo Erba HRGC 5360 fitted with a 30-m DB-5 fused silica capillary column and flame ionisation detector. The temperature programme used an initial temperature of 40°C (1 min) up to a final temperature of 300°C ramped at 5°C/min. The temperature was then held at 300°C for 20 min. GC–MS was performed using a Hewlett-Packard 5890 gas chromatograph equipped with a Hewlett-Packard 5970 mass selective detector (MSD). The MSD was operated in scan mode with a 70 eV ionising potential and ion source temperature of 300°C. The GC was operated in the splitless injection mode with an injector temperature of 250°C. The temperature programme was from 40°C to 300°C at 5°C/min and held at 300°C for 20 min.

2.2.2.2. Metal content of sediments. Freeze-dried sediment samples were ground to obtain < 500 and > 500 μm fractions. The 0.3-g of a < 500 μm fraction was placed in a pre-cleaned Teflon bomb together with 4 ml of concentrated nitric acid and 2 ml of 30% hydrogen peroxide. The sediment was allowed to pre-digest overnight before a microwave heating programme was performed (2 × 1 min sessions on medium power). The contents from each bomb was then quantitatively transferred to volumetric flasks and the metals determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) [24]. A certified reference material, PACS-1 (National Research Council, Canada) was run along side to validate the procedure.

2.2.2.3. Metal content of sediment slurries. In order to investigate the biological effects from a sediment suspended in SW, slurries were prepared from a dried "general sample". This "general sample" was prepared by mixing together equal amounts of the sediment from the six sites. This was justified on the grounds that the physical and chemical characteristics varied little across the sampling area. From the known metal content of the "general sample", increasing concentrations of sediment slurries were prepared covering the range of 0–1.6 g/l. Analysis of these waters, before and after use, for arsenic and copper content was performed by ICP-mass spectrometry (ICP-MS) [24]. A control slurry comprising a sediment of low available metal content suspended in SW was prepared for comparison.

2.3. Determination of developmental, cellular and genotoxicity

Following collection, in situ physico-chemical and subsequent chemical characterisation, the samples were used to determine the direct toxicity on embryo–larval stages of the *M. edulis*. Because the collection sites were quite close to each other spatially and as will be described later, did not differ substantially in physical or chemical characteristics, in order to evaluate the developmental, cellular and potential genotoxicity of the sediment samples, equal amounts of sample collected from six different sites was mixed to prepare a "general sample". This "general sample" was used in the determination of direct toxicity tests. In brief, the fertilised gametes were obtained and allowed to grow for 12 h, as
described in Section 2.1, following which they were transferred to beakers containing pre-determined weights of sediment samples in SW, based on the semi-logarithmic series routinely used for aquatic ecotoxicity testing [25]. The sediment samples were well-mixed and stirred in the filtered, clean SW for at least 12 h prior to use. The growing embryos were placed into the water column of the beakers (containing sediment samples) separated by a 30-μm nylon mesh sieve. The embryos were exposed for 6 h (approximately one and a half cell cycles) and for 8 h (approximately two cell cycles) in the presence of BrdU at 15(±1)°C. At the end of the exposure periods (6 and 8 h) embryos were processed for metaphase preparation, SCD staining and, eventually for cytogenetic analyses (i.e. evaluation of PRI, SCEs and CAbs). Also, at the end of the 8-h exposure period sub-samples from each treatment were transferred to clean SW and allowed to grow until 48 h (at 15 ± 1°C). They were then analysed for survival and developmental effects. The embryos which were either dead or could not reach to typical “D-shaped” structure at this time (48 h), were considered to be abnormal as per the criteria recommended by American Society for Testing of Materials (ASTM) [26] and adopted in earlier studies using the marine worm, P. dumerilii [27,28]. This exercise provided information on the developmental toxicity and helped to determine the MTD for the sediment samples on the embryo-larval stage of M. edulis prior to determination of potential genotoxicity. A summarised experimental protocol for the evaluation of developmental, cytotoxic and genotoxic effects is given in Fig. 2.

3. Results

3.1. Validation of in vivo test system

The cytotoxicity of the reference mutagens (MMS and B(a)P) were evaluated in all the experiments using the PRI endpoint. For each reference chemical, the PRI results from both the SCEs and CAbs experiments were observed to be similar at comparable concentrations and therefore, only the PRI data gen-
erated from the SCEs experiments are presented. In the SW controls for both the chemicals in the SCEs experiments, the PRI ranged from 2.12 to 2.72. In each of the exposure regimes, an inverse concentration relationship was observed for this end point (Fig. 3). A significant increase for the induction of SCEs in the embryo-larvae of *M. edulis* was also observed at these concentrations. In SW controls, the SCEs ranged from 0 to 3/cell and from 0 to 2/cell for solvent control (DMF for B[a]P only); up to 18 and 31/cell in the embryo-larvae exposed to B[a]P and MMS, respectively (Fig. 4). At higher concentrations, the intercellular range of SCEs distribution varied considerably among the cells (0–18 and 0–31 for B[a]P and MMS, respectively, as represented by large error bars in Fig. 4. This probably reflects the interindividual differences of uptake and metabolism of test chemicals at higher concentrations. The karyotype of *M. edulis* (2n = 28) facilitated the analysis of both CAbs and chromatid type aberrations (Fig. 5). There was also a concentration-dependent increase for the induction of CAbs as a measure of genotoxicity. Compared to SW controls, in which the percentage aberrant cells ranged between 0 and 3, cells exposed to highest concentrations of B[a]P and MMS showed 8% and 17% aberrant cells, respectively (Fig. 6). The aberrations scored were predominantly chromosome and chromatid breaks (deletions).
The aberrations were expressed as percent aberrant cells and total aberrations (chromosome plus chromatid type excluding gaps) based on at least 100 complete metaphases.

3.2. Physico-chemical and chemical characteristics of environmental samples

3.2.1. Physico-chemical characteristics

The on-site physico-chemical characteristics, i.e., redox potential, pH and dissolved oxygen, together with the grain size analysis of the sediment samples are summarised for sites 1, 3 and 6 in Table 1. It is clear that the sediments are anoxic, having large negative redox values and close to 0% dissolved oxygen content. Depth profiles from 1 to 10 cm show the expected increase in negative values from −150 to −450 mV. The pH shows a neutral value around 7.0 at 5-cm depth. The particle size distribution of the sediment is partly dependent upon the presence of organic matter. Distributions, for example, of sites 1 and 3 show 85%–95% < 500 μm with some 40%–60% < 63 μm (without organic matter) which drops to 80% < 500 μm and 32% < 63 μm (with organic matter).

3.2.2. Chemical characterisation

3.2.2.1. Organic Content. The gravimetric determination of organic content of the sediments by an ashing technique produced a range of values from 3.4% to 6.6% (wet weight) across the six sites. When equal amounts of samples from different sites were mixed together to produce a “general sample”, a value of 5.99 ± 0.15% was obtained.

The fractionated organic extracts (aliphatic and aromatic sub-samples) were analysed by GC-FID and GC-MSD to qualitatively estimate the range of organic species present. In a number of cases the bulk of these sub-sample extracts produced an unresolved complex mixture (UCM), appearing between 25 and 60 min and in the case of aliphatic extracts, appeared to contain components of a high molecular mass. Where possible, identification was made using GC-MS and these results are shown in Table 2. Besides a series of n-alkanes detected in the aliphatic

| Table 1 |
|---|---|---|---|---|
| In situ physico-chemical and grain size analysis of the sediment samples |  |  |  |  |
| Site no. | Redox potential (mV) | Dissolved oxygen (%) | pH | Grain size (% of total sediment) |
|  |  |  |  | < 500 μm | < 63 μm |
| 1 | −400 | 0 | 6.8 | 95 | 60 |
| 3 | −450 | 0 | 7.0 | 85 | 40 |
| 6 | −30 | 8 | 7.0 | 50 | 10 |
Table 2
Qualitative and quantitative estimations of aliphatic and aromatic hydrocarbons from the sediment samples using a Soxhlet extraction technique and HRGC with MS

<table>
<thead>
<tr>
<th>Sub-sample</th>
<th>Compounds</th>
<th>Concentration (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliphatic</td>
<td>tridecane</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>octadecane</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>general alkane</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>heneicosane</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>hopane</td>
<td>6.2</td>
</tr>
<tr>
<td>Aromatic</td>
<td>benzoic acid</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td>naphthalene</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>phenanthrene</td>
<td>425.0</td>
</tr>
<tr>
<td></td>
<td>benzenemethanol</td>
<td>34.5</td>
</tr>
<tr>
<td></td>
<td>benzene</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>fluoranthene</td>
<td>40.9</td>
</tr>
<tr>
<td></td>
<td>plasticizer</td>
<td>1830.0</td>
</tr>
</tbody>
</table>

extract, a definite presence from polyaromatic hydrocarbons (PAHs) was found in the aromatic extract comprising naphthalenes, fluoranthenes and phenanthrenes and their alkyl derivatives. However, the major component identified in these extracts was a phthalate plasticizer.

3.2.2.2. Determination of metals. The mean concentrations of metals determined in the sediment samples collected from the three sites together with three individual sites values are shown in Table 3. The levels represent the “available” metal content rather than the total. The latter would require digestion of the harder alumino-silicate material which would only release the minor occluded components over a geological time scale.

Table 3
Results of the metal analysis of Brixham harbour sediments using ICP–AES (mg/kg)

<table>
<thead>
<tr>
<th>Metal</th>
<th>Site 1</th>
<th>Site 3</th>
<th>Site 6</th>
<th>Mean ± SD</th>
<th>General</th>
</tr>
</thead>
<tbody>
<tr>
<td>As</td>
<td>196.00</td>
<td>193.00</td>
<td>215.00</td>
<td>205.00 ± 9.11</td>
<td>210.00</td>
</tr>
<tr>
<td>Cd</td>
<td>4.10</td>
<td>3.14</td>
<td>1.25</td>
<td>2.69 ± 1.14</td>
<td>3.50</td>
</tr>
<tr>
<td>Cr</td>
<td>41.80</td>
<td>35.50</td>
<td>26.90</td>
<td>37.38 ± 5.90</td>
<td>37.60</td>
</tr>
<tr>
<td>Cu</td>
<td>650.00</td>
<td>427.00</td>
<td>251.00</td>
<td>453.67 ± 132.36</td>
<td>464.00</td>
</tr>
<tr>
<td>Fe</td>
<td>19520.00</td>
<td>13498.00</td>
<td>6105.00</td>
<td>13831.33 ± 5373.00</td>
<td>11671.00</td>
</tr>
<tr>
<td>Pb</td>
<td>414.00</td>
<td>311.00</td>
<td>202.00</td>
<td>315.50 ± 73.44</td>
<td>325.00</td>
</tr>
<tr>
<td>Mn</td>
<td>129.00</td>
<td>133.00</td>
<td>128.00</td>
<td>143.33 ± 20.69</td>
<td>142.00</td>
</tr>
<tr>
<td>Ni</td>
<td>60.00</td>
<td>55.30</td>
<td>51.50</td>
<td>59.63 ± 6.65</td>
<td>66.40</td>
</tr>
<tr>
<td>Sn</td>
<td>158.00</td>
<td>170.00</td>
<td>177.00</td>
<td>169.83 ± 13.10</td>
<td>166.00</td>
</tr>
</tbody>
</table>

3.3. Developmental, cellular and genotoxicity of the environmental sample

Following an initial “range finding” experiment, it was found that a sediment concentration of 1.6 g of sediment/l SW had 94.9% abnormal embryo–larvae, as per the criterion described earlier in Section 2.3. Fig. 7 describes typical D-shaped (normal embryo–larvae) and dead and abnormal embryos after 48 h (15°C). At 15°C in 48 h, in the experimental conditions, in SW controls approximately 90% of embryo–larvae had reached the “D-shaped” structure whereas abnormal individuals were distinctly under-developed. At the higher concentrations, some of the test organisms appeared as a shrunken mass of necrotic tissue within the outer membrane. These features again allowed the ready identification of normal vs. abnormal individuals in each sub-sample and allowed the percentage of normal individuals to be calculated as an index of developmental toxicity for each treatment [27,28] (Fig. 8).

At the end of exposure periods (6 and 8 h), sub-samples of the growing embryo–larvae were collected and processed for metaphase spread preparations and staining, as described in Section 2.1. As mentioned, the evaluation of developmental toxicity of the sediment sample was complemented with cytotoxicity by analysing PRI for both SCEs and CAs experiments. A significant reduction for PRI value was observed at the highest concentration of sediment used in both the experiments. Again, the PRI results from both the SCEs and CAs experiments were observed to be similar at comparable concentrations, therefore, only the PRI data gener-
Rg 7 Determination of developmental toxicity in the embryo-larval stages of *M. edulis*: (a) typical 'D-shell' normal larva aged 48 h, (b) abnormal embryo-larva aged 48 h and (c) dead embryo-larvae (15 ± 1°C).

ated from the SCEs experiments are presented. From Fig. 9, it is apparent that the PRI value for SW and sediment controls was almost similar (2.2) whereas for the top concentration of sediment, it dropped to 1.2. This cytotoxic effect very neatly complements the developmental toxicity results which had helped in determining the MTD for the sample. Conversely, a concentration-dependent increase for the induction of SCEs was observed. The spontaneous (base) level of SCEs in SW and sediment controls were exceedingly low (mean value: 1.5 ± 1.2 and 2.6 ± 1.5/cell) whereas, at the top concentration, a two-fold increase (mean value: 4.8 ± 2.7 SCEs/cell) for the induction of SCEs was found (Fig. 9a). Based on the analysis of at least 100 complete metaphases per treatment, the frequency of percent aberrant cells (excluding cells with only gap-type aberrations) was found to be three and four, whereas the total aberrations (excluding gaps) ranged between three and nine for SW and sediment controls, respectively. There were also marked concentration-related increase in terms of both percent aberrant cells and total aberrations for the embryo-larval stages exposed to the sediment samples. A concurrent positive control using a single concentration of MMS (1.0 × 10^{-3} M) showed the maximum level of induced-aberrations,
Fig. 9. The effects of different concentrations of sediment sample on (a) PRI and SCE and (b) the induction of CAbs, in the embryo–larvae of the marine mussel, *M. edulis*.

(i.e., 17% aberrant cells and 34 total aberrations) compared to the highest concentration of sediment sample used (i.e., 11% aberrant cells and 22 total aberrations) (Fig. 9b).

4. Discussion

4.1. Validation of the in vivo test system

The embryo–larval stages of the marine mussel provided evidence of being a reproducible and sensitive model for the detection of mutagens present in SW. Although originally applied for application to actively proliferating and highly differentiated mammalian cells in vitro, the PRI provided useful means of quantifying the cytotoxicity of the test compounds to the embryo–larvae in vivo. However, in contrast to highly differentiated mammalian cells growing under in vitro conditions, the PRI for the embryo–larvae probably represents an average figure, based on a mixed population of growing, differentiating and cells undergoing programmed cell death with differing mitotic rates under in vivo conditions, over the exposure periods described. It is therefore difficult to ascertain the total number of cells (and cellular divisions) representing a 12–20-h-old embryo–larvae. The number of complete metaphases with convenient chromosomal morphology also varied from larvae to larvae, typically in the range of 0–5. Both the direct-acting alkylating agent, MMS and indirect acting (compounds requiring metabolic activation) B[a]P were observed to induce significant dose-responses in terms of both SCEs and CAbs. Although a significant dose-response relationship for MMS-induced SCEs was observed ($R^2 = 0.769$), compared to B[a]P, it was not very cogent (Fig. 4). Probably a different concentration range of MMS would have given a better picture of dose-effect relationships. The activation of B[a]P into active metabolite capable of inducing cytogenetic damage, in particular, is interesting, bearing in mind the relevance of PAHs contamination of the marine environment due to increasing oil spills and leaks. While there is evidence that adult mussels and embryos of fish possess an active cytochrome P-450 enzyme system [29] there is a paucity of information for the embryo–larval stages of these organisms to transform inactive PAHs into active xenobiotics. Earlier cytogenetic studies have, however indicated that the embryo–larval stage of this organism is capable of activating cyclophosphamide, a pro-mutagen via the cytochrome P-450 pathway [11]. Cytogenetic studies on the marine worms and sea urchins, have also provided indirect evidence that the embryo–larvae of marine invertebrates are capable of transforming promutagens, cyclophosphamide and B[a]P [19,30], supporting the present observation. Representative SCEs data from this and other reported marine invertebrate studies are summarised in Table 4, which suggests that embryo–larvae of *M. edulis* are comparatively sensitive to the reference chemicals under our experimental conditions.

The present validation study, differs from earlier studies carried out on the embryo–larvae of *M. edulis* [10–12] in several ways. Firstly, bearing in
Table 4
Comparative sensitivity of reference mutagens and contaminants for the induction of SCEs/ccII in the embryo-larval stages of different marine invertebrates. Concentration of named mutagens/contaminants in parenthesis (NR: not reported)

<table>
<thead>
<tr>
<th>Reference mutagen/contaminant</th>
<th>Species and number of SCEs/ccII</th>
<th>Concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMS</td>
<td>M. edulis*</td>
<td>31 (1.0 × 10^-3)</td>
</tr>
<tr>
<td></td>
<td>M. edulis*</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>P. dumerili*</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>N. arenaceodentata*</td>
<td>NR</td>
</tr>
<tr>
<td>B(a)P</td>
<td>M. edulis*</td>
<td>18 (1.0 × 10^-3)</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>M. edulis*</td>
<td>6 (1.6 g/l)</td>
</tr>
<tr>
<td></td>
<td>P. dumerili*</td>
<td>1.9 (1.0 × 10^-4)</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>M. edulis*</td>
<td>20 (5.0 × 10^-6)</td>
</tr>
<tr>
<td></td>
<td>P. dumerili*</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>40 (6.0 × 10^-6)</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>15 (1.0 × 10^-3)</td>
</tr>
<tr>
<td></td>
<td>Sediment sample</td>
<td>7.0 (1.0 × 10^-3)</td>
</tr>
</tbody>
</table>

*Present study.
1Harrison and Jones [10].
2Jha et al. [19].
3Pesch et al. [44].
4Calculation based on SCE/chromosome (2n = 28).

mind that the induction of lesions into cellular DNA and its subsequent conversion into cytogenetic damage is a cell cycle dependent phenomenon, we exposed the growing cells only for a required period of time (either two (8 h) or one and half cell cycle time (6 h) for SCEs and CAbs analyses, respectively), whereas the earlier studies, without taking into consideration the cell cycle factor, exposed the cells on a flat rate basis for 12 h to analyse only the induction of SCEs. Secondly, the methodology adopted to prepare metaphase spreads in this study facilitated the analysis of complete metaphases rather than individual chromosomes. The difficulties in obtaining complete metaphases from embryo–larvae of mussels have been acknowledged by earlier workers [10,12] which required them to analyse the induction of SCEs on per chromosome rather than per cell basis. Our observations are, however, based on the analyses of per cell or overall genome basis. Thirdly, in view of the ongoing debate over the biological significance of SCEs due to lack of direct association between their induction and an adverse cellular and health outcome leading to limited value in risk assessment [15,16], we also analysed the induction of CAbs from metaphase spreads, which is considered to be the most robust end point due to its known biological significance [15,16].

4.2. Physico-chemical and chemical characteristics of the environmental samples

It is known that the anoxic nature of sediments, with a large negative redox potential, as observed in this study can lead to the reduction of sulphate via a sulphite to sulphide. This reduction process, associated with diagenesis in the sediments, will produce, if the pH is correct, a sulphide smell in the harbour. The breakdown of organic matter including the detritus from human and animal material found in the harbour area, prior to mineralisation, would have further added to the cocktail of degradation products. The pH range of the sediments varied between neutrality and slight acidity and this may in part be due to the presence of organic acids resulting from breakdown of the organic matter. Sediments with the smallest grain size and highest organic content had the highest contamination levels, supporting several earlier observations [31].

Most of the contaminants originating from anthropogenic activities close by are eventually deposited in the sediments. A common feature of contaminated sediments in urban harbours, in different parts of the world is the presence of UCM of both lighter and heavier organic species, as observed in the GC analyses of our sediment samples [32,33]. While the lighter UCM could well be the result of contamination of light fuel oils, in this case most probably diesel, the heavier components of the UCM are likely to originate from a different source, probably from a heavier oil, such as lubricating oil. The potential source of such contamination includes recreational and commercial boat traffic. The aromatic sub-samples, which contained a variety of PAHs including low molecular species such as phenanthrenes, fluoranthenes and naphthalenes, are
again indicative of diesel fuel pollution. The relatively high concentrations present in all the samples of a complex plasticizer requires further investigation.

Sediments are an important storage compartment for metals released into surface waters. Furthermore, because sediments act as sinks for these metals, they reflect water quality and record anthropogenic emissions. It was necessary to determine the extent of possible metal contamination in the harbour. It is noted that the application of anti-fouling paints could be a possible source for some of these metals present in elevated levels in the harbour. In addition to organo-tin compounds, Cu and Zn compounds have also been widely used as anti-fouling agents. The leaching action of SW on the anti-fouling painted surface is known to release the biocides into the water column, and thence to sediments, where they could be detrimental to both the target and non-target organisms [34]. Such processes would have the most impact in areas where large numbers of vessels are gathered. Historic metalloferrous mining activities in the south-west England, has led to very high levels of As in some soils. However, there are several anthropogenic sources (e.g. agricultural, livestock, medicine, electronics etc.) by which arsenicals can be deposited into the sediments [35]. Overall, taking into consideration the elevated levels of metals in this area and present sediment quality criteria in Europe and/or in the USA [36,37], the study showed that Brixham harbour sediment had suffered elevated levels of contaminants.

4.3 Developmental, cellular and genotoxicity of the environmental sample

Although analytical chemistry tests have been useful in providing an estimate of the degree of contamination, the fraction of the total contamination available to the organisms can only be determined with biological assays. Sediment toxicity to aquatic biota, in particular, the bivalve embryo bioassay, have been widely adapted to deal with sediment elutriates as an useful biological tool for marine environmental quality assessment [38,39]. The developmental toxicity results in our study confirmed the sensitive measure of toxicant response in the embryo–larvae of *M. edulis*. This also facilitated the determination of MTD for genotoxic evaluation of the sediment samples. The developmental and cytotoxicity results complemented the genotoxic response at lower levels of biological organisation, as more sensitive and robust end points. Needless to mention, cytogenetic assays, based on metaphase analyses, provided a specific mechanism of effect at DNA level, which would have direct implications for the developmental, and subsequently reproductive success of the exposed organism. Bearing in mind the challenges and difficulties of evaluating the population or community level consequences [2] the individual level consequences in terms of developmental and survival effects following toxicant exposure could be taken as a sensitive parameter in achieving the goals of eco-genotoxicology.

Identification of causative agent(s) responsible for toxic effects is a common problem in the assessment of complex environmental samples. Many of these agents, particularly the PAHs and heavy metals are known to have genotoxic potential. Although some of these metals (e.g. Zn and Cu) are essential for life and could occur in high concentrations in the marine environment, many of them (e.g. Ni, As, Cd) affect DNA either by generating reactive oxygen species (ROS) or by directly binding to DNA and/or the repair enzymes. These metals therefore not only influence the induction of S-phase dependent and independent genetic lesions but are also known to potentiate the lesions induced by other physical and chemical agents [40,41]. The concentration of total metals in the sediments however could not be considered as a reliable indicator of biological effects since it does not take metal bioavailability into account [42]. Even though the relationship between toxicity and metal accumulation is not always constant, body concentrations of the metals would probably have been more reliable predictors of toxicity. Due to the small size and quantity of the embryo–larvae used in our study, it was not technically feasible to use them for analytical studies. However, when SW spiked with sediments samples were analysed for two metals: As, a known genotoxic agent [40,41] and Cu, a non-genotoxic agent, it was found that irrespective of the concentrations of these metals in the sediments samples, the rate of leaching (As vs. Cu) was different. When comparing the arsenic concentration in the sediments and the control sample,
Table 5
Chemical analyses of the SW samples spiked with sediment samples

<table>
<thead>
<tr>
<th>Treatments</th>
<th>As (µg/l)</th>
<th>Cu (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW control</td>
<td>&lt; 5</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Control sediment</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>1.6-g sediment</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>0.5-g sediment</td>
<td>10</td>
<td>23</td>
</tr>
</tbody>
</table>

there was little difference seen from their leaching capabilities as shown in Table 5. When the Cu concentration in the sediments and control samples were compared, the levels were near double that of As (Table 3). This suggested that irrespective of the concentrations of the contaminants in the sediment sample, their presence in the water column and therefore bioavailability to the pelagic embryo-larval stage could be different. Various factors, including speciation have been attributed to influence the bioavailability of metals. Our results however supported the presence of bioavailable substances in the sediment samples which proved to be highly toxic to the early life stages of marine organisms under laboratory conditions. The impact of sediment toxicity on invertebrates, leading to altered growth and survival in laboratory bioassays also suggests that such toxic exposures in the natural conditions could not only be relevant to benthic or intertidal invertebrates but can be indicative of effects further up in the food chain.

5. Conclusions

In this study, we made an attempt to further validate an in vivo test system using sensitive life stages of an ecologically relevant organism by overcoming some of the earlier limitations. Using this validated test system we also attempted to link different levels of organisation while elucidating cause-effect coupling. Collection and analyses of environmental samples from a moderately contaminated site in this study also emphasised the need for developing discriminating procedures for sampling and investigating the effects of contamination on the health of organisms from pristine and polluted sites. The study led us to conclude that properly validated in vivo systems could be as sensitive as chemical systems to evaluate the toxic and genotoxic potential of complex environmental samples. While such laboratory-based bioassays have certain inherent limitations, in contrast to mere chemical analyses, they at least provide the potential integral effects of complex environmental samples on the ecosystem.

Our study also emphasises the need for adequately assessing the short and long-term impacts of dredge disposal. At present, toxicity testing is not required for the licensing of off-shore disposal of dredge material in different parts of the world, including USA [8,43]. Environmental Quality Standards (EQS) for dredged material, which aims to protect the receiving environment against negative impacts are based on an ecotoxicological evaluation of only a limited number of compounds (e.g. metals, PAHs, PCBs, etc.). In an industrial environment, a suite of potentially toxic agents, in low or undetectable concentrations, may be present that are not included in the EQS. As shown in our study, disposal of such contaminated sediments in huge quantities can cause significant effects to the short and long-term survival of the biota. The study therefore explains the importance of the application of bioassays in the overall assessment. In addition, there is also a need to understand the predictability of chronic effects from acute bioassays at different levels of biological complexity which would be important in assessing the long-term effects on the health of populations or the integrity of the ecosystem.

Acknowledgements

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[33] M.C. Kenny, T.J. McDonald, G.J. Deaou, S.J. McDonald, Hydrocarbon contamination on the Antarctic Penin-


Fitness Parameters and DNA Effects Are Sensitive Indicators of Copper-Induced Toxicity in *Daphnia magna*

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This study compared the effects occurring at molecular and population levels in *Daphnia magna* exposed to copper concentrations in the range of 15–120 μg/L. The qualitative and quantitative modifications arising in random amplified polymorphic DNA (RAPD) profiles as a measure of DNA effects were compared with a number of key ecological fitness parameters, namely, the age-specific survival, age-specific fecundity, net reproductive rate, and intrinsic rate of population increase. Results suggested that growth, reproduction, and most of the fitness parameters as well as genomic template stability (a qualitative measure reflecting changes in RAPD profiles) were significantly affected at copper concentrations of 90 and 120 μg/L. Among the fitness parameters, the age-specific fecundity and net reproductive rate were the most sensitive parameters of toxicity. Changes in RAPD patterns generally occurred at copper concentrations of 90 and 120 μg/L, but with one primer, changes significantly arose at all copper concentrations. Overall, molecular and population parameters compared well and represented a sensitive means to measure toxicity induced by copper in *Daphnia magna*. In conclusion, the measurement of parameters at both molecular and population levels is valuable for investigating the specific effects of agents interacting with DNA. Ultimately, this methodology may allow the ecotoxicological examination of the link between molecular alterations and measurable adverse effects at higher levels of biological organization.

**Key Words:** copper; RAPD; DNA effects; genomic template stability; fitness parameters; *Daphnia magna*.

Although the detection of DNA damage has been widely studied using a number of laboratory methods, to the best of our knowledge, the study of DNA lesions and their consequences at higher levels of biological organization has seldom been attempted (Anderson and Wild, 1994; Jha, 1998). Not only can genotoxins shorten the life expectancy of organisms, but they can also result in alterations in population dynamics. Thus, they can have an effect at both intra- and inter-species biodiversity (Anderson and Wild, 1994; Depledge, 1998; Würgler and Kramers, 1992). Such changes may initiate direct and adverse ecological consequences. In this context, research is needed to improve our understanding of the consequences of DNA damage and mutations at the population level. While neoplasia in invertebrate species is considered to be low in comparison with tumor incidences reported in fish (Couch and Harshbarger, 1985), it is not to say that genotoxic agents are without effects. Kurelec (1993) suggested that, in invertebrates, genetic damage is manifested as a suite of pathological changes, the so-called "genotoxic disease syndrome." In this context, the effect of genotoxins on growth, fecundity, and mortality are particularly relevant.

In this study, the effects of copper were evaluated at the population and molecular levels in an invertebrate species. Copper is essential to living systems, in part through its fundamental role in electron transport, respiration, growth, and development (Linder, 1991). This essential element is associated with key enzymes and/or proteins, such as superoxide dismutase (Atienzar et al., 1998; Fridovich, 1995), metallothioneins (Hamer, 1986), and cytochrome c oxidase (Steffens et al., 1987) which are well represented in invertebrates (Linder, 1991). In vertebrate cells, copper has been implicated in the stabilization of chromosomes and it may be involved in transcription and/or specific transcription-stimulatory events affected by hormones (Linder, 1991). However, numerous studies have also reported that copper induces toxicity (Conradi and Depledge, 1998; Koivisto and Ketola, 1995) including genotoxicity (Eichorn and Shin, 1968; Lloyd and Phillips, 1999). For instance, the binding of copper to DNA bases unwinds the double-helix (Eichorn and Shin, 1968) and DNA damage can be generated. Lloyd and Phillips (1999) reported that the binding of copper to DNA is necessary for the generation of double-strand breaks, 8-hydroxydeoxyguanosine, and putative intrastrand cross-links in Fenton reactions. The generation of genomic DNA profiles, using randomly primed polymerase chain reaction (PCR) protocols such as the arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990) and random amplified polymorphic DNA (RAPD) (Williams et al., 1990) has proved valuable in many areas of biomedical research. RAPD and AP-PCR are powerful tools for gene mapping, population, pedigree analysis, phylogenetic studies, and strain identification (Grayson et al., 2000; Liu et al., 1999; Tinker et al., 1993). In addition, their use in survey-
ing genomic DNA for evidence of various types of damage and mutation suggest that they may potentially form the basis of novel genotoxicological assays for the detection of DNA damage and mutations (Atienzar, 2000; Atienzar et al., 1999, 2000a; Becerill et al., 1999; Ionov et al., 1993; Kohno et al., 1994; Kubota et al., 1992, 1995; Lopez et al., 1999; Peinado et al., 1999; Savva, 1998; Shimada and Shima, 1998). Despite earlier criticism of the RAPD assay (Ellsworth et al., 1993; Khanda et al., 1997), subsequent reports have demonstrated that after rigorous optimization of the PCR parameters, the assay performs well, in terms of number of amplified bands, product yield, and clarity of the profiles, with a wide range of organisms including bacteria, plants, as well as invertebrate and vertebrate animals (Atienzar et al., 2000b).

In the context of the information provided above, the objectives of this study have been to determine whether the RAPD assay could detect copper-induced DNA effects in the fresh water flea, *Daphnia magna*, under in vivo conditions, to measure fitness and Darwinian parameters in this key species for ecotoxicological studies, and to assess how these endpoints (i.e., changes in RAPD profile and fitness parameters) compare in terms of detection of toxicity.

**MATERIALS AND METHODS**

**Culture of Daphnia magna.** *Daphnia magna* (D. magna, clone 5) were maintained in Elendt's medium (Elendt and Bieio, 1990), at a temperature of 20 ± 2°C with a photoperiod of 16 h light (1000 lux):8 h dark. Animals were fed with the alga *Chlorella vulgaris* (1.2-2.4 × 10³ cells per daphnid per day) and a booster solution of Frhipack microencapsulated food (Salt Lake Brine Shrimp, Granville, UT) as described in Atienzar et al. (1999).

**Preparation of test solutions.** Test solutions for acute and chronic exposures were prepared from stock solutions of copper (in the form of CuCl₂, Sigma, Poole, UK) 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 liter of M7 medium).

**Toxicity tests.** The acute toxicity of copper 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 copper equivalent to 10, 25, 62.5, 156.25, or 390.62 μg/L. Animals were fed (see culture of the organism) during the test, and surviving animals were counted after 48 h of LC₅₀ and its 95% confidence level.

The chronic toxicity of copper to *D. magna* was assessed under the same experimental conditions as for the acute toxicity tests. Animals were exposed to copper concentrations of 15, 30, 60, 90, or 120 μg/L for 15 days. Surviving animals were counted on days 1, 3, 5, 8, 10, 12, and 15. Mortibund, non-swimming animals were removed from the culture at regular intervals on and between counting days. Animals were stored at -80°C prior to DNA extraction and RAPD profiling. As M7 medium contains 2 μg/mL copper, all concentrations previously mentioned in acute and chronic toxicity tests can be corrected by dividing 2 to each of the values. However, as this is negligible, non-rectified values have been used throughout the paper.

**Growth and reproductive measurements.** The length of every *D. magna* (except for the experiment [day 1] where 20 *D. magna* were measured) surviving after 48 h at days 1, 3, 5, 8, 10, 12, and 15 was measured by deo capture and image analysis using a Quantimet 570 image analyzer (Cambridge Instruments, Cambridge, UK). Neonates were counted and recovered at daily intervals. The length of newborn neonates (generally 10 animals per replicate) released at first generation was also determined.

**Calculation of fitness parameters.** The intrinsic rate of natural increase of the *D. magna* population, *r₉*, was calculated using Lotka's equation:

\[ r₉ = \frac{m}{1 - \frac{m}{S_m}} \]

where *m* is the age-specific survival (number of living females on day *x*/number of females at start of life table); *S_m* is the age-specific fecundity (number of newborn individuals produced on day *x*/number of living females and 0.50 of age); *Rₚ* is the net reproductive rate (*Rₚ*) can be calculated using the following formula:

\[ Rₚ = \sum_{x=1}^{∞} m_x + \sum_{x=1}^{∞} U_x \]  

where *U_x* is the realized fecundity. Minimum generation time (*Tₘᵢₜ*) and inter-brood time (*Bᵢ*) represent the time between birth and the deposition of the first batch of offspring, and the time between clusters or broods, respectively.

**Generation of D. magna DNA profiles using RAPD.** Total DNA from *D. magna* was extracted and purified using a conventional phenol/chloroform method as described in Atienzar et al. (1999). The extracted DNA was electrophoresed on 1.2% (w/v) agarose gels using a Tris-Borate-EDTA (TBE) buffer system (1X TBE = 90 mM Tris-base, 90 mM boric acid, and 2 mM EDTA) at 100 V for 2 h. The DNA concentration of each sample was determined by comparison with known concentrations of Lambda phage DNA (Sigma, Poole, UK). DNA profiles of *D. magna* were generated in RAPD reactions performed in a reaction volume of 25 μL containing 2 μM 10-mer primer (OPA9, OPB1, OPB5, OPB6, OPB7, OPB8, OPB10, OPB11, OPB12, OPB14, or OPB17, Operon Technologies, Southampstead, UK; sequences for each primer can be obtained from the website at http://www.operon.com) 0.33 mM dNTP, 5.11 mM MgCl₂, 20 ng DNA, 2.8 units of *Thermus aquaticus* DNA polymerase and 1X reaction buffer (10 mM Tris-Cl, pH 8.8, 50 mM KCl, 0.08% Nonidet P40), and 2.5 μg bovine serum albumin. These primers were used because they allowed satisfactory RAPD profiles to be obtained with different species belonging to the bacterial, vegetal, and animal kingdoms and in particular with *D. magna* (Atienzar et al., 2000b). Thermal cycling parameters consisted of a 5- min denaturation (95°C) followed by 40 cycles of 1-min denaturation (95°C), 1-min annealing at 50°C, and a 1-min extension (74°C) (with the final extension period adjusted to 10 min). All PCR chemicals were from Immunogenon International (Sunderland, UK) except when otherwise mentioned.

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 cyanol, Sigma, Poole, UK) and 12-15 μL of this solution was loaded onto the agarose gel. PCR products were electrophoresed on 1.2% agarose gel using a TBE system buffer (see above) at 90 V for 6 h, stained with ethidium bromide (0.015% (w/v)), visualized under UV light, and photographed using a Polaroid CO5 camera system (Eastman Kodak, New York). The image of the gel was saved electronically for further analysis when necessary. The intensity of selected bands was determined using the Kodak Digital Science™ 1 D (Eastman Kodak, New York). For comparison, DNA molecular size marker (GeneRuler™ 100bp DNA ladder plus, Immunogenon International, Sunderland, UK) was used. Bands visualized were from top to bottom: 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp.

**Estimation of genomic template stability and transformation of the data.** Each obvious change observed in RAPD patterns (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 5 primers (OPA9, OPB7, OPB6, OPB10, and OPB17, representing approximately 50% of the primer used) which showed clear changes in RAPD. Primers which did not produce changes in RAPD profiles or which were too difficult to score were not used to calculate the genomic template stability (GTS). GTS (%) was calculated by the formula 100 - (100*n/a), where *a* is the number of bands detected in control DNA profiles and *n* the average number of changes in DNA profiles. To compare the sensitivity of each parameter (GTS, *r₉*, and *Rₚ*), changes in these values were calculated as a percentage of their control value (set to 100%).

**Statistical analyses.** The 48 h LC₅₀ value and the 95% confidence limit were calculated using the Logit method and the package software SPSS 6.1 for
Changes in Growth, Reproduction and Mortality

Demographic trends for the populations of *D. magna* exposed to differing concentrations of copper are shown in Table 1. In the range 15–120 μg/l copper, consistent reductions occurred in the maximum number of eggs per female and maximum body size. In contrast, the longevity and the number of broods were only affected at 120 μg/l of copper. *D. magna* exposed to the highest copper concentration could not survive for more than 10 days whereas animals from the other groups survived throughout the experiment (Table 1, Fig. 1A). The mean period for *D. magna* to become ovigerous was identical among groups, and animals exposed to gradual increases in copper concentrations displayed the same lime between broods except for the animals exposed to 120 μg/l copper (Table 1). The populations growing at 120 and 90 μg/l copper had a smaller body size than controls (*p < 0.001*), and there were no significant differences between control and other copper-exposed groups (Fig. 1B). In addition, the sizes of sub-24-h juveniles at days 8 and 10 were similar among all groups (*p < 0.01*, data not shown). Finally, the total number of offspring was significantly reduced at 90 and 120 μg/l copper (*p < 0.01*; Fig. 1C, Table 1) compared to the control.

**RAPD Profiling**

DNA amplified was extracted from healthy organisms aged 15 days from all groups except for *D. magna* exposed to 120 μg/l copper. Genetic material obtained from those latter animals was carried out on weak but living animals aged 10 days. 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 2 were generated using 4 primers and a mixture of 4 individuals from each replicate. Profiles generated by these primers revealed 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 (Fig. 2) show some of the obvious modifications. Although some changes in RAPD profiles arose...
ATIENZAR ET AL.

A) to II

TInit (Dap)

1000

IKO;

100

Fifteen and 30 μg/l copper (e.g. bands 9-2, 7-3 [increase in band intensity], 9-4 [disappearance of bands]), most of the modifications occurred in patterns of D. magna exposed to 60, 90, and 120 μg/l copper. Extra bands generally appeared for some or all of the 3 highest 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 in the patterns generated by the animals exposed to the 2 lowest copper concentrations. In contrast, every type of modification was well represented in the patterns produced by D. magna exposed to the 3 highest copper concentrations. Figure 3 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 μg/l copper, respectively, compared to control intensities (curve b). In contrast, the average intensity of the remaining bands (40%) increased at all copper concentrations (curve a). Finally, some of the modifications (e.g. bands 14-1, 14-2, and 14-3) generated by a mixture of D. magna (Fig. 2D) generally followed the same tendency than those obtained from single individuals in both replicates (data not shown). For instance, the intensity of band 14-1 was generally brighter at 120 μg/l copper, whereas the intensity of band 14-2 progressively decreased in the range 60–120 μg/l copper. Moreover, band 14-3 appeared in profiles generated by the D. magna exposed to 90 and 120 μg/l copper.

Comparison of Fitness Parameters with Genomic Template Stability

To compare the sensitivity of the fitness parameters presented in Table 1, changes in each factor were calculated as a percentage of their control value (set to 100%). All the parameters presented in Table 1 were measured throughout the experiment except for the population of D. magna exposed to 120 μg/l copper, which were carried out for 10 days. Changes in RAPD profiles were expressed as reductions in GTS (a qualitative measure reflecting the obvious changes to the number and intensity of DNA bands in DNA patterns generated by toxicant-exposed D. magna) in relation to profiles obtained from control animals. GTS calculated with 5 primers (OPA9, OPB7, OPB8, OPB10, and OPB17) is presented in Table 2. As already mentioned, primers that did not produce any changes in RAPD profiles or that were too difficult to score were not used to calculate the GTS. A comparison between GTS and fitness parameters is presented in Figure 4. Results reveal that all parameters measured were significantly different at 90 and 120 μg/l copper (p < 0.05) except Ir, which was only significantly different at the highest copper concentration. The general tendency of all measured parameters was a progressive decrease with rising copper concentration, except for the age-specific fecundity which was significantly higher than control at 15 μg/l copper (p < 0.05). Overall among fitness parameters, m, and Rr were the most sensitive parameters. Although GTS calculated with 5 primers was only significantly different at 90 and 120 μg/l copper, GTS calculated from single 10-mer primer OPA9 was significantly different from control at all copper concentrations (p < 0.05).
MOLECULAR AND POPULATION EFFECTS IN COPPER EXPOSED DAPHNIA

DISCUSSION

Despite growing concern over the presence of genotoxins in the aquatic environment, there is a lack of knowledge pertaining to the potential consequences of DNA damage and mutations at population level in the aquatic biota. In this study, changes in RAPD profiles that reflect DNA effects were compared to fitness parameters in *D. magna* exposed to copper concentrations in the range 15-120 μg/l. DNA effects include DNA damage as well as mutations and possibly other effects (see below) at the DNA level, which can be induced by chemical or physical agents that directly and/or indirectly interact with genomic DNA. Parameters measured at molecular (GTS) and population (growth, reproduction, fitness parameters) levels were mainly affected at 90 and 120 μg/l copper.

Among the fitness parameters, the age-specific fecundity and net reproductive rate were the most sensitive parameters of toxicity as they were also significantly different at 15 and 60 μg/l copper. In contrast, age-specific survival was only significantly different at the highest copper concentration, suggesting that mortality is not a sensitive parameter. Although GTS calculated with 5 primers was only significantly affected at 90 and 120 μg/l copper, it is important to mention that one primer significantly detected DNA effects at all copper concentrations tested. Thus, the RAPD assay can be particularly sensitive. Overall, molecular and population parameters represented a sensitive means to measure toxicity induced by copper in *D. magna*. Similarly, Sadinski et al. (1995) reported that DNA adducts and micronuclei can be sensitive measures of sublethal DNA damage, as well as possible short-term indicators of
FIG. 3. Variation in band intensities selected from RAPD profiles of *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 their own control value (set to 100%). As band intensity followed 2 different patterns, average and standard deviation were calculated with 11 (squares, curve a) and 17 (triangle, curve b) bands.

indirect effects on fitness in amphibians exposed to benzo(a)pyrene [B(a)P]. Atienzar et al. (1999) reported that B(a)P-induced DNA effects also compared favorably with the traditional indices of fitness in *Daphnia magna*. Studies have also shown that cytogenetic damage correlates with development and survival of early life stages of marine invertebrates, which could have detrimental consequences at the higher levels of biological organization (Jha et al., 2000).

The measure of molecular and population parameters present several advantages. First of all, in ecotoxicology, it is fundamental to accumulate data at different levels of biological organization in order to fully understand the effect of a toxicant in organisms. Secondly, the measure of some parameters at the population level 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. In the present study, it seems that for the 2 highest copper concentrations, DNA replication was significantly reduced due to a higher level of DNA damage. On the other hand, as growth and reproduction displayed similar values compared to the control in the range 15–60 μg/l copper, it could be assumed that DNA lesions were efficiently repaired and that DNA replication was not significantly inhibited.

With respect to copper toxicity, it has been reported that lethal effects on *Daphnia* occurred at 10 μg/l copper (Baird et al., 1991). In another study, Winner and Farell (1976) revealed that copper toxicity was increased at concentrations beyond 40 μg/l. However, the great variability among different studies makes direct comparisons difficult, owing to differences in medium quality, amounts of food, and the use of different clones of the same species (Koivisto and Ketola, 1995; Meador, 1991; Soares, 1992). In the present study, 15 μg/l copper stimulated reproduction, since age-specific fecundity was significantly higher than control, as previously reported in other studies (Dave, 1984). However, the total number of neonates generated by the population of *D. magna* exposed to 0 and 15 μg/l copper were statistically identical, because the number of surviving animals decreased at 15 μg/l copper in comparison to the control and other groups (except for animals exposed to 120 μg/l copper). Thus, despite the significant increase in reproduction (m*) at 15 μg/l copper in comparison to the control, overall there was no beneficial effect in *D. magna* exposed to the lower copper concentration. The stimulation in reproduction can be explained by hormesis, an unspecific stimulation by a chemical or physical agent (Luckey, 1975).

<table>
<thead>
<tr>
<th>Cu (μg/l)</th>
<th>OPA9</th>
<th>OPB7</th>
<th>OPB8</th>
<th>OPB10</th>
<th>OPB17</th>
<th>Mean</th>
<th>SD</th>
</tr>
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TABLE 2
Genomic Template Stability Calculated with Five 10-mer Primers
decrease in growth following sublethal copper exposure has also been demonstrated in various species of crustaceans (e.g., Conradi and Depledge, 1998). By attaining reproductive maturity at a smaller body size, D. magna are able to buffer the impact of lower body growth rate on the age at the first reproduction (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 (Enserink et al., 1993) but was due to a direct toxic effect of copper.

The second objective of the study was to evaluate the potential of the RAPD method to detect copper-mediated DNA effects in D. magna. Recently, the RAPD technique has been successfully used to detect DNA effects induced by benzo(a)pyrene (Atienzar et al., 1999), mitomycin C (Becerril et al., 1999), ultraviolet radiation (Atienzar et al., 2000a), and 17β-estradiol (estrogen)/4-n-nonylphenol (xenoesrogen) (Atienzar, 2000; manuscript submitted) in aquatic species under in vitro and in vivo conditions. In this study, D. magna were exposed to copper because it is known to induce a range of DNA damage such as single- and double-strand breaks, modified bases, abasic sites, DNA-protein cross-links, and even bulky adducts representing intrastrand dimerization of adjacent purine bases (dimers) (Carmichael et al., 1992; Lloyd and Phillips, 1999). Copper, as with other transition metals, catalyses the Fenton type reduction of hydrogen peroxide to form hydroxyl radical, one of the most reactive radical oxygen species (Drouin et al., 1996).

RAPD profiles detect alterations to genomic DNA through the use of arbitrarily primed PCR reactions. These effects include changes in oligonucleotide priming sites and variations in the activity of the Taq DNA polymerase. In the present study, variation in band intensity, disappearance of bands, and appearance of new PCR products occurred in profiles generated from the exposed organisms. Copper-induced DNA damage, e.g., Cu(I)-DNA complex, bulky adducts, oxidized bases, may significantly interfere with the PCR events. 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 (Pritz et al., 1990). In addition, dimers can alter the structure of the DNA (Hanawalt, 1998; Wang et al., 1993). If so, such structural changes are likely to have a significant effect on the kinetics of PCR events. New PCR products can be amplified because
some sites become accessible to the primers after structural change or because the same mutations have occurred in the genome. A loss of an amplicon can only be the outcome if the same structural changes occur in 75–90% of the cells or if the same mutations arise in the same percentage of cells (Atienzar, 2000). However, it is very unlikely that mutations occur in a large portion of cells, because most of the DNA damage (which can lead to mutations during DNA replication) will be efficiently repaired. DNA lesions such as bulky adducts are expected to have detrimental effects on RAPD profiles. Not only can they induce structural changes, but they can also reduce the polymerization of the DNA and/or block the Taq DNA polymerase (Nelson et al., 1996), which will result in a decrease in band-intensity, or alternatively, in a disappearance of amplified products in the case of extensive DNA damage (Atienzar, 2000). As already mentioned, new PCR products may reveal a change in the DNA sequence due to point mutations and/or large rearrangements. A single point mutation within the primer site can generate significant changes in RAPD patterns (Williams et al., 1990; Atienzar, 2000). Nevertheless, the RAPD assay also has the potential to detect point mutations outside the priming site, as they can induce structural changes (Bowditch et al., 1993). It is also well known that DNA repair and replication of damaged DNA can lead to point mutations (Livneh et al., 1993). Due to the rapid growth of D. magna (Fig. 1B) and the presence of DNA damage, it is very likely that such mutations occurred after replication. Our preliminary results suggest that 10–25% of the cells need to be affected by mutation and/or DNA damage to allow a visible change in the RAPD profile (Atienzar, 2000). As “hot spot” interactions between DNA and copper have been reported in the literature (Pruitz et al., 1990; Rodriguez et al., 1995), it is likely that hot spot mutations are generated following DNA replication.

Although DNA damage and mutations induced by genotoxins are likely to be the main factors affecting RAPD profiles, our preliminary results suggest that other factors may also contribute to changes in patterns but probably to a lesser extent (manuscript submitted; Atienzar, 2000). These factors could be responsible for some of the changes occurring in control RAPD patterns (see, for instance, Figs. 2A and 2D). Numerous studies have shown that gene expression correlates with a change in DNA structure (Kohwi and Kohwi-Shigematsu, 1991; Wolfe and Hayes, 1999), which in turn may induce changes in RAPD profiles. Other parameters such as genomic amplification and rearrangement, which are components of normal cellular development, may also influence RAPD profiles. There is also a possibility that DNA damage (e.g., 8-hydroxyguanine) in control animals could conceivably be induced as a result of the normal metabolic processes that maintain steady-state levels of genetic damage (Hanawalt, 1998) and may therefore contribute to differential RAPD profiles. In addition, cell physiology, which is known to have a direct influence on DNA (Oshimura and Barrett, 1986), may also have an effect on RAPD profiles. Therefore, the changes that occur in control RAPD patterns could be explained by a change in one or a combination of the factors previously mentioned between both replicates.

The RAPD technique clearly shows promise in the detection of pollutant-induced DNA effects. The main advantages of the RAPD method lie in its rapidity, applicability to any organism (since no information on the nucleotide sequence, cell cycle, or chromosome complement is required), and sensitivity to detect a wide range of DNA damage and mutations. However, RAPD is a qualitative method and the nature and amount of DNA effects can only be speculated. Thus, in the context of the evaluation of pollutant-induced DNA effects, a powerful approach would be to perform the RAPD method to obtain qualitative data that would give an overview of the DNA effects, and then to use more specific methods such as the 3P-postlabeling, DNA strand break measurements (comet assay), or cytogenetic assays to generate quantitative data.

In conclusion, fitness parameters and DNA effects are sensitive markers to detect copper-induced toxicity in D. magna. Thus, the RAPD assay to detect DNA effects in conjunction with other biomarkers from higher levels of biological organization would prove a powerful ecotoxicological tool.

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MOLGENE AND POPULATION EFFECTS IN COPPER EXPOSED DAPHNIA


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