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University of Plymouth

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Integrated biomarker and molecular responses in marine bivalve following exposure to environmental contaminants: Implications for human and environmental health

by

Yanan Di

A thesis submitted to the Plymouth University in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biomedical and Biological Sciences
Faculty of Science
In collaboration with Marine Biology Association (UK)

December 2012
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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

This study was financed with the aid of a studentship from the Overseas Research Students Awards Scheme (ORSAS) and carried out in collaboration with Marine Biology Association (MBA, UK) and Plymouth Marine Laboratory.

Relevant scientific seminars and conferences were regularly attended at with work was often presented; external institutions were visited for consultation purposes and several papers prepared for publication.

Presentation and Conferences attended were in Appendix V.

Publications (or presentation of other forms of creative and performing working) attached in Appendix VI

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<tbody>
<tr>
<td>18S</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>ADG</td>
<td>Adipogranular</td>
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<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
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<tr>
<td>ASPP</td>
<td>Apoptosis stimulating protein of P53</td>
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<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
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<tr>
<td>ATR</td>
<td>ATM-related factor</td>
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<td>B(α)P</td>
<td>Benzo(α)pyrene</td>
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<td>BaA</td>
<td>Benz(α)anthracene</td>
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<tr>
<td>EDCs</td>
<td>Endocrine Disrupting Chemicals</td>
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<td>EFTEM</td>
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<td>Epoxide hydrolase</td>
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<td>ENPs</td>
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<td>ERICA</td>
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<td>ESCODD</td>
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<td>HT</td>
<td>Tritiated hydrogen gas</td>
</tr>
<tr>
<td>HTO</td>
<td>Tritiated water</td>
</tr>
<tr>
<td>IAEA</td>
<td>International Atomic Energy Agency</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial Methylated Spirit</td>
</tr>
<tr>
<td>IRs</td>
<td>Ionising radiations</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LMPA</td>
<td>Low melting point agarose</td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MC</td>
<td>Methylchrysene</td>
</tr>
<tr>
<td>MCDE</td>
<td>Methylchrysene diol epoxide</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute clone 2</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse Embryo fibroblast</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCB</td>
<td>Nanosized carbon black</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>NMA</td>
<td>Normal melting agarose</td>
</tr>
<tr>
<td>NPP</td>
<td>Nuclear power plant</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>OBT</td>
<td>Organically bound tritium</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for Economic Cooperation and Development</td>
</tr>
<tr>
<td>p21/WAF1</td>
<td>Cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>P38</td>
<td>P38-MAPK</td>
</tr>
<tr>
<td>PAHs</td>
<td>Polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP-ribose) Polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline buffer</td>
</tr>
<tr>
<td>PCBs</td>
<td>Polychlorinated Biphenyls</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Ph d10</td>
<td>Phenanthrene d10</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>POPs</td>
<td>Persistent organic pollutants</td>
</tr>
<tr>
<td>PXXP</td>
<td>Proline-rich domain</td>
</tr>
<tr>
<td>RAF</td>
<td>A serine/threonine kinase protein</td>
</tr>
<tr>
<td>RAPD</td>
<td>Randomly amplified polymorphic DNA</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>Ref-1</td>
<td>Redox effector factor 1</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S.E.M</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SAM</td>
<td>Sterile alpha motif</td>
</tr>
<tr>
<td>SBs</td>
<td>Strand Break</td>
</tr>
<tr>
<td>SCE</td>
<td>Sister chromatid exchange</td>
</tr>
<tr>
<td>SCGE</td>
<td>Single cell gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>tGSH</td>
<td>Total glutathione</td>
</tr>
<tr>
<td>TS</td>
<td>Trebarwith Strand, Cornwall, UK</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VCT</td>
<td>Vesicular Connective Tissue</td>
</tr>
</tbody>
</table>
ABSTRACT

Integrated biomarker and molecular responses in marine bivalve following exposure to environmental contaminants: Implications for human and environmental health

Yanan Di

Environmental genotoxicants/carcinogens in the aquatic environment induce complex detrimental impacts on aquatic organisms. Integrated approach is required to comprehensively assess such impacts in terms of animal and environmental health. Biomarkers at different levels of biological organisations, including clearance rates (at individual level), histopathological alterations (at tissue level), DNA strand breaks using comet assay (at cellular level) and transcriptional alterations of key genes determined by real-time quantitative PCR (at molecular level), were designed and applied to analyse the impacts in marine bivalve: *Mytilus* sp., a classic bio-indicator species for environmental health monitoring, following exposure to selected environmental contaminants (i.e. Benzo(α)Pyrene (B(α)P), C_{60} fullerene, and tritium). The performance of biomarkers changed in response to exposures with higher sensitivity for DNA strand breaks and transcriptional alterations of selected genes. In particular, genes representing 7 gene groups (tumour suppressor gene - *p53*, proto-oncogene - *ras*, cytochrome P450 family - *cyp4y1*, MAPKs family - *jnk*, Bcl-2 family - *bax*, MDM2 - *mdm2* and RAD family - *rad51*) showed tissue- and chemical-specific expression profiles under selected experimental conditions. A comparison revealed similarity of interplay among these highly conserved genes with mammalian models, suggesting *Mytilus* sp. could be an ideal model for signalling research in the carcinogenesis process. To my knowledge, this is the first research to detect tissue specific transcriptional alteration of *p53* and *ras* genes in the organism for the analysis of detrimental impacts induced by different environmental genotoxicant/carcinogen exposures. Furthermore, the study is also the first one to include genes which can interplay with each other to function in the regulation of DNA repair, apoptosis and cell cycle arrest in marine organisms. The results suggested that the applied integrated biomarker approach is a comprehensive and sensitive method to monitor environmental health and, marine bivalve *Mytilus* sp. is an ideal model organism for research on genotoxicant induced carcinogenesis and will shed light on the mechanisms of this complex process in higher organisms, including humans.
CHAPTER 1

General introduction
1.1 Aquatic environment, pollutants and our health

It is now generally accepted that our health is dependent upon the health of the environment. The environment is, however, a complex system with a variety of abiotic and biotic components, including air, water, soil, food, plants and animals, with whom we share the limited biosphere. Severe negative effects on the quality of human life can be directly or indirectly related with the deterioration in the health of any of these components (Jha, 2004). The aquatic environment, which covers two-thirds of the planet is inhabited by the majority of extant species in different ecological niches (Gray, 1991). It plays vital roles for ecosystem functioning and human health as it is involved in many human activities and is an important source for human food. As a consequence of human population growth, industrial development and new technique investment (e.g., nano-technique), the production, consumption and disposal of anthropogenic chemicals and wastes continue to be increasingly discharged in the aquatic environment. The accumulation of these anthropogenic contaminants can potentially induce genotoxic and carcinogenic effects in the aquatic organisms (Jha, 2004). The assessment of potential impacts of these contaminants in aquatic organisms induced by these increasingly wide range and quantity of contaminants is urgently required to protect human and environmental health.

There are different groups of anthropogenic contaminants present in the environment, broadly classified as (a) organics (b) metals and (c) radionuclides. In addition, there are some contaminants which could be classified as organometallics (e.g. anti-fouling paints). Potential detrimental impacts of these contaminants need to be determined in the natural biota by applying a range of biological or biomarkers responses. It should also be noted that in the real environment these contaminants occur in all probable combinations and they can interact among themselves in varieties of ways (i.e.
synergistically, antagonistically, additively) to induce a biological response. Environmental contaminants manifest their toxicity in a variety of ways and often a contaminant could act simultaneously as genotoxicant, neurotoxicant, immunotoxicant or reprotoxicant (Jha, 2008). Of particular interest for biologists and the regulatory communities have been the potential impacts of those contaminants which are capable of inducing damage in the genetic material, the DNA (Moore, 1985; Phillips and Arlt, 2009). As aquatic organisms are an important source of food for humans, there is also a risk to our health when these organisms are exposed to environmental toxicants, including genotoxic and carcinogenic substances (Harvey, 1991; Parache et al., 2011; Urban et al., 2009). While induction of genetic damage has been correlated with the induction of cancer, teratogenic and hereditary effects in humans, it also has implications for the short and long-term survival of the natural biota (Jha, 2004, 2008; Solomon and Sibley, 2002).

Three groups of chemicals have captured attention in this study. Polycyclic aromatic hydrocarbons (PAHs) are, perhaps, the first recognized environmental carcinogens which cannot degrade easily under natural conditions (Haritash and Kaushik, 2009). Engineered or manufactured nanoparticles (ENPs) are the product of rapidly developed nanotechnology and can interact with a variety of other environmental contaminants. Ionising radiations (IRs) are mainly released from different anthropogenic sources including nuclear power plants and in common with human health arena, are known to induce efficiently genetic damage and other effects in the natural biota.

1.1.1 PAHs and benzo(α)pyrene (B(α)P)

PAHs are a pervasive and abundant class of environmental pollutants found in air, water, food, and waste sites (Ross and Nesnow, 1999). They are aromatic hydrocarbons with two or more fused benzene rings and can be formed during the thermal decomposition
of organic molecules and their subsequent recombination (Haritash and Kaushik, 2009). The common environmentally relevant PAHs and their properties are listed in Table 1.1. PAHs in the environment are contributed from both natural and anthropogenic sources. Naturally, forest and rangeland fires, oil spills and seeps, volcanic eruptions and exudates from trees can release PAHs into the environment. Anthropogenic sources include burning of fossil fuel, coal tar, wood, garbage, refuse, used lubricating oil and oil filters, municipal solid waste incineration, petroleum spills and discharge (Kaushik and Haritash, 2006).

### Table 1.1 Common environmentally relevant PAHs: their structures and properties

<table>
<thead>
<tr>
<th>PAH</th>
<th>Formular(^A)</th>
<th>Molecular Weight(^A)</th>
<th>CAS registry NO(^A)</th>
<th>Chemical structure(^B)</th>
<th>Solubility (µg/L)(^C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>C(<em>{10})H(</em>{8})</td>
<td>128</td>
<td>92-20-3</td>
<td></td>
<td>31300</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>C(<em>{14})H(</em>{10})</td>
<td>178</td>
<td>85-01-8</td>
<td></td>
<td>1070</td>
</tr>
<tr>
<td>Anthracene</td>
<td>C(<em>{14})H(</em>{10})</td>
<td>178</td>
<td>120-12-7</td>
<td></td>
<td>44.6</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>C(<em>{16})H(</em>{10})</td>
<td>202</td>
<td>206-44-0</td>
<td></td>
<td>4.90</td>
</tr>
<tr>
<td>Pyrene</td>
<td>C(<em>{16})H(</em>{10})</td>
<td>202</td>
<td>129-00-0</td>
<td></td>
<td>5.17</td>
</tr>
<tr>
<td>Chrysene</td>
<td>C(<em>{18})H(</em>{12})</td>
<td>228</td>
<td>218-01-9</td>
<td></td>
<td>5.61</td>
</tr>
<tr>
<td>Benzo((\alpha))anthracene</td>
<td>C(<em>{18})H(</em>{12})</td>
<td>228</td>
<td>56-55-3</td>
<td></td>
<td>5.61</td>
</tr>
<tr>
<td>Benzo((\alpha))pyrene</td>
<td>C(<em>{20})H(</em>{12})</td>
<td>252</td>
<td>50-32-8</td>
<td></td>
<td>6.04</td>
</tr>
</tbody>
</table>

\(A\): Boström et al. (2002), \(B\): Xue and Warshawsky (2005), \(C\): Dipple et al. (1987)
In marine systems, pollution by petroleum and its derivatives is one of the greatest environmental concerns as annual inputs are estimated to be in the range of million tons per year for the global ocean, with around 28% contaminating highly productive coastal waters (GESAMP, 1990). PAHs, as the main component of petroleum and crude oil, have been evaluated by the International Agency for Research on Cancer (IARC) and have been deemed to be associated with increased cancer cases, both in human and experimental animals (Dipple et al., 1984; Harvey, 1991; Warshawsky, 1992).

In the marine environment, the fate of PAHs in an organism can be divided into four stages: absorption, distribution, biotransformation and excretion. Absorption of PAHs depends on molecular properties (lipophilicity, size and polarity/charge) and the features of the organism. Distribution of PAHs is mainly determined by the route of uptake and tissue lipid levels. Lipophilic compounds are readily taken up into the tissues of living organisms and are accumulated in these tissues (bioaccumulation). Generally, the bioaccumulation increases as tissue lipid levels increase in an organism. After PAHs have been distributed among the tissues, metabolic reactions are initiated by enzymes. Metabolic enzymes have a wide tissue distribution but the highest activities are generally found in tissues involved with food processing (Livingstone, 1991). Finally, the chemicals pass through excretion involving elimination of waste metabolic products and other non-useful materials.

The relative planar highly conjugated aromatic structures of PAHs makes the metabolic activation is required for them to exert their mutagenic/carcinogenic effects during bioaccumulation and tissue distribution of these chemicals in aquatic organisms (Harvey, 1991; Miller and Miller, 1966). The mechanisms of metabolic activation are complex. Presently, the bay/fjord region dihydrodiol epoxides metabolic pathway for PAHs has been established with experimental evidence (Xue and Warshawsky, 2005).
The distinction between bay and fjord region is based on the structural difference in the critical area of epoxide formation, involving either a crowded fjord region or a sterically less hindered bay region (Buterin et al., 2000). It involves three enzyme-mediated reactions: firstly, oxidation of a double bond catalysed by cytochrome P450 enzymes (CYPs) to unstable arene oxides; secondly, hydrolysis of the arene oxides by microsomal epoxide hydrolase (EH) to trans dihydrodiols; finally, a second CYP-catalysed oxidation at the double bond adjacent to the diol function to generate a vicinal diol-epoxide (DE). This pathway can lead to sterically-hindered bay or fjord region DE. The bay/fjord region DE are electrophiles capable of binding to DNA while some of the DE stereoisomers of PAHs are found to ultimately be carcinogens (Figure 1.1). Apart from B(α)P, studies on metabolism, DNA binding, mutagenicity, carcinogenicity, and cell transformation have indicated that many other PAHs such as, chrysene (Mordquist et al., 1981), 5-methylchrysene (Hecht et al., 1986), phenanthrene, benzo[c]phenanthrene (Dipple et al., 1987), benz[α]anthracene (Levin et al., 1978), 7,12-dimethylbenz[α]anthracene (Slaga et al., 1979), and dibenzo[α,l]pyrene (Ralston et al., 1994) are metabolized through this pathway (Figure 1.2).

Figure 1.1. Bay region dihydrodiol epoxides pathway for B(α)P metabolisation.
Figure 1.2 Major enantiomers of bay/fjord region dihydrodiol epoxides of selected PAHs

Despite an abundance of convincing data that the bay/fjord DE pathway plays an essential role in metabolism, evidence is accumulating to suggest that there is no longer a single mechanism dominating metabolic activation of PAHs as a whole. Two other
pathways: radical cation and activation through PAH-o-quinone pathways have been proposed to be involved in metabolic activation and detoxification (Bolton et al., 2000; Cavalieri and Rogan, 1995; Penning et al., 1999; Penning et al., 1996). Xue and Warshawsky (2005) have thoroughly reviewed all three pathways and their functions in PAHs biotransformation. It is likely that all of the metabolic activation pathways may contribute to PAHs metabolism in organisms. Following the biotransformation of PAHs to their DE form, they can interact with DNA strands and to form DNA adducts with a high preference of the exocyclic amino groups of deoxyguanosine (dG) and deoxyadenosine (dA) as demonstrated by both in vivo and in vitro experiments (Geacintov et al., 1997; Graslund and Jernstrom, 1989; Harvey, 1985; Jerina et al., 1991) (Figure 1.3). Unless removed by DNA repair processes, the resulting adduct may give rise to mutations following DNA replication and in turn to cause a series of abnormalities in organisms, such as tissue structure damages, immune system dysfunctions, DNA strand breaks and transcriptional alterations in key genes. The impacts of PAHs in marine biota is summarised in Table 1.2. Among all the PAHs, B(α)P is the most well studied one with respect to both its metabolic activation pathways and carcinogenic effects on human and other experimental animals. Since early 1930s, an amount corresponding to a few grams of B(α)P was isolated from 2 tons of pitch and was shown to cause tumours in rodents (Harvey, 1985). B(α)P has been frequently selected as representative genotoxic/carcinogenic PAH in research on environmental pollutants induced hazards to animal health. Therefore, B(α)P was selected in this project to provide promising DNA damage response in organisms, and simultaneously responses at other levels of biological organisation (e.g., transcriptional alteration at molecular level, histopathological and physiological effects at tissue and individual levels respectively) can be detected.
Table 1.2 Summary of PAHs induced biological responses in different aquatic organisms

<table>
<thead>
<tr>
<th>Group</th>
<th>Species</th>
<th>Experimental conditions/PAH contaminated site</th>
<th>Biological Responses observed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish</td>
<td>Coregonus lavaretus</td>
<td>Whitefish exposed to B(α)P (10 mg/kg body weight (BW)) for 48 h</td>
<td>P53 is moderately inducible by B(α)P in tissue of liver, brain and kidney of whitefish</td>
<td>Brzuzan et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>Oryzias latipes</td>
<td>Japanese medaka exposed to B(α)P (200 µg/g BW) for 48 h</td>
<td>Significant induction of CYP1A expression within lymphoid tissue after exposure</td>
<td>Carlson et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>Fundulus heterocitus</td>
<td>Fish collected from PAHs contaminated site at Elizabeth River, Portsmouth, VA, USA</td>
<td>High level of PAH concentration in collected fish tissues. Increased DNA damage in blood measured by flow cytometric method was observed compared to samples collected at reference site</td>
<td>Jung et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>Danio rerio</td>
<td>Fish embryos exposed to 0, 0.05, 0.5, 5 and 50 nmol/L pyrene up to 72 h post-fertilisation</td>
<td>Disruption of normal cardiac development and altered expressions of defective cardiac differentiation related genes (e.g., NKx2.5 and Bmp2b)</td>
<td>Zhang et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Oncorhynchus mykiss</td>
<td>Cell line RTG-2 derived from gonadal tissue of rainbow trout exposed to B(α)P at concentration of 0.05, 0.1 and 0.5 µg/mL over period up to 30 days</td>
<td>DNA fingerprints were analysed by random amplified polymorphic DNA (RAPD) technique. Both qualitative and quantitative analysis showed an increase in the instability in the DNA fingerprint of exposed cells over a time- and concentration-dependent manner</td>
<td>Castaño and Becerril (2004)</td>
</tr>
<tr>
<td></td>
<td>Fundulus heteroclitus</td>
<td>Fish embryos were exposed to waterborne B(α)P at 10 and 100 µg/L and both mRNA expression and enzyme activity of glycine N-methyltransferase (GNMT) were determined</td>
<td>B(α)P exposure altered GNMT expression at both mRNA and enzyme activity level</td>
<td>Fang et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>Anjuilla anjuilla</td>
<td>Fish were exposed to B(α)P (0, 0.1 and 0.3 µM) over a period of 4 weeks</td>
<td>An increase in DNA damage was found by comet assay. No point mutations or change in ras gene expression level were detected when compared to control samples</td>
<td>Nogueira et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>Platichthys flesus</td>
<td>Fish were treated with a single intraperitoneal injection of B(α)P at dose of 20 mg/kg for up to 21 days</td>
<td>A specific mutational events at the adenine base of TaqI registration site of exon 8 of the flounder p53 gene at codon 243 was observed after exposure</td>
<td>Sueiro et al. (2000)</td>
</tr>
<tr>
<td><strong>Mussel</strong></td>
<td><strong>Mytilus edulis</strong></td>
<td>Mussels were exposed to fluoranthene and B(α)P at 1 and 6 µg/L respectively for up to 4 weeks</td>
<td>Reduced feeding activity and alterations of antioxidant enzymes superoxide dismutase and catalase activities</td>
<td>Eertman <em>et al.</em> (1995)</td>
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<tr>
<td><strong>Mytilus edulis</strong></td>
<td>Mussels were fed with B(α)P-dosed <em>Isochrysis galbana</em> (0.5 and 20 ppb B(α)P) for 14 days</td>
<td>Increased DNA strand breaks after laboratory exposure to B(α)P-dosed algae</td>
<td>Large <em>et al.</em> (2002)</td>
<td></td>
</tr>
<tr>
<td><strong>Mytilus edulis</strong></td>
<td>Isolated digestive gland cell mixtures were exposed to B(α)P (50 µM) for 1 h in dark</td>
<td>B(α)P induced DNA strand breakage was observed and indicated to be cytochrome P450-catalysed and to occur via the production of B(α)P quinones</td>
<td>Mitchelmore and Chipman (1998a)</td>
<td></td>
</tr>
<tr>
<td><strong>Mytilus edulis</strong></td>
<td>Mussels collected from PAH contaminated site in Norway</td>
<td>Significant change in histopathological and histochemical parameters</td>
<td>Aarab <em>et al.</em> (2008)</td>
<td></td>
</tr>
<tr>
<td><strong>Mytilus edulis</strong></td>
<td>Mussels were exposed to single dose of 1 ppb B(α)P under subtidal (SC) or tidal conditions (TC) over a period of 14 days</td>
<td>Uptake and oxidative toxicity of water-born B(α)P in mantle and whole tissue of mussels were analysed and indicated that increased B(α)P mediated toxicity in mantle lipid was due to the interactive effect of the tidal cycle of immersion/emersion of B(α)P mediated oxidative damage</td>
<td>Durand <em>et al.</em> (2002)</td>
<td></td>
</tr>
<tr>
<td><strong>Mytilus galloprovincialis</strong></td>
<td>Mussels were exposed to B(α)P (50 mg/kg dry weight/day) for up to 28 days. Gill DNA was extracted for the analysis of DNA adduct</td>
<td>B(α)P related DNA adducts in gill were positively correlated with B(α)P concentration in whole mussel, and were produced in a time-dependent manner relative to exposure</td>
<td>Akcha <em>et al.</em> (2000)</td>
<td></td>
</tr>
<tr>
<td><strong>Mytilus galloprovincialis</strong></td>
<td>Mussels were exposed to a wide dose-range (0.5-1000 ppb) of B(α)P over a period of 3 days</td>
<td>Evidence of the formation of DNA adducts, oxidative DNA damage, and changes in CYP1A-immunopositive protein levels were observed</td>
<td>Canova <em>et al.</em> (1998)</td>
<td></td>
</tr>
<tr>
<td><strong>Mytilus galloprovincialis</strong></td>
<td>Mussels were treated with 0.5-100 µg/L of B(α)P for 2 and 3 days</td>
<td>DNA adducts in the gill tissue were observed in a reproducible dose-dependent increase pattern</td>
<td>Venier and Canova (1996)</td>
<td></td>
</tr>
<tr>
<td><strong>Perna viridis</strong></td>
<td>Mussels were exposed to B(α)P (0, 0.3, 3 and 30 µg/L) for up to 12 days</td>
<td>Comet assay indicated an increase in the proportion of strand breaks occurred generally with increasing B(α)P concentration. Monitoring the frequency of micronucleus development in mussel haemocytes indicated both dose- and time- response relationship within the exposure period</td>
<td>Siu <em>et al.</em> (2004)</td>
<td></td>
</tr>
<tr>
<td>Organism</td>
<td>Description</td>
<td>Observations</td>
<td>References</td>
<td></td>
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<td>------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------</td>
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<td>-------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><em>Perna viridis</em></td>
<td>Mussels were exposed to 0, 0.3, 3 and 30 µg/L B(α)P over a period of 24 days. Mussels were collected on day 0, 1, 3, 6, 12, 18 and 24, and the levels of DNA adducts and DNA strand break in hepatopancreas tissue monitored</td>
<td>Complex alteration of DNA strand breaks and DNA adduct level were observed after exposure. For example, increased DNA strand breaks were observed in mussels exposed to 0.3 and 3 µg/L after 1 day of exposure and reached to the peak after exposure to 0.3 µg/L for 3 days and 3 µg/L for 6 days. Increasing B(α)P concentrations resulted in elevated DNA adduct levels after 3-6 days of exposure, but this pattern of dose-related increase disappeared after 12 days</td>
<td>Ching <em>et al.</em> (2001)</td>
<td></td>
</tr>
<tr>
<td><em>Perna viridis</em></td>
<td>Mussels were transplanted from clean site to PAHs polluted sites (B(α)P concentration assessed) for 30 days field exposure</td>
<td>Induced antioxidant parameters in mussels tissues at PAH contaminated site</td>
<td>Cheung <em>et al.</em> (2001)</td>
<td></td>
</tr>
<tr>
<td>Water fly</td>
<td><em>Daphnia magna</em> Organisms were exposed to 50 µg/L B(α)P for 3 or 6 days and were allowed to recover in clean medium for 12 or 9 days respectively</td>
<td>Qualitative and quantitative changes were observed in the RAPD profile generated from both B(α)P exposed and recovered <em>Daphnia</em>. Some of the RAPD changes disappeared at the end of both recovery experiments suggested the DNA effects were fully repaired or reversed</td>
<td>Atienzar and Jha (2004)</td>
<td></td>
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<tr>
<td>Diatom</td>
<td><em>Thalassiosira pseudonana</em> Diatoms were exposed to three PAHs (pyrene: 2.5-500 µg/L, fluoranthene: 3-3000 µg/L and B(α)P: 0.05-328 µg/L) either as single compound or as mixture for 24 h</td>
<td>Genes (total of 8) expression were analysed by Real-time PCR. Two genes <em>lacsA</em> and <em>sil3</em> were strongly influenced by the PAHs and other genes were slightly down-regulated by PAHs</td>
<td>Bopp and Lettieri (2007)</td>
<td></td>
</tr>
<tr>
<td>Marine worm</td>
<td><em>Platynereis dumerilii</em> 12-h-old embryos were exposed to B(α)P (0.5% (v/v)) for 12 h (SCE analysis) or 8 h (CA analysis)</td>
<td>Sister chromatid exchanges (SCE) and chromosomal aberrations (CAS) were observed for mutagen B(α)P exposure</td>
<td>Jha <em>et al.</em> (1996)</td>
<td></td>
</tr>
<tr>
<td>Clam</td>
<td><em>Macoma balthica</em> Clams were collected from PAH, PCB and heavy metals contaminated site at Sydney Harbour, Nova Scotia</td>
<td>Histopathologic and histochemical responses alterations were observed from clams collected from the contaminated site</td>
<td>Tay <em>et al.</em> (2003)</td>
<td></td>
</tr>
<tr>
<td><em>Meretrix meretrix</em></td>
<td>Embryo-larval stage of clam were exposed to B(α)P and Aroclor 1254 to 6.25-1,600 µg/L for 96 h</td>
<td>The extreme toxicity of B(α)P and Aroclor 1254 were observe in clam embryos and larvae. Aroclor 1254 was more toxic than B(α)P to embryos and larvae</td>
<td>Wang <em>et al.</em> (2012)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.3 Diagrammatic representation of DNA adduct formation following B(α)P exposure and its fate following replication and repair. Blue dot indicates benzo(α)pyrene diol epoxide (BPDE). Red circle highlights occurrence of mutation generated by BPDE induced DNA adduct.
1.1.2 Nanoparticles (NPs) and $C_{60}$ fullerene ($C_{60}$)

NPs are defined by the U.S. National Nanotechnology Initiative as materials that have at least one dimension in the 1 to 100 nm range (Initiative, 2011). Nano-sized materials are naturally present from forest fires and volcanoes, as viral particles, biogenic magnetite, and even protein molecules such as ferritin (Oberdörster, 2004). Recently, manufactured or engineered nanoparticles have been widely applied for a large number of industrial and commercial purposes because of their large surface area to mass ratio which makes them unique compared to their bulk counterpart.

These particles are introduced into the environment in varying amounts from multi-tons of carbon black and fumed silica for plastic fillers, car tyres and house insulation; kilograms in sunscreens, tooth paste, cosmetics, sanitary ware coatings; and micrograms in antifouling as fluorescent quantum dots in biological imaging (Hoet et al., 2004). In mammalian studies, both in vitro and in vivo cytotoxicity studies have demonstrated oxidative stress, inflammation conditions, cytokine production, cytoskeletal changes, altered vesicular trafficking, apoptosis, as well as changes in gene expression and cell signalling in response to different types of NPs (Ding et al., 2005; Hussain et al., 2005; Ju-Nam and Lead, 2008; Magrez et al., 2006; Owen and Depledge, 2005; Tian et al., 2006), probably through the generation of reactive oxygen species (ROS) to induce breakdown of membrane lipids and the DNA (Clutton, 1997). After uptake, several studies have reported that NPs can be translocated from the lungs into the blood in hamster (Nemmar et al., 2001) and can thereby move to other organs and tissues, raising concern that they may cause oxidative stress-mediated toxicity in whole biological systems (Samet et al., 2004). The negative biological outcomes have also been observed in marine organisms. Canesi et al. (2010) has demonstrated that haemocytes of *Mytilus galloprovincialis* following in vitro exposure to suspensions of
selected commercial nanomaterials (C$_{60}$, n-TiO$_2$, n-SiO$_2$) stimulated lysosomal damage, oxidative stress and toxic cell injury. Oberdörster (2004) reported that juvenile largemouth bass exposed to 0.5 ppm aqueous uncoated C$_{60}$ for 48 h had a significant increase in lipid peroxidation of the brain, and glutathione (GSH) depletion in the gill. The metabolism of NPs in organisms, however, is still unknown. The CYPs are known to metabolise xenobiotics by hydroxylation, and since hydroxylated fullerene is less toxic than neat fullerene (Sayes et al., 2001), this could lead to detoxification. Significantly increased hepatic levels of some CYP2-like isozymes (e.g., CYP2K, CYP2M) were reported in fathead minnow after C$_{60}$ exposure (Zhu et al., 2006), suggesting that they may function in metabolising C$_{60}$ or in repairing lipid peroxidation by metabolising endogenous compounds such as medium to long-chain fatty acids (Oberdörster, 2004; Zhu et al., 2006). However, a conflicting result was also reported by Oberdörster et al. (2006) who observed both mRNA and protein levels for the CYP isozymes CYP2K and CYP2M were not significantly altered compared to control in largemouth bass following exposure to C$_{60}$. Although the metabolic kinetics are not very well understood, it is suggested that severely negative biological outcomes can occur when NPs bind to DNA, impact its shape which could ultimately lead to diseased conditions, including cancer (Zhao et al., 2005).

Among all the NPs, C$_{60}$, the smallest form belonging to a family of carbon allotropes in the shape of a hollow sphere (Yang et al., 2010), has been employed in a number of biological applications, such as free radical scavengers (Dugan et al., 1997), photo-induced DNA cleavage agents (Tokuyama et al., 1993), inhibitors of HIV-1 protease (Friedman et al., 1993), and cytotoxic agents for human cells (HDF, HepG2, and NHA) (Sayes et al., 2005). Upon discharge in the aquatic or marine environment, the single C$_{60}$ will aggregate together due to its low solubility. The aggregates are reported in diameters from 5 to 500 nm (Andrievsky et al., 1999) and allow for concentrations up to
100 mg/L in water system, which is 11 orders of magnitude more than the estimated single C\textsubscript{60} solubility (Fortner \textit{et al.}, 2005). Apart from largely increased concentration after aggregation, the formed structure can lead to the molecular entrapment inside, such as PAHs (Yang and Xing, 2007). The interaction between C\textsubscript{60} and PAHs would also alter the properties and bioavailability of each chemical. This potential has been the main drive of this study.

\textbf{1.1.3 Ionising radiations (IRs) and tritium}

Ionising radiations have played important roles in modern civilisation, but several accidents including the one caused by the earthquake-tsunami at Japan’s Fukushima Diichi nuclear power plant (NPP) in 2011 have raised concern about the potential hazard of large amount of radionuclides released into environment. In addition, authorised discharges from nuclear installations around the world have also raised public, scientific and regulatory concern with respect to human and environmental health (Dallas \textit{et al.}, 2012). Radionuclide exposure may cause major alterations to the structure and function of biological macromolecules, such as lipids, proteins, and nucleic acids which can in turn produce irreversible effects such as carcinogenesis (Stein \textit{et al.}, 1994). It can also affect fecundity (Theodorakis \textit{et al.}, 1997), immune function (Hurks \textit{et al.}, 1995), or deplete cellular energy stores (Pereira \textit{et al.}, 2011).

As a low-energy \(\beta\) emitter, tritium can be released into the environment directly from NPPs process, and it should be noted that fusion power reactors using tritium as a fuel may well become widely used in coming years, suggesting an increase in tritium discharge in the marine environment. In addition, tritium is used in the manufacture of radionuclide-labeled materials for application in medicine, research and industry, and can be released from such manufacturing plants (notably that in Cardiff, UK, operated by GE Healthcare) and in the use and disposal of these materials (AGIR, 2007). Tritium
has also been used in luminous paint employed in some wristwatches and compasses, and in emergency exit signs, gun-sights, and ‘Trimphones’. Discharges can be in the form of tritiated water, liquid of vapour (HTO), tritiated hydrogen gas (HT), or organically bound forms (OBT). Marine organisms are consistently exposed to such large scale release of tritium which could potentially produce damage at different levels of biological organisation. Adam-Guillermin et al. (2012) has recently reviewed the genotoxic and reprotoxic effects of tritium on aquatic animals, indicating it damages DNA integrity and reproductive ability in both aquatic vertebrates and invertebrates.

Although the International Atomic Energy Agency (IAEA) suggests that there is no convincing scientific evidence that chronic dose below 1 mGy/d will harm animal or plant populations and that, in particular, effects on aquatic populations are not expected at doses lower than 10 mGy/d or 400 µGy/h ((IAEA), 1992), there is still lack of information to confirm whether low dose delivered by radionuclides could induce effects in the organisms. Also, tritium has been reported to have a higher biological response effect compared to other released radionuclides (AGIR, 2007). Therefore, it is likely that the response will be altered after tritium exposure in comparison to other radionuclides at comparable doses.

1.2 Marine bivalve – *Mytilus sp.*

Bivalve mussels of the genus *Mytilus* are distributed widely in temperate waters of both hemispheres (Gosling, 1992) and have been used for many years in aquaculture and environmental monitoring programmes (Venier et al., 2006). They attach by byssal threads to each other, rocks and pylons of docks where they grow in clumps. They are ecologically important since many other marine organisms, including shore birds, sea stars and crabs, feed on them. Humans also consume mussels and therefore, any pollutant which can accumulate in mussel tissues has the potential to enter higher
trophic level organisms via the food chain. Mussels are sessile, which makes them useful as a marine bio-indicators since they stay in one place and their health status yields information relevant to a particular location (Dondero et al., 2006). Mussels have been used widely in environmental monitoring so there is much information available for comparison purposes. For example, the “Mussel watch program” in the United States has been using these organisms in monitoring programmes for over 30 years to indicate the health of the Great Lakes, estuarine and coastal waters using a variety of indicators, including size of the mussels, condition index, stage of reproduction, diseases and other pathologies (Cajaraville et al., 2000). In addition, mussels’ lifestyle is stationary filter feeding, inhabiting the benthic environment where pollutants usually end up and accumulate. Mussels readily bioaccumulate both organic (Moore, 1985) and metal (Viarengo, 1985) pollutants, therefore often rendering them more accurate in situ bioindicators of pollution than fish (Cajaraville et al., 2000). Therefore, mussels provide many advantages as a model to assess toxic effect of environmental pollutants in organisms.

Three species, *M. edulis*, *M. galloprovincialis*, and *M. trossulus*, are closely related and practically indistinguishable by morphological characteristics only (Inoue et al., 1995). In common, *Mytilus sp.* has an open circulatory system where the organs are bathed in haemolymph. The impact of marine pollutants is thought to be significantly different in various organ/tissues along with their own biological function variability. For example, gill tissue and digestive gland of mussels play important roles in food collection, absorption and digestion. Chronic exposure of mussels to pollutants in water and sediments may ultimately impair their nutrient absorption ability and compromise their growth and reproduction (Smital et al., 2004). Given the high filtering capacity of mussels, gill tissues are not only continuously in contact with pollutants in waters, but they may also concentrate pollutants contained therein due to the remarkably high
volume of water that they filter (Au, 2004). As mentioned earlier, *Mytilus sp.* have already been applied in marine environment biomonitoring programmes (Aarab et al., 2008; Goldberg, 1986), and sub-lethal biological responses or biomarkers have already been proposed for this sentinel species to monitor levels of environmental pollutants. In addition, occurrence of leukaemia and gonadal neoplasia has been reported in mussels (Barber, 2004; Ciocan and Sunila, 2005; Galimany and Sunila, 2008; Muttray et al., 2008). Externally, such mussels appear normal, but they are lethargic, withdraw slowly into the shell, have flaccid siphons, and eventually die (Walker et al., 2009), which provide a perfect model for cancer related research, especially in carcinogen induced cancer development.

### 1.3 Basic biomarkers or biological responses

Biomarkers can provide an indication of the sub-lethal impacts of stressors, as well as the biochemical mechanism that may be affected by them, and an ‘early warning’ of population level impacts (Vasseur and Cossu-Leguille, 2003). A variety of molecular, biochemical, physiological, histo-cytopathological, organism, population and community responses may be used to identify exposure to certain chemicals, providing information on spatial and temporal changes in the concentration of contaminants, and indicating environmental quality or occurrence of adverse ecological consequences (Au, 2004). For any biomonitoring program, a tool box of biomarkers should be applied, including whole organism measurements, such as growth rate, sex ratio; diagnosis at the tissue level using histology; cell function tests, such as adhesion, phagocytosis, as well as molecular biomarkers. Adopting an integrated approach, in this study, the following biomarkers were selected to assess chemical induced impacts in *Mytilus sp.*
1.3.1 Clearance rate (diagnosis of physiological condition of whole organism)

The clearance rates of bivalves have been used by researchers for many years as a simple and robust physiological indicator of the overall health of the organism, often as a component for ‘scope for growth’ (Widdows and Salkeld, 1993). Normal feeding in *Mytilus sp.* and other filter-feeding bivalves with laterofrontal cirri depends on the cirri-trapping principle (Riisgård *et al.*, 1996) where bands of lateral cilia produce the main water transport through inter-filamentary canals of the gill while suspended particles are separated from the main currents and transferred onto the frontal surface by the action of the laterofrontal cirri. In the clearance rate assay (sometimes termed feeding rate) of filter-feeding organisms, food consumption is measured in terms of the rate at which a given volume of water is cleared of food particles per unit time (Widdows *et al.*, 1995). The alteration in feeding rate can reflect the health condition of test organisms (Canty *et al.*, 2009).

1.3.2 Histopathological alteration (diagnosis at the tissue level)

Histopathological alterations (of aquatic organisms) are powerful indicators to provide health assessments of individuals and of populations since they incorporate measurements of reproductive and metabolic condition, and allow for the detection of a range of pathogens that may affect morbidity and mortality (Bignell *et al.*, 2008). In addition to its role in providing a “baseline” measure of health, histopathology analysis has been employed to investigate the changes related to PAH, PolyChlorinated Biphenyls (PCBs) and heavy metal exposure in mussels (Marigomez *et al.*, 2006). However, since histopathology has also been used to investigate the effects of disease and parasite infestation in wild mussel populations (Powell *et al.*, 1999), care must be taken when using histopathology as a biomarker of contaminant exposure *per se* because the changes can be the indicators of both parasite infestation and chemical-
induced responses. In this respect, histopathology can be considered as a tool to provide supporting information for measures (biomarkers) that specifically aim to assess historic exposure to, or effect of, a contaminant.

1.3.3 DNA strand breaks (diagnosis at the cellular level)

DNA single- and double-strand breaks may occur via a number of mechanisms (Mitchelmore and Chipman, 1998a). In addition to production of overt strand breakage by, for example, IR, strand breaks (SBs) can also be produced via alkali labile sites, e.g., as produced by ultraviolet light, reactive oxygen and other reactive intermediates (Lindahl and Ljungquist, 1975). Strand breakage may also arise through the action of excision repair enzymes (Speit and Hartmann, 1999). Consequently measurement of SB offers a mean of measuring exposure to a wide range of genotoxic chemicals or radionuclides that may modify DNA in different ways. It is important to recognise that SB may occur via mechanisms not related to direct genotoxicity of a chemical. The activation of enzymes such as endonucleases and topoisomerases, e.g., through elevation of intracellular free calcium (McConkey et al., 1988), may give rise to SB caused by the increases in the level of endogenous reactants such as superoxide radical and nitric oxide (Meneghini, 1988). Thus, as well as detecting direct effects of xenobiotic substances, SB may also reflect impairment of normal cell redox status although a recent study demonstrated a negative response in the comet assay (tool to measure DNA strand breaks) to three cytotoxic non-mutagenic carcinogenic compounds in V79 Chinese hamster cells and human white blood cells (Hartmann and Speit, 1997). It is for reasons of non-specificity that SB has limitations for the genotoxicity screening of chemicals. However, the non-specific nature of the assay coupled with high sensitivity and applicability to many cell types may be considered favourable for environmental monitoring, provided that a more detailed understanding of the causes of
any observed effect are pursued (Frenzilli et al., 2008; Jha, 2008). It has been demonstrated that various isolated cells (e.g. gill, digestive gland, haemocyte) from aquatic organisms responded to B(α)P to induce SB through its metabolic activation (Bihari et al., 1990; Shugart, 1988). Therefore, using DNA strand breaks as a biomarker will give a promising result of genotoxicant-induced effects.

At present, a number of different approaches has been made to detect SB. Singh et al. (1988) adapted a number of previous methods to develop a method of single cell gel electrophoresis (SCGE) under alkaline conditions to emphasise the occurrence of DNA single-strand breaks, with applications in genotoxicity testing, human biomonitoring and molecular epidemiology, eco-genotoxicology, as well as fundamental research in DNA damage and repair (Collins, 2004). The assay detects DNA strand breaks at single cell level by measuring the migration of DNA from immobilised nuclear DNA. The advantages of using this technique for assessing DNA damage in aquatic organisms over other techniques are: DNA damage in single cells is measured; only a small number of cells are required (<10,000); the technique can be carried-out on virtually any eukaryotic cell type; and the assay is very sensitive and reproducible if performed under optimised conditions (Frenzilli et al., 2008; Jha, 2008; Lee and Steinert, 2003).

1.4 Molecular biomarkers in relation to DNA repair and carcinogenesis

Carcinogenesis is a multistep, multi-mechanism process involving genotoxic events (mutations); altered gene expression at the transcriptional, translational, and post-translational levels (epigenetic events); and altered cell turn-over rate and survival (proliferation and apoptosis) (Hanahan and Weinberg, 2000). Operationally, tumour initiation encompasses several distinct requirements, which for chemical carcinogens
include the compound (reactive per se or reactive following metabolism) reacting with DNA and thus causing DNA damage. In many cases these changes consist of DNA adducts, covalent bonding of chemicals with DNA bases. Following DNA replication, the DNA damage caused by these reactive agents may be fixed as a mutation, such as PAHs induced adducts can cause transversion of GC → AT (Harris, 1991; Hollstein et al., 1991). A mutation or altered expression in one of a few critical genes in cell is considered a key event in the carcinogenesis process. An increasing amount of evidence suggests that two classes of genes including tumour suppressor genes and proto-oncogenes involved in signal transduction, DNA repair, and cell proliferation and differentiation are crucial to carcinogenesis (Boström et al., 2002; Kinzler and Vogelstein, 1998). In general the accumulation of genetic damage in the form of activated proto-oncogenes and inactivated tumour suppressor genes is the driving force in the evolution of a normal cell to a malignant cell. In addition, mutations in genes involved in the DNA repair, replication and nucleotide metabolism could also play a significant role in this multistage process (Papis et al., 2011).

1.4.1 Tumour suppressor gene p53

Over last 20 years, 35 tumour suppressor genes have been identified and cloned according to their cancer-preventive effects (such as Rb, p53, cd95) (Weinberg, 2007). Among them, the P53 is one of the most intensely investigated proteins which can regulate several major cellular functions including gene transcription, DNA synthesis, DNA repair, cell cycle regulation, apoptosis and cell death (Gomez-Lazaro et al., 2004). The wild-type P53 protein is short-lived (half-life 10-30 min) and constitutively expressed at low levels in most cell types (Culmsee and Mattson, 2005). It is comprised of 393 amino acids that are arranged in four main domains: transactivation domain, DNA binding domain, oligomerization domain and basic domain, with different
functions of each domain as summarised in Figure 1.4 (Millau et al., 2008; Muller-Tiemann et al., 1998; Nicholls et al., 2002; Stommel and Wahl, 2004; Walker and Levine, 1996; Wang et al., 1993; Weinberg et al., 2004). Two P53 homologues, P63 and P73, as protein family members, have been identified and sequenced in a wide range of vertebrates, including mice and humans. Both P63 and P73 can activate P53-regulated genes, and their over-expression induces apoptosis (Levrero et al., 2000). However, unlike P53, P63 and P73 contain a sterile alpha motif (SAM) within their C terminal extensions which cause their different functions in protein interaction and developmental regulation (Schultz et al., 1997). For example, P63 maintains epithelial stem cells and P73 functions primarily in neurological and inflammatory development (Graziano and De Laurenzi, 2011; Moll and Slade, 2004). Both P63 and P73 levels are at elevated levels throughout vertebrate embryo development, whereas P53 expression decreases after the central nervous system matures. Furthermore, not all DNA damaging agents induce P73; mutations of either p73 or p63 in human cancers are infrequent and p73-deficient mice are not prone to spontaneous tumours (Levrero et al., 2000).
Figure 1.4 Diagrammatic representation of P53 showing four main domains which play different roles in its function (adapted from Culmsee and Mattson (2005)). The N-terminal region contains the transactivation domain divided into 3 sections: AD1 (residues 1-42), AD2 (residues 43-63) and a proline-rich domain PXXP (P indicates proline and X indicates any other amino acid). The DNA-binding domain (residues 102-292) contains two classes of protein binding site: class 1- Apoptosis stimulating protein of P53 (ASPP) and class 2- P53 homologues P63 and P73. The oligomerization domain (residues 323-356) contains a nuclear export signal (NES). The basic domain, located at the end of C-terminal region (residues 363-393), contains two nuclear localization signal (NLS).
Two proteins, murine double minute clone 2 (MDM2) and c-Jun N-terminal Kinase (JNK) are responsible for the constitutive instability of P53. MDM2 binds P53 at distinct sites at the N-terminus and acts as an E3-ubiquitin ligase targeting P53 for degradation by the 26S proteasome (Pluquet and Hainaut, 2001). By binding to P53, MDM2 not only earmarks the protein for degradation but also conceals the transcription activation domain and mediates P53 export from the nucleus into the cytoplasm (Tao and Levine, 1999). P53 can also activate MDM2, defining a feedback loop in which MDM2 controls the level, extent and duration of P53 protein activation (Wu et al., 1993). JNK belongs to Mitogen-activated protein kinases (MAPKs) which mainly function in signal transduction. JNK plays two distinct roles in the control of P53 activity. When inactive, this kinase binds to P53 and targets P53 for degradation by the proteasome. Expression of a constitutively activated JNK, or activation of the upstream kinase MEKK1, increases the level of P53 by allowing escape from degradation (Fuchs et al., 1998b). In contrast, active JNK phosphorylates P53 on threonine 81 and participates in its activation (Buschmann et al., 2001). Complexes between P53 and JNK are preferentially found in G0/G1 phase of cell cycle, in contrast with MDM2-P53 complexes, which are mostly detectable in S and G2/M phases. It has been proposed that JNK may essentially act as a regulator of basal levels of P53 protein in non-stressed cells (Fuchs et al., 1998a).

Under conditions in which the DNA is damaged, P53 mediates DNA repair, cell cycle arrest and apoptosis, largely through transactivation of specific target genes. The diversity functions of P53 are reflected in the wide variety of target genes that are selectively trans-activated depending on the type and severity of the stress stimulus and on the specific amino acid residues of P53 that are phosphorylated or acetylated (Culmsee and Mattson, 2005). Increase of P53 via DNA damage leads to cell cycle arrest in G1 and/or G2, allowing time for DNA repair to take place, and then induces
apoptosis if excess DNA damage is occurring (Levine, 1997). Cell cycle arrest involves
the transcriptional activation of p21/WAF1, a cyclin-dependent kinase inhibitor (Chen
et al., 1996). In the early phase of the P53 response to DNA damage, P53 is stabilised
by phosphorylation of specific residues which impedes the inhibitory effect of MDM2
binding. This allows P53 to activate cell cycle checkpoints giving further time for DNA
repair.

There are 5 major DNA repair pathways, including homologous recombination (HR),
non-homologous end joining (NHEJ); nucleotide excision repair (NER); base excision
repair (BER); and mismatch repair (MMR) (Wood et al., 2001). P53 can directly
interact with proteins involved in HR, NER and BER, for example: BRCA1, ATM,
ATR, DNA-PK, Ref-1, PARP-1 etc., in response to DNA damage (Achanta et al., 2001;
Chen et al., 1999; Evans et al., 2000; Shiloh, 2001; Weslerska-Gadek et al., 1999).
However, if the DNA damage is excessive, P53 can mediate apoptosis by two distinct
mechanisms which are the transcriptional up-regulation of Bax (belonging to Bcl-2
family) (Chipuk et al., 2010) and direct interaction of P53 with mitochondria (Levine,
1997). Therefore, upon DNA damage, p53 participates in a series of regulation in
determining cell fate (Figure 1.5).
Figure 1.5 Diagrammatic representation showing p53-dependent DNA repair, cell cycle arrest and apoptosis as interdependent process in a stressed cell. Under stress (e.g.: hypoxia, excitotoxicity, genotoxic stress and oxidative stress), extracellular signals cross the cell membrane to active P53 directly or indirectly via the c-Jun N-terminal Kinase (JNK) mediated signal pathway. Activated P53 can promote a cyclin-dependent kinase inhibitor p21/WAF to arrest cell at cell cycle check point for DNA repair. Activated P53 can also interact with a series of DNA repair related effectors participating in different DNA repair pathways. For example, P53 can react with breast cancer-associated gene 1 (BRCA1), ataxia telangiectasia mutated (ATM), ATM-related factor (ATR), DNA protein kinase (DNA-PK), poly(ADP-ribose) Polymerase (PARP) and redox effector factor-1 (Ref-1), and function in DNA repair pathways directly including homologous recombinational repair (HRR), nucleotide excision repair (NER) and base excision repair (BER). P53 can also interact with B-cell lymphoma 2 (Bcl-2) family members (e.g: Bcl-2 associated X factor (Bax)) to function in apoptosis. The intercellular level of P53 can be regulated by murine double minute clone 2 (MDM2) and JNK.
Although the important role of P53 has been well established in mammalian models, its actual function in marine organisms has still been not elucidated. Along with the development of molecular techniques, recently *p53* has been cloned from a variety of aquatic organisms, including fish, clam, mussels, oyster and squid. Highly conserved homogenous sequences have been shown among different species, including human, suggesting similar function of P53 is potentially conserved in a phylogenetically disparate group of organisms (Farcy *et al.*, 2008; Kelley *et al.*, 2001; Muttray *et al.*, 2005). *p53* has been widely used as biomarker to indicate genotoxicant-induced responses in fish (Table 1.3) but no direct evidence to link function of *p53* with fish neoplasia development has been established. Walker and Van Beneden’s group has focused their interesting work elucidating development of leukeamia in clam haemocytes. Mutation and expression alteration of *p53* were observed in this naturally occurring cancer model species, suggesting *p53* indeed functions in carcinogenesis and that the pathway it mediated is similar to that in human. Other studies using *p53* as bioindicator in clams have shown clear relationships among environmental pollution, leukemic haemocytes and *p53* expression alteration, however, these data are limited and highly site-specific (Walker *et al.*, 2011). Apart from clam, *p53* has been cloned from mussels, oyster and squid and its expression has been shown to respond to a variety of changes in environmental conditions, indicating that *p53* could be a sensitive biomarker for the application in environmental biomonitoring. However, studies focussing on *p53* function in DNA repair, cell cycle arrest and apoptosis are still limited, mainly because of lack of information about other *p53* mediated molecules and suitable stable study cell lines for aquatic organisms as established for mammalian studies.
Table 1.3 Summary of application of p53 as biomarkers in aquatic organisms

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Species</th>
<th>Experimental conditions</th>
<th>Main reported results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish</td>
<td>Coregonus lavaretus</td>
<td>Fish were injected with different concentration of microcystin-LR (0, 10 and 100 µg/kg of body weight) and then sacrificed at 0, 8, 24, 48 or 72 h later. Liver tissues were collected for investigation</td>
<td>Up-regulated expression of p53 in liver after induction of microcystin-LR</td>
<td>Brzuza et al. (2009)</td>
</tr>
<tr>
<td>Fish</td>
<td>Platichthys flesus</td>
<td>Wild-type or mutant Val147Glu flounder TP53 cDNA cloned in the multicopy vector pAD5 and then expressed in the yeast strain YPH-p21. Yeast transformants were cultured at a range of temperature (20-35 °C) to investigate temperature impact to P53 function</td>
<td>P53 function as temperature-sensitive transcription factor, indicating stress response function</td>
<td>Cachot et al. (2004b)</td>
</tr>
<tr>
<td>Fish</td>
<td>Kryptolebias marmoratus</td>
<td>Fish were exposed to bisphenol A (600 µg/L), 4-nanlyphenol (300 µg/L), and 4-tert-octylphenol (300 µg/L) for 96 h</td>
<td>Up-regulated p53 after endocrine disrupting chemicals exposure</td>
<td>Lee et al. (2008b)</td>
</tr>
<tr>
<td>Fish</td>
<td>Oryzias latipes</td>
<td>3 medaka cell lines (MES1, OLF and OLME) were UV-irradiated at 12, 25 and 100 J/m² for OLF, MES1 and OLME respectively. And 10 medaka fry were subjected to UV irradiation at 900 J/m². The fry and cells were collected for investigation after 0, 6, 12, 24, 48 and 96 h UV irradiation</td>
<td>No effect of UV irradiation on the expression of p53 in cell cultures as well as in fry</td>
<td>Chen et al. (2001)</td>
</tr>
<tr>
<td>Fish</td>
<td>Coregonus lavaretus</td>
<td>Whitefish exposed to B(α)P (10 mg/kg body weight (BW)) for 48 h</td>
<td>Up-regulated p53 in tissues after exposure to B(α)P</td>
<td>Brzuza et al. (2006)</td>
</tr>
<tr>
<td>Fish</td>
<td>Platichthys flesus</td>
<td>Functional analysis of chemically-induced p53 mutation (FACIM) assay was conducted and used to evaluate the mutagenesis of the flounder TP53 exposed in vitro to BPDE (0.01-2 µM) for 1 or 24 h</td>
<td>Dose-dependent increase of P53 mutation rate with increasing concentrations of BPDE</td>
<td>Cachot et al. (2004a)</td>
</tr>
<tr>
<td>Organism</td>
<td>Description</td>
<td>Methodology</td>
<td>Reference</td>
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<td><strong>Platichthys flesus</strong></td>
<td>Fish were treated with a single intraperitoneal injection of B(α)P at dose of 20 mg/kg for up to 21 days.</td>
<td>Mutation in <em>p53</em> gene at the adenine base in the <em>Taq</em>I restriction site of exon 8 after exposure to B(α)P</td>
<td>Sueiro et al. (2000)</td>
<td></td>
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<tr>
<td><strong>Clam</strong></td>
<td>Leukemic clam haemocytes were treated with leptomycin B, topoisomerase II poisons and wheat germ agglutinin <em>in vitro</em> to assess the cytotoxicity, immunocytochemistry and apoptosis in relation to <em>p53</em> function.</td>
<td>Intact <em>P53</em> pathway in leukemic clam haemocytes and maintenance of this tumour phenotype requires nuclear absence of <em>P53</em></td>
<td>Bottger et al. (2008)</td>
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<td><strong>Mya arenaria</strong></td>
<td>Plasmids expressing either Hsp53 (human) and Map53 (clam) were introduced by transient transfection into the p53-null H1229 cell line, and then functionality was assessed.</td>
<td>Similar function in clam <em>P53</em> with human <em>P53</em></td>
<td>Holbrook et al. (2009)</td>
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<td><strong>Clam</strong></td>
<td>Clam collected from an endemic zone for neoplasia. The ploidy of haemocytes was assessed for each clam using flow cytometry and <em>p53/p73</em> gene expression was analysed by real-time PCR.</td>
<td>Evidence of a cytoplasmic <em>P53</em> sequestration mechanisms in clam haemocytic neoplasia</td>
<td>Siah et al. (2008)</td>
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<td><strong>Mya arenaria</strong></td>
<td>Homologues for human <em>p53</em> and <em>p73</em> gene were cloned by RACE PCR and expression patterns for their corresponding protein analysed in tissues from normal and leukemic softshell clams by western blots.</td>
<td>Identification of homologues for <em>p53</em> and <em>p73</em> in softshell clam</td>
<td>Kelley et al. (2001)</td>
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<tr>
<td><strong>Clam</strong></td>
<td>Leukaemia cells were identified using a murine monoclonal antibody, 1E10 which recognizes a leukaemia-specific protein expressed by tumour cells, and mutant P53 protein was detected using a murine monoclonal antibody (PAb240) which reacts with mutant P53.</td>
<td>Mutant <em>p53</em> protein with AC to G transversion at the end of exon 6 in leukaemia cells</td>
<td>Barker et al. (1997)</td>
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<tr>
<td><strong>Mussel</strong></td>
<td>RNA was extracted from neoplastic and normal blood samples from <em>Mytilus trossulus</em> for mutation analysis and gene expression analysed by real-time PCR.</td>
<td>Mutation and changes in expression in neoplastic blood sample</td>
<td>Ciocan et al. (2005)</td>
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<tr>
<td>Organism</td>
<td>Description</td>
<td>Methodology</td>
<td>Result</td>
<td>Reference</td>
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<td><em>Mytilus trossulus</em></td>
<td>Single nucleotide polymorphisms (SNPs) in the coding region sequence of <em>p53</em>-like cDNA from mussels were detected to investigate the association of <em>p53</em> sequence with haemic neoplasia.</td>
<td>Identification of mussel haemic neoplasia associated variations in <em>p53</em>-like cDNA sequence.</td>
<td>Vassilenko et al. (2010)</td>
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<tr>
<td><em>Mytilus sp.</em></td>
<td>Mussels were exposed to B(α)P at 75 nM for 24 h. Digestive gland and haemocytes were collected for <em>p53</em> expression analysis.</td>
<td>Identification of <em>p53</em>-like mRNA isoform in <em>Mytilus sp.</em>.</td>
<td>Banni et al. (2009)</td>
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<td><em>Mytilus sp.</em></td>
<td>Haemocyte of mussels were collected to identify the <em>p53</em>-like isoform: TAp63/73 and ∆Np63/73 using real-time PCR.</td>
<td>Identification of <em>p53</em>-like mRNA isoform in <em>Mytilus sp.</em>.</td>
<td>Muttray et al. (2008)</td>
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<td><em>Mytilus sp.</em></td>
<td><em>p53</em> genes in two species of <em>Mytius edulis</em> and <em>Mytilus trossulus</em> were identified and characterized by real-time PCR with degenerate and specific primers to conserved regions of the gene.</td>
<td>Identification of <em>p53</em> in both <em>Mytilus sp.</em>.</td>
<td>Muttray et al. (2005)</td>
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<td><em>Crassostrea gigas</em></td>
<td>RNA was extracted from oyster collected from four locations along the French coasts. <em>p53</em> transcription expression pattern was analysed by real-time PCR.</td>
<td>Variations at <em>p53</em> mRNA associated with seasonal cycle at different sampling sites.</td>
<td>Farcy et al. (2008)</td>
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<td><em>Euprymna scolopes</em></td>
<td><em>p63</em>-like transcripts were identified by degenerate RT-PCR and RACE PCR. Immunocytochemistry and Western blots using antibody to analyse function of P53 family members.</td>
<td>P53 family members (P63) activation in response to symbiont-induced signal.</td>
<td>Micheal et al. (2006)</td>
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1.4.2 Proto-oncogene *ras*

Proto-oncogenes are expressed during "regulated growth", such as during embryogenesis, wound healing, regeneration of damaged liver, and stimulation of cell mitosis by growth factors. Proto-oncogenes are highly conserved, being detected in species as divergent as yeast, drosophila, and humans. These genes encode for growth factors (e.g. *sis, int*), growth factor receptors with tyrosine kinase activity (*erbB, fms, met, ros* etc.), regulatory proteins in signal transduction (*ras, gsp, gip*), non-receptor tyrosine kinases (*abl, yes, src*), serine/threonine kinases (*mos, raf*), and transcription factors (*myc, fox, c-jun*) (Anderson *et al.*, 1992). The encoded proteins play a crucial role in cellular growth and differentiation (Cantley *et al.*, 1991; Hunter, 1991) and in apoptosis or programmed cell death (Amsterdam and Selvaraj, 1997). Viral oncogenes arise by recombination between cellular proto-oncogenes and the genome of non-transforming retroviruses (Bishop, 1991). Proto-oncogenes can also be activated to cancer-causing oncogenes by mechanisms independent of retroviral involvement. These mechanisms include point mutations and gross DNA rearrangements such as translocation and gene amplification, and it is these mechanisms that generate the oncogenes observed in human and rodent tumours (Halazonetis *et al.*, 2008).

Among all proto-oncogenes, *ras* is the most widely studied. First *H-Ras* were named after the discovery of transforming Harvey rat sarcoma virus which encoded an oncogene hijacked from its host in 1964 (Harvey, 1964). A similar virus was isolated in 1970 and was named the Kirsten rat sarcoma virus or *K-ras* (Kirsten *et al.*, 1970). In 1982, the human genes homologous to the viral genes were elucidated and were designated *c-H-ras* and *c-K-ras* (Chang *et al.*, 1982), and subsequently the third and final Ras family member was isolated from human neuroblastoma samples and termed *c-N-Ras* (Chang *et al.*, 1982). Ras proteins are 21 kDa molecular and can switch the
cycle between the inactive GDP-bound conformation and the active GTP-bound conformation. Guanine nucleotide exchange factors (GEFs) activate Ras by catalysing the release of GDP, facilitating GTP binding due to its 10-fold higher concentration than GDP in the cytosol (Bos et al., 2007). GTP binding induces a conformational change in the Ras switch domains, revealing an effector binding site (Krengel et al., 1990). Ras has an intrinsic GTPase activity that will return the protein to the inactive GDP-bound state. This process is normally slow unless enhanced by GTPase-activating protein (GAP). GAP can enhance the GTPase activity of Ras by at least a factor of $4 \times 10^3$ (Anderson et al., 1992). The members of the Ras-GTP family are crucial players in many signalling networks connecting a great variety of upstream signals to an even wider set of downstream effectors (Figure 1.6). These effectors are involved in the pathways linked to the functional control of a great assortment of cellular outcomes, including cell cycle progression, growth, migration, cytoskeletal changes and apoptosis. The crosstalk between this plethora of signalling pathways and others controlled by different sets of signalling molecules creates molecular networks whose balance is crucial to determine the final outcome of cellular responses (Rajalingam et al., 2007). For example, the Raf family of serine/threonine kinases consists of three members: ARaf, BRaf, and CRaf (Gardner et al., 1993). Following activation by Ras, Raf kinases phosphorylate MAPK family member MEK1 and MEK2, resulting in their activation. MEK proteins subsequently phosphorylate extracellular regulated MAPK (ERK) (ERK1 and ERK2) in the cytoplasm, resulting in their activation and nuclear translocation. ERK kinases have a variety of cytoplasmic and nuclear targets, importantly including ETS transcription factors (containing erythroblast transformation specific (ETS) domain) to regulate the expression of many pro-proliferative genes (DeNicola and Tuveson, 2009).
Figure 1.6 Diagrammatic representation showing proto-oncogene Ras signal pathway in the cell. Upon receiving the signal from a growth factor, Ras changes its conformation from inactive guanosine diphosphate (GDP) to the activated guanosine triphosphate (GTP) form. The activated Ras can react with RAF (a serine/threonine kinase protein) and then pass the signal to initiate gene transcription via a series of mitogen-activated protein kinases (MAPKs) family members, including MAPK kinase (MEK) and extracellular signal-regulated protein kinase (ERK). The conformational change can be regulated by guanine nucleotide exchange factor (GEF) and GTPase-activating protein (GAP).
Oncogenic mutation appears to be the most exclusive mechanism linking \textit{ras} genes to human tumour development. \textit{In vivo}, activating point mutations have been observed in codons 12, 13, 61, 117 and 146 of Ras (Bos, 1988). The main biochemical difference between oncogenic Ras with mutations in codons 12, 13, or 61 and wild-type Ras is the ability of GAP to induce GTP hydrolysis in the active Ras-GTP complex (Scheffzek \textit{et al.}, 1997). In comparison, the mutations in codons 117 and 146 increase the GDP:GTP exchange rate and thus increase the amount of cellular Ras-GTP complex in the absence of an external signal (Krengel \textit{et al.}, 1990). K-Ras is the most commonly mutated member of the Ras family, present in over 90% of ductal pancreatic cancers, 40-50% colorectal cancers, and 30% of non-small cell lung cancers (Downward, 2003). Apart from mutation, overexpression of \textit{K-ras} and \textit{N-ras} was found in bladder carcinomas and \textit{H-ras} was frequently overexpressed in transitional cell carcinomas (Fernandez-Medarde and Santos, 2011). Therefore, Ras is activated through mutation and changes in expression in mammalian cells. The activation of \textit{ras} genes is not an obligatory event but when it occurs it can contribute to both early and advanced stages of human carcinogenesis.

Recently, \textit{ras} genes have been cloned from several fish species, such as tomcod (\textit{Microgadus tomcod}) (Wirgin \textit{et al.}, 1989), winter flounder (\textit{Pseudopleuronectes americanus}) (McMahon \textit{et al.}, 1990), dragonet (\textit{Callionymus lyra}) (Vincent \textit{et al.}, 1998), English sole (\textit{Pleuronectes vetulus}) (Peck-Miller \textit{et al.}, 1998), and applied as a key biomarker to investigate carcinogenesis mechanisms in fish. Mutation in \textit{K-ras} has been observed in the hermaphroditic fish \textit{Rivulus marmoratus} after exposure to endocrine disruptor 4-nonylphenol under laboratory conditions (Lee \textit{et al.}, 2006). Two types of \textit{K-ras} have been cloned with different lengths in the same research work. Both of them showed tissue-specific expression pattern, for example, the longer \textit{K-ras}...
showed higher expression in gonad and intestine and the shorter form was expressed in highest levels in liver of fish. Another study exposed the same fish species to both bisphenol A and 4-nonylphenyl and found up-regulation of N-ras after exposure (Lee et al., 2008a). Tissue specific expression was also reported with the highest levels in fish brain. In contrast, Nogueira et al. (2006) exposed the European eel Anguilla Anguilla to B(α)P in a laboratory experiment; no point mutations or changes in ras gene expression levels were detected when compared to control samples. The relationship between ras and carcinogenesis is still a puzzle in fish model. Similarly, little is known about ras mutation or expression alteration in invertebrates. Ciocan et al. (2006) reported the presence of ras gene mutations and changes in expression from neoplastic haemolymph samples drived from Mytilus trossulus. Point mutation of ras was also reported in Mytilus galloprovincialis after exposure to 12.5% No. 4 fuel-oil under laboratory conditions when compared to control or environmental samples (Lima et al., 2008). Interestingly, this mutation point at codon 35 does not belong to the common mutation hot-spots as discovered in mammalian systems. Recently, Ruiz et al. (2012) have reported a down-regulation of ras expression in Mytilus edulis collected from a PAHs contaminated site, however, no mutation was detected under the same conditions. A cDNA of ras isolated from the shrimp Penaeus japonicas was cloned into E. coli and the protein showed the capability to bind with both GDP and GTP, confirming the similarity of Ras function as observed in humans (Huang and Chuang, 1998). The studies of Ras in marine organisms provide the clue that Ras shares the similar function and mechanism among different species. However, more studies in terms of the crucial role of Ras in carcinogenesis should be pursued in details.

Along with increasingly available genetic information and the development of molecular techniques, molecular biomarkers are important to be included in
biomonitoring. Molecular biomarkers can indicate physiological deregulation associated with the development of undesired outcomes (such as disease and death) at an early stage. Given their highly conserved sequences and important roles in cell regulation, \(p53\) and \(ras\) are considered to have several advantages as biomarkers at molecular level. Firstly, the genetic information is available in a wide range of organisms. It is therefore easier to share study outcomes of \(p53\) and \(ras\) genes, such as cDNA sequences, and make comparison within a range of studies. Secondly, there is evidence that the metabolic activation of B(\(\alpha\))P is proved to induce DNA adducts in humans and rodents (Akcha et al., 2000; Halldórsson et al., 2008; Medeiros et al., 2008). The formation of persistent B(\(\alpha\))P-DNA adducts in target tissues has been shown to be closely related to induced mutations in the \(p53\) and \(ras\) genes (Chaturvedi and Larkshman, 1996). Thirdly, mutations of these two genes have been demonstrated to be closely related with tumour formation and their regulation pathways are relatively well-studied in organism at different ecological levels.

At present, haemic proliferative disorders have been described in several bivalve molluscs, including the softshell clam, \(Mya arenaria\) (Barker et al., 1997) and marine mussels \(Mytilus\ sp\). As filter-feeders, bivalve molluscs are chronically exposed to and concentrate numerous environmental pollutants. Leukaemia in bivalve molluscs may result from several biological phenomena. Firstly, they may filter contaminated sediments and/or water containing carcinogens (e.g. B(\(\alpha\))P). Secondly, they may be exposed to chemicals which behave as tumour promoters. Thirdly, they may simultaneously be exposed to compounds which are xenobiotics (e.g., NPs, IRs). The disease is classified as a leukaemia because the malignant cells divide continuously in the haemolymph (Barker et al., 1997). As the disease progress, leukemic cells penetrate multiple tissues such as the connective tissue, gonads, mantle and foot through blood
Although the origin of tumours in each tissue is not yet clear, both haemocytes and solid gonadal neoplasia have indeed been discovered in mussels living in contaminated environments. In addition, the disease can be transferred from one animal to another via the interaction between organisms (e.g. food chain). Therefore, defining common mechanisms of tumour formation in all animal systems, including invertebrates is required. To date, p53 and ras have been sequenced in various animals, including human, fish, clams and mussels. The amino acid sequences between human and Mytilus sp. shows high conservation for both proteins (Ciocan and Rotchell, 2005; Kelley et al., 2001; Muttray et al., 2005). It is possible to relate carcinogenic chemicals with tumour formation related molecular biomarkers in the laboratory condition.

Against the backdrop of the above information, the main hypotheses of this project are: (1) selected environmental pollutants (B(α)P, C_{60} and tritium) can induce biological responses at different levels of biological organisation in marine mussels Mytilus sp.. The diverse sensitivities of these responses can provide the information for environmental monitoring programme; (2) molecular biomarkers in terms of alterations in gene expression can be a robust and reliable tool to link molecular responses to carcinogenesis processes in the organism, providing early warning for animal and environment health. In response to environmental contaminant induced stress (e.g. DNA damage), the level of gene expression will be altered to cope with the stress. The expression of the tumour suppressor gene p53 is expected to increase under stress, and then, trigger a series of cell defence apparatus related to the initiation of DNA repair, cell cycle arrest and apoptosis. In contrast, the proto-oncogene ras should not exhibit significantly up-regulated expression alteration if no mutation in the genes is induced and tumours developed in organisms. Overexpression of ras, however, is also expected.
if the stress is too excessive for cells to cope with or if the DNA defence mechanism is not efficient. This overexpression may be an indicator of the early stage of tumour development. The objectives of this project were to establish an integrated approach which includes biomarkers, from molecular to individual levels; to assess environmental pollutant-induced responses in *Mytilus sp.* and to understand the function and mechanism of key genes in relation to carcinogenesis in different tissues.
CHAPTER 2

Materials and methods
2.1 Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich Ltd. (Poole, Dorset, UK) and Fisher Scientific Ltd. (Loughborough, Leicestershire, UK), except if stated otherwise. All the solutions/ reagents used in the studies are listed in Appendix I.

2.2 Mussels collection and culture

Mussels (*Mytilus sp.*) of similar shell length (40-50 mm) were collected at low tide from Trebarwith Strand (North Cornwall, UK, 50°38.747’N; 4°45.881’W, Figure 2.1), a relatively clean site where the mussels are found in a healthy condition. After collection, they were immediately transported to the laboratory in a cool box (less than 2 h) and were placed in aerated tanks with filtered (< 10 µm) seawater (three animals per 2 L seawater), where they were kept at 15 °C, and fed daily with the microalga, *Isochrysis galbana* (Liquifry, Interpet, Dorking, UK) at a final concentration of 100,000 algal cells per L seawater. Water was changed daily, where appropriate. At least 14 days were allowed for the mussels to acclimatise before their use in the experiments. In no cases were spawning animals used in the experiments.
2.3 Determination of clearance rate

The clearance rate as a measure of potential physiological effects following exposure to different chemicals was determined as routinely carried out in our laboratory by counting the rate at which mussels fed on algae particles which were suspended in seawater (Al-Subiai et al., 2012; Canty et al., 2009; Coughlan, 1969). Briefly, individual mussels were placed in separate 400 mL glass beakers, each containing 350 mL of seawater. A $12 \times 6$ mm magnetic stirrer was added to each beaker and the vessels were placed on 2 separate 15-point magnetic stirrers, along with 2 identical beakers without mussels for system controls (Figure 2.2). Mussels were allowed to acclimatise for 15 min prior to the addition of 500 μL of Isochrysis algal suspension (final concentration 20,000 algal cells per mL per beaker). The beakers were stirred manually
with a glass rod; then a 20 mL aliquot of water was collected using a glass syringe. This procedure was repeated after 30 min. Water samples were analysed on a Beckham™ Coulter Particle Size and Count Analyser (Z2, USA), with a 100 μm aperture tube fitted and set to count particles between 4.0–10.0 μm in diameter. The clearance rate of the mussels was then calculated using the following equation (E 2.1):

$$ CR = \frac{V(\log_{e} C_1 - \log_{e} C_2)}{T} $$  

E 2.1

Where CR = clearance rate, V = volume of water, C1 and C2 = algal concentration at beginning and end of time interval T.

Figure 2.2 Clearance rate measurement of individual mussel.
2.4 Tissue dissection and histopathological analysis

Mussel shells were opened by severance of the adductor muscle followed by removal of the tissue mass. Samples of adductor muscle, gill, digestive gland and mantle (gonad) were dissected (Figure 2.3) and a portion was preserved in RNALater® (Qiagen, Sussex, UK) for RNA isolation (section 2.7). Another portion of the tissues was placed in histological cassettes and preserved in 10% neutral, buffered formalin and fixed for at least 48 h for histopathology analysis. They were then stored until processing for routine histological examination at room temperature (Figure 2.4) (Bignell et al., 2011; Humason, 1972; Sheir et al., 2010). In general, the preserved tissues were processed using an automatic tissue processor (Leica TP 1020, Nussloch, Germany) where the tissues in cassettes were placed in sample baskets which were automatically immersed into a series of solution tanks according to a pre-set programme for mussel tissues (Table 2.1). After dehydration and clearing, tissues were left transparent, ready for paraffin infiltration. They were then transferred to the paraffin oven (58–60 °C) (Leica, Nussloch, Germany) and were embedded in paraffin blocks and sectioned to a thickness of 5–8 μm by microtome (Leica, Nussloch, Germany). Sectioned tissues were placed on glass slides, dried at room temperature overnight, cleared with xylene, and dehydrated with ethanol. Prepared slides were then stained with haematoxylin (Mayer’s haematoxylin, formula in Appendix I) for 40 min and eosin (1% in 1% calcium nitrate) for 1 min. Slides were examined by light microscopy using an Olympus Vanox-T microscope and photographed using a digital camera (Olympus camedia C-2020 Z) at total magnifications of x100 and x400.
Figure 2.3 Dissection of mussels to obtain different tissues/organs.

Table 2.1 Programme (specific to mussels) for tissue processor

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Purpose</th>
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<tr>
<td>70 % Alcohol</td>
<td>12 h</td>
<td>Dehydration</td>
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<tr>
<td>90 % Alcohol</td>
<td>2 h</td>
<td></td>
</tr>
<tr>
<td>Industrial Methylated Spirit (IMS)</td>
<td>2 h</td>
<td></td>
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<tr>
<td>Absolute alcohol (No. 1)</td>
<td>2 h</td>
<td></td>
</tr>
<tr>
<td>Absolute alcohol (No. 2)</td>
<td>2 h</td>
<td>Clearing</td>
</tr>
<tr>
<td>Xylene (No. 1)</td>
<td>1 h</td>
<td></td>
</tr>
<tr>
<td>Xylene (No. 2)</td>
<td>30 min</td>
<td></td>
</tr>
<tr>
<td>Xylene (No. 3)</td>
<td>30 min</td>
<td></td>
</tr>
<tr>
<td>Wax (No. 1)</td>
<td>1 h</td>
<td>Embedding</td>
</tr>
<tr>
<td>Wax (No. 2)</td>
<td>30 min</td>
<td></td>
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</table>
Figure 2.4 Flow chart to illustrate different steps involved in histopathological analysis of the tissues/organs.
2.5 Determination of programmed cell death or apoptosis in haemocytes using the ‘dual stain’ technique

Haemolymph (200 µL) samples were obtained from the mussels using a 1 mL syringe attached to a 21 gauge needle inserted into the posterior adductor muscle and the samples were transferred to 1.5 mL microcentrifuge tubes (Figure 2.5). Samples were then centrifuged at 9600 x g (relative centrifugal force) for 2 min to pellet the cells (haemocytes). The supernatant was removed and cell pellets were re-suspended in a series of concentrations of H₂O₂ (10 to 10000 µM) by repeated pipetting before incubation for 30 min at 4 °C in the dark. Exposed cell suspensions were then centrifuged at 9600 x g for 2 min and the supernatants were discarded. Cell pellets were re-suspended in 1 mL ice-cold phosphate buffered saline buffer (PBS) [137 mM NaCl, 2.7 mM KCl, 100 mM Na₂HPO₄, 2 mM KH₂PO₄, pH=7.4]. Propidium Iodide (PI) was added to the cell suspension at a final concentration of 5 µg/mL and the mixture was incubated at room temperature for 15 min. Cells were then fixed in 4% paraformaldehyde (substituted by ice-cold acetone) for 20 min at room temperature. Following fixation, 4′,6′-DiAmidino-2-Phenyl Indole (DAPI) was added to cell suspension at final concentration of 5 µg/mL and the mixture was incubated at room temperature for 5 min. Cells were then washed in distilled water to remove excess dye and re-suspended in 200 µL distilled water. Suspended haemocytes (100 µL) were then placed on microscope slides to dry at room temperature in the dark. Dried slides were then analysed using the fluorescence microscope (Leica DMR HCS, UK). Counts for three cell morphotypes were undertaken, including red cells, dark blue cells and bright blue cells. The results are shown in Appendix II.
2.6 Determination of cell viability

Cell viability was determined by cell staining methods as described by Cheung et al. (2006) with the modification of changing the stain to Trypan blue. Basically, haemolymph (100 µL) obtained from mussels was centrifuged at 9600 × g for 2 min. Supernatant was discarded and cell pellets were washed in 100 µL PBS twice before resuspension in 100 µL PBS. Trypan blue solution (40 µL, 0.4% v/v from stock solution, diluted with distilled water) was added into 10 µL prepared cell suspension. The mixture was allowed to settle for 3 min (at least) at room temperature for dead cells to be stained by Trypan blue. Cells were then counted using a haemocytometer under a light microscope (Leitz Laborlux S microscope, UK). Cell viability was calculated using E 2.2.

\[
\text{Cell viability (\%)} = \frac{\text{Total cell number} - \text{Blue cell number}}{\text{Total cell number}} \times 100\% 
\]  

E 2.2
2.7 Determination of DNA strand breaks in the haemocytes of mussels

Single cell gel electrophoresis or the comet assay was carried out to determine DNA strand breaks (Figure 2.6) (Jha, 2008; Kumaravel and Jha, 2006). Briefly, haemolymph (200 µL) samples were obtained from posterior adductor muscle from individual mussels and then centrifuged at 9600 × g for 2 min and the supernatant was discarded and replaced with 200 µL 0.75% (w/v) low melting point agarose (LMPA). The mixture was then applied as two drops of 100 µL to slides pre-coated with 1.5% normal melting agarose (NMA) and air dried for 24 hours before the comet assay. Coverslips were placed over each drop and gels were allowed to set at 4 °C for 1 h. When the gels had solidified to form duplicated microgels, coverslips were gently removed and the slides were immersed for 1 h in cold (4 °C) lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% N-Lauroyl-sarcosine, 1% Triton X 100, 10% DMSO, pH=10] to remove membranes and histones from DNA. After the lysis period, slides were placed in a horizontal electrophoresis unit (TS-COMET-RB, Thistle Scientific, Norway) containing freshly prepared electrophoresis buffer [0.3 M NaOH, 1 mM EDTA, pH>13]. The DNA was allowed to unwind for 30 min to denature before electrophoresis proceeded at 25 V for 30 min. The slides were removed from the electrophoresis tank and gently immersed in neutralization buffer [0.4 M Tris base, pH=7.5] for 5 min and this step was repeated three times followed by rinsing with distilled water for 10 min and left it to dry for overnight. Finally, to visualize comets, 40 µL of ethidium bromide (EB, 20 µg/L) stain was applied to each microgel. Nucleus were randomly selected and measured by video capture and image analysis using Komet 5.0 software (Kinetic Imaging, Liverpool, UK) with 50 nuclei scored per microgel. The software provided measurements for different parameters (e.g. Olive tail moment, tail length etc.) but
DNA percentage in tail was considered as the reliable parameter to evaluate DNA strand breaks (Kumaravel and Jha, 2006).

Figure 2.6 Demonstration of main steps involved in the comet assay.
2.8 RNA isolation from tissues and haemocytes for gene expression analyses

Haemocytes were collected from posterior adductor muscle before digestive gland, adductor muscle, gill and mantle tissues were dissected from individual mussels. All dissected tissues and haemocytes were preserved in RNALater® (Qiagen, Sussex, UK) following the manufacturer’s instructions. Total RNA was extracted from different tissues and haemocytes (starting tissue weight; 20–30 mg) using the RNeasy Mini Kit (Qiagen, Sussex, UK) according to the manufacturer’s instructions and RNA was eluted with 50 μL of RNase free water. RNA concentration and quality was determined using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany) according to manufacturer’s instructions. Purified RNA was then stored at -80 °C freezer until further use.

2.9 Reverse-transcription

RNA (1 mg) was treated with RNase-free DNase (Promega, Southampton, UK) according to the manufacturer’s instructions. Treated RNA concentration and purity were then measured a second time using a NanoDrop® ND-1000 UV-vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) (Figure 2.7). A known concentration of RNA (0.1 to 100 ng) was used for reverse transcription using SuperScript™ II reverse transcriptase kit (Invitrogen, Paisley, UK) and Oligo (dT) primers, according to the manufacturer’s instructions. Resulting cDNA samples were stored at -20 °C for future application.
2.10 Design of primers and optimisation of PCR condition

Primers for target genes (length between 60-300 bp) were designed either according to existing literature or using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA). In general, the length of each primer was between 15-30 bp. The 5’ and 3’ ends should not contain many guanines or cytosines together to prevent the primers folding on themselves and to avoid G/C clamps. Designed primers were obtained from Eurofins MWG operon (Ebersberg, Germany) and diluted to the required concentration using molecular biology grade water (Thermo Scientific, UK) to make the final concentration of 100 µM according to the manufacturer’s instructions. Primer stocks were stored at -20 °C. Working stocks (10 µM) were prepared by dilution of the primary stocks to avoid repeated thawing and freezing of stock.

Standard PCR was performed to confirm the correct amplification of the primers and to determine the best conditions to yield good PCR products, i.e. no primer dimers etc. (Reed et al., 2003). Reaction volumes of 25 µL were used following the recommended solution combinations by the GoTaq® FlexiDNA polymerase manufacturer, which contained 2 µL cDNA template, 1 µL each primer (10 µM), 2.5 µL dNTPs (2.5 mM), 5 µL 5× Buffer, 1.5 µL Mg²⁺, 0.1 µL GoTaq FlexiDNA polymerase (Promega, UK), and 11.9 µL of water. PCR amplification was performed following the Taq polymerase manufacturer’s instructions as follows: 10 min at 95 °C, and 35 cycles of 15 s at 95 °C, 30 s at selected annealing temperature and 20 s at 72 °C, and 5 min at 72 °C. PCR amplification was performed in a Gradient Palm-Cycler™ PCR machine (Corbett Life Science) (Figure 2.7) with a range of annealing temperatures tested for each target gene. The range of annealing temperature was decided according to the temperature given by the Primer Premier 5.0 software ± 5 °C.
Agarose gel electrophoresis was performed using a BioRad Sub-cell horizontal system (Figure 2.7) for submerged gel electrophoresis. The principle of using electrophoresis to separate and identify nucleic acid was thoroughly described by Violy (2000). In practice, agarose gels (2%) were prepared with 2.4 g agarose (Promega Corporation analytical grade) dissolved in 120 mL 1 × TAE electrophoresis buffer by heating. The solution was cooled to approximate 60 °C and EB (1 µL, 20 µg/L) was added and mixed thoroughly. The agarose was then poured into the holding tray ensuring that the teeth of the Teflon comb were immersed, and allowed to set for approximate 30 min at room temperature prior to removal of the comb and submerging into the electrophoresis buffer in the tank. PCR products (8 µL) were combined with Orange G loading solution (2 µL) and then loaded into the wells of the gel. HyperLadder™ V ladder (Bioline, UK) was also loaded into the gel in order to size the DNA fragments. A voltage of 100 V was then applied to the gel and stopped when the dye had migrated an appropriate distance through the gel. Gels were examined and photographed on a G:Box EF gel imaging and analysis system (Syngene, USA) (Figure 2.7). The primers and PCR conditions which produced clear single bands of the desired size were selected for future application.
2.11 Sequencing of PCR products

Sequencing was performed to confirm the identity of amplified PCR products as described by Mount (2004). PCR product (2.5 µL) was incubated with 1 µL of Exosap-IT solution (GE Healthcare, UK) at 37 °C for 15 min, followed by incubation at 80 °C for 15 min to remove any contaminating single-stranded DNA. Samples were then pre-sequenced using the ABI Big dye v3.1 sequencing kit (Applied Biosystems, USA) in 20 µL reaction mixture containing 1 µL ABI premix solution, 3.5 µL 5x Buffer, 1 µL forward primer and 11 µL water. Sequencing amplification was performed as follows: 1 min at 96 °C, 25 cycles of 10 s at 96 °C, 5 s at confirmed annealing temperature for each gene and 4 min at 60 °C. Sequencing products were then purified by ethanol/EDTA precipitation. Samples were briefly spin down before adding 5 µL of 125 mM EDTA and 60 µL of 100% ethanol. The mixture was mixed by inverting for 4 times before incubation for 15 min at room temperature. After incubation, samples were centrifuged for 30 min at 6000 x g. The supernatant was removed before adding 60 µL 70% ethanol and was centrifuged a further time for 15 min at 6000 x g. The supernatants were discarded and samples were left to air dry at 37 °C for at least 4 h. Precipitated sequences were sent to Source Bioscience (Cambridge, UK) for final sequencing. The results were analysed by Chromas software (Technelysium, AU) and subjected to Basic Local Alignment Search Tool (BLAST) to confirm the correct fragment had been amplified.
2.12 Quantitative real-time PCR for the analysis of gene expression

Real-time PCR relies upon the detection and quantification of a target gene expression by using a fluorescent reporter, the signal of which increases in direct proportion to amount of PCR product in a reaction (Heid et al., 1996). In this study, real-time PCR was performed using the SensiMix™ NoRef Kit (Quantace, UK) and Rotor-Gene 6000 real-time qPCR system (Qiagen, UK) (Figure 2.7) using primers specific to target genes. Real time PCR was performed in triplicate for each sample in a 25 μL reaction volume containing 2 μL of cDNA sample, 1 μL of each primer (10 μM), 12.5 μL SensiMix NoRef solution (containing reaction buffer, heat-activated Taq DNA Polymerase, dNTPs, 6 mM MgCl₂, stabilizers) and 0.5 μL SYBR Green I. PCR amplification was performed as follows: 10 min at 95 °C, and 35 cycles of 15 s at 95 °C, 30 s at confirmed annealing temperature and 15 s at 72 °C. PCR products were subjected to melt curve analysis (ramping from 72 °C to 95 °C, rising by 1 degree each step, and acquisition to green fluorescence channel), and selected samples were electrophoresed to verify that a single product was present. Control reactions were performed with no cDNA template (blank control) and no reverse transcribed RNA (negative control) to ensure no background DNA contamination in the samples after DNase treatment. The value of Ct (cycle threshold), which is defined as the number of cycles required for the fluorescent signal to cross the threshold, was analysed for quantification.

Quantification of RNA transcription by real-time PCR can be either relative or absolute. Absolute quantification, also known as the standard curve method, requires the construction of an absolute standard curve that produces a linear relationship between the Ct and known starting concentrations of cDNA. Determination of the copy numbers of RNA transcripts of unknowns is based then on their Ct value (Heid et al., 1996). Nevertheless, the generation of reliable standard material precisely quantified is very
time consuming and the amplification efficiencies of the target cDNA and the cDNA used in the calibration curve have to be identical. In relative quantitation, changes in gene expression are compared to an external standard and/or a reference sample (housekeeping gene). There are many mathematical models to calculate the gene expression from relative quantitation assays (Wong and Medrano, 2005). The comparative Ct methods (‘delta Ct’, E 2.3 and ‘delta delta Ct’, E 2.4) are based on the comparison of the distinct cycle differences (Livak and Schmittgen, 2001).

\[
\Delta Ct = Ct \text{ value of target gene} - Ct \text{ value of reference gene}
\]

\[
2^{-\Delta Ct} = \frac{2^{\text{target gene (Ct control–Ct treated)}}}{2^{\text{reference gene (Ct control–Ct treated)}}}
\]

The main disadvantage of the comparative Ct methods is that they assume equal efficiencies (calculated from a standard curve) of target and reference genes. An efficiency corrected method that accounts for the differences in amplification efficiencies of the target and reference genes has been developed (Pfaffl, 2001; Pfaffl et al., 2002). The main disadvantage of these methods is that they do not take into account run-to-run variances. For more precise results, averages of efficiencies should be taken running different standard curves at separate times.
Figure 2.7 Flow chart showing major steps involved in gene expression analysis.
2.13 Statistic analyses

Statistical analyses were carried out with the aid of Minitab V15 statistical package (Minitab Inc., USA). Significant differences between untreated control and treated or exposed mussels were studied using the Student’s t-test and one-way analysis of variance (ANOVA) after testing for normality of the data and homogeneity of variances. All values are provided as means ± S.E.M (standard error of mean). Significance was established at P<0.05.
CHAPTER 3

An integrated biomarker approach to evaluate the impact of B(α)P in marine mussels: A natural model organism for human cancer research
CHAPTER 3

3.1 INTRODUCTION

Cancer or malignancy is one of the major diseases responsible for the death of human populations, at least in the western world. This disease arises due to accumulation of mutations in the somatic cells of our body. Both tumour suppressor gene and proto-oncogene involved in the multistage process of carcinogenesis. Mutational inactivation of tumour suppressor genes and activation of oncogenes are two of the most frequently observed and thoroughly studied molecular pathways in human cancer research (Bos, 1989; Greenblatt et al., 1994; Solomon et al., 2010). p53, as a representative tumour suppressor gene, serves as the major cellular barrier against cancer development. In almost all human cancers, the p53 pathway is impaired (Weisz et al., 2007). Approximately 50% of tumours sustain mutations in the p53 gene itself, whereas the other half maintains a wild-type p53 but acquires other genetic or epigenetic alterations that compromise the p53 responses (Levine, 1997; Muttray et al., 2008). DNA damage or cellular stress can activate the P53 protein via the orchestrated action of multiple post-translational modifications which either increase the stability of P53 or directly enhance its DNA-binding affinity, leading to arrest of cell cycle progression to allow DNA repair (Warshawsky, 1992).

The ras proto-oncogene encodes low molecular weight guanine nucleotide-binding proteins that cycle between inactive GDP-bound and active GTP-bound forms (Buday and Downward, 2008). Activated ras oncogenes are frequently detected in human and other animal tumours with point mutations occurring within exons 1 and 2; codons 12, 13 and 61 (Lee et al., 2008a; Lima et al., 2008). When this gene suffers a mutation in one of above mentioned codons, the encoded protein inhibits GTPase activity, leading to aberrant cell proliferation, altered cell checkpoint control and cell differentiation (Ross and Nesnow, 1999; Rotchell et al., 2001). Recent studies concerning the
development of carcinogenesis in mammals and fish reported mutant forms of ras in a 
large proportion and wide variety of tumours (Nogueira et al., 2006; Sakuma et al., 2004). About 20% of most types of human malignancies have also been found to 
contain an altered ras sequence (Rajalingam et al., 2007).

Due to the worldwide distribution and ecological habitat of mussels, they are 
extensively used for environmental toxicity and genotoxicity studies (Jha, 2004). Similarly, their worldwide availability could make them sensitive as an important model 
organism for characterising haemic proliferate disorders. At present, these disorders 
have been described in invertebrates, particularly bivalve molluscs, including the soft-
shell clam, Mya arenaria and marine mussels Mytilus sp.. Leukaemia, also known as 
haemic neoplasia, and gonadal neoplasia have been reported in blue mussel Mytilus sp. 
around the world (Ciocan and Sunila, 2005; Galimany and Sunila, 2008). In this context, 
ocurrence of malignancies in bivalve molluscs has also been correlated with higher 
incidence of tumours in human populations who were exposed to increased use of 
herbicides (Van Beneden, 1994). Defining common mechanisms of tumour formation in 
mussels, therefore, could provide valuable information for human cancer research. 
Furthermore, both the gene sequences for p53 and ras have recently become available 
for Mytilus sp. and these show highly conserved sequences across phylogenetically 
different group of organisms, including humans (Rotchell et al., 2009). Therefore, a 
study to elucidate expression of tumour-regulating genes in mussels is both timely and 
warranted, especially when seeking to develop a non-human model to explore the 
underlying mechanisms of neoplasia.

Benzo(α)Pyrene (B(α)P), a representative Polycyclic Aromatic Hydrocarbon (PAH), is 
the most extensively studied genotoxicant and carcinogen in aquatic organisms (Venier 
and Canova, 1996). Various groups of aquatic organisms collected from PAH
contaminated sites, including the European flounder *Platichthys flesus*, the oyster *Crassostrea virginica* and the marine mussel *Mytilus sp.*, have been reported to have a greater incidence of neoplastic disease compared to samples collected from uncontaminated sites (Barber, 2004; Depledge, 1998; Lyons *et al.*, 2004). The main hypotheses of this chapter is that B(α)P as a genotoxic component can induce a series responses in *Mytilus sp.* (i.e. physiological alteration, histopathological abnormalities, DNA strand breaks). In association with DNA strand breaks, the change in expression of key carcinogenesis related genes (i.e. *p53* and *ras*) will be a sensitive and robust biomarker for the investigation of B(α)P induced impact in mussels. This chapter aims to elucidate the gene expression profiles of *p53* and *ras* in *Mytilus sp.* following exposure to B(α)P. An integrated approach with biomarkers at different biological levels of organisation were also analysed prior to gene expression analysis. Gene expression was analysed in different tissues as occurrences of neoplasia have been found to have different susceptibilities among tissues (Greenblatt *et al.*, 1994). To our knowledge, this is the first study to evaluate the tissue-specific expression profile of tumour-regulating genes (*p53* and *ras*) in the blue mussel. Attempts have also been made to link biomarker responses at different levels of biological organisation in mussels following B(α)P exposure.
3.2 MATERIALS AND METHODS

3.2.1 Optimisation of comet assay conditions by in vitro H$_2$O$_2$ exposure

Mussels were collected and maintained as described in Chapter 2.2. Haemolymph (300 µL) samples were collected from the posterior adductor muscle of each mussel and centrifuged as described in Chapter 2.7. The supernatant was removed and replaced with 300 µL of H$_2$O$_2$ solution (diluted with physiological saline), obtaining final nominal concentrations of 0, 10, 50, 100, 200, 500 and 1000 µM H$_2$O$_2$. This concentration range was based on an earlier study using *M. edulis* (Cheung et al., 2006). The samples were then incubated for 30 min at 4 °C in the dark followed by centrifugation at 9600 x g for 2 min. The supernatant was removed and cells were re-suspended in 300 µL of physiological saline. Cell suspensions (100 µL) were applied for cell viability analyses as described in Chapter 2.6 to indicate that majority haemocytes (over 90%) used in the comet assay were alive after exposure to H$_2$O$_2$ to minimize the error caused by dead cells which could increase the proportion of non-chemical induced DNA damage.

The remaining 200 µL cell suspension was applied for optimisation of comet assay conditions. The detailed procedure is described in Chapter 2.7 with adjustments of experimental parameters, such as lysing time, electrophoresis voltage and time, etc., trialed to determine the best comet assay conditions for haemocytes from *Mytilus sp.*.

3.2.2 Optimisation of toxicity of carrier solvent (i.e. acetone) following in vivo exposure

B(α)P is a highly hydrophobic and persistent PAH. In order to achieve more effective dispersion in seawater, acetone was selected as a carrier solvent. However, acetone itself may have an impact on biomarker responses in invertebrates (Cowgill and Milazzo,
1991; LeBlanc and Suprenant, 1983; Zhang and Baer, 2000). The maximum acetone concentration as recommended by the Organization for Economic Cooperation and Development (OECD) for a carrier solvent is 100 μL/L (79 mg/L, 0.01% v/v) (Hutchinson et al., 2006). Three concentrations of acetone (0.005%, 0.01% and 0.1% v/v) were selected to evaluate if acetone can cause significant physiological and genotoxic responses in mussels.

The experimental set-up for the 6-day static exposure is shown in Figure 3.1. Before exposure to the solvent, clearance rates were determined to establish the baseline physiological status of collected mussels. The exposures were then carried out for 6 days at 15 °C and food was withheld during the exposure period. Beakers (2 L) were used as exposure vessels, each containing 1.8 L of seawater (Filtered to 10 μm) and containing one mussel. The experiment was designed based on previous exposure experiments using *Mytilus sp.* in our laboratory with modifications (Al-Subiai et al., 2011; Jha et al., 2005). Vessels were individually aerated using Pasteur pipettes and were sealed with Parafilm™ to minimize the evaporation of acetone. Exposure vessels were checked for mortalities on daily basis. Concurrent controls were also carried out using the same conditions with the exception of no acetone being introduced to the seawater. After 6 days exposure, mussels were collected for clearance rate and the comet assay analyses as described in Chapter 2.3 and 2.7, respectively.
Figure 3.1 Experimental design to determine the potential toxic effects of acetone in mussels.

3.2.3 Exposure of mussels to B(α)P in vivo

B(α)P (range from 0.28 – 5 mg) was dissolved in 5 mL of acetone, and then added to seawater to achieve final nominal B(α)P concentration of 5.6, 56 and 100 µg/L and a final acetone concentration of 0.01% (v/v). The concentration of B(α)P added to the test media represented ‘trace’, ‘intermediate’, and ‘maximum’ concentrations, which typify the range according to literature that induce a response in mussels (Halldórsson et al., 2008; Skarphéinsdóttir et al., 2003). The exposure vessels (in triplicates) were 12 L glass tanks, each containing 10 L of filtered seawater and 10 mussels. The tanks were individually aerated using air stones and were covered with glass to avoid evaporation of B(α)P (Figure 3.2). The tanks were checked for any mortality on daily basis. Seawater quality parameters were checked daily in each tank by measuring dissolved oxygen (96 ± 0.3 %), pH (7.8 ± 0.02), total ammonia (0.04 ± 0.02 mg/L), temperature (15 ± 1 °C) and salinity (31.5 ± 0.15‰) (Multi 340i/SET, WTW, Weilheim, Germany). Water was changed at day 6 and the same concentration of B(α)P was introduced into
each tank as at the start of the experiment. Mussels were only fed 1 day prior to the start of the experiment and there was no feeding during the whole exposure period. Controls were carried out using the same conditions except for the introduction of 0.01% (v/v) acetone only. Triplicate mussels were sampled from each tank after 6 and 12 days exposure. Further detail is indicated in Figure 3.3.

Figure 3.2 Exposure of mussels in the tanks under standard laboratory conditions.

Figure 3.3 Overall experimental design to evaluate impact of B(α)P at different levels of biological organisation in mussels.
3.2.4 Determination of B(α)P concentration in seawater using gas chromatography and mass spectrometry (GC-MS)

Water samples (9 mL) were collected into glass vials and dichloromethane (DCM) (1 mL, HPLC grade, Rathburn Chemicals Ltd., UK) was added. Phenanthrene d10 (1.1 µg in 10 µL dichloromethane) was then added as an internal standard. Following thorough shaking, the mixtures were stored in the dark at 4 °C. Immediately prior to analyses, the DCM layers were removed into glass micro-vials. 2 µL aliquots of the sample extracts were analysed using an Agilent Technologies 6890 N Network GC system interfaced with an Agilent 5973 series Mass Selective detector. A DB-5MS (crosslinked 5% phenyl methyl siloxane) capillary column (30 m) with a film thickness of 0.25 µm and internal diameter 0.25 mm was used for separation, with helium as a carrier gas (maintained at a constant flow rate of 1 mL/min). Extracts were injected splitless, with the injector maintained at 280 °C. The oven temperature programme was 40 °C for 2 min and then increased at 6 °C/min to a final temperature of 300 °C, where it was held for 4 min. The mass spectrometer was operated in electron impact mode (at 70 eV) with the ion source and quadruple analyser temperatures fixed at 230 °C and 150 °C, respectively. Samples were screened for B(α)P and phenanthrene d10 using selected ion monitoring, in which the target ions were 252 and 188, respectively (according to molecular mass). Full scan GC-MS was performed for confirmational purposes. Prior to sample extract analyses, the system was calibrated using authentic standards. With each batch of samples, a solvent blank, a standard mixture and a procedural blank were run in sequence for quality assurance purposes. B(α)P concentration was calculated based on internal standards.
3.2.5 Determination of clearance rate, histopathological effects and DNA strand breaks

Clearance rate was determined prior to haemolymph collection as described in Chapter 2.3. A total of 6 mussels from each treatment were sampled for histopathological analysis. Tissue samples of adductor muscle, gill, digestive gland and mantle were dissected and processed as described in Chapter 2.4. Haemolymph from a total of 9 mussels from each treatment were collected for the comet assay analysis as described in Chapter 2.7.

3.2.6 Gene expression analyses

3.2.6.1 Optimisation of gene expression analyses under control condition

Digestive gland, adductor muscle, gill and mantle tissues from untreated mussels were dissected for RNA isolation. Total RNA from dissected organs/tissues were extracted as described in Chapter 2.8. Serial dilutions of RNA (0.1 ng, 1 ng, 10 ng and 100 ng) were reverse transcribed to cDNA as described in Chapter 2.9. Primers for the target genes p53, ras and reference genes actin and 18S rRNA were designed according to existing literature (Ciocan et al., 2006; Dondero et al., 2006; Muttray et al., 2008) and PCR were performed as mentioned in Chapter 2.10 to determine the suitable annealing temperature for each gene (Table 3.1). All cDNA samples from the dilution series of extracted RNA generated a single product for each gene after gel verification by electrophoresis. Amplicons for each gene were additionally sequenced to confirm the amplification of the correct target gene as described in Chapter 2.11.

3.2.6.2 Tissue specific gene expression analyses after B(α)P exposure

Tissues were dissected from total of 6 mussels from each treatment after both 6 and 12 days exposure to different concentrations of B(α)P. Total RNA from respective tissue
was extracted, cleaned and reverse transcribed (10 ng of RNA) to cDNA as described in Chapters 2.8 and 2.9. Real-time PCR for the target genes: \textit{p53} and \textit{ras} and reference gene \textit{actin} were performed in triplicate for each sample as described in Chapter 2.12.

A validation experiment to verify the efficiencies were approximately equal for both the target genes (i.e. \textit{p53} and \textit{ras}) and the reference gene (i.e. \textit{actin}) was performed. A dilution series of different input amount (1 ng, 10 ng, 50 ng and 100 ng) of target and endogenous control cDNA obtained from gill RNA extracts of control mussels were prepared and then subjected to qPCR. After performing a run, the log of the input amount (a dilution series) vs. $\Delta$Ct (Ct value of target gene – Ct value of reference gene) was plotted, and a linear regression was used to approximate a line through the data. As a guideline, the absolute value of the slope of log input amount vs. $\Delta$Ct should be less than 0.1 (Brzuzan \textit{et al.}, 2006).
### Table 3.1 Primers designed for each gene and their PCR reaction conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Tested $T_A$ Range (°C)</th>
<th>Selected $T_A$ (°C)</th>
<th>Amplicon size (bp)</th>
<th>Genbank accession NO.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p53$</td>
<td>F 5’ TGTGTAGACTGAGGGATTCATTGG 3’</td>
<td>52-62</td>
<td>55</td>
<td>151</td>
<td>AY579472</td>
<td>Muttray et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>R 5’ TTCACCTTCTTCATCAGTTTGT 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ras</td>
<td>F 5’ ATGACGGAATAACAGCT 3’</td>
<td>45-55</td>
<td>50</td>
<td>231</td>
<td>AY679522</td>
<td>Ciocan et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>R 5’ TCCTTCTCCGTGTCAT 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>actin</td>
<td>F 5’ CTCTTGGATGGGAGCGAGGAGA 3’</td>
<td>52-62</td>
<td>55</td>
<td>118</td>
<td>Ab257134</td>
<td>Dondero et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>R 5’ AGGATGGTTGGAATAGTGATT 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$18S rRNA$</td>
<td>F 5’ AACTTTTGCTGATCAGCGACG 3’</td>
<td>52-62</td>
<td>55</td>
<td>150</td>
<td>DQ640512</td>
<td>Muttray et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>R 5’ CGTTTCTCATGCTCCCTCTC 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$T_A$: Anneal temperature for PCR; F: Forward primer; R: Reverse primer.
3.3 RESULTS

3.3.1 Optimisation of the comet assay in haemocytes following exposure to H$_2$O$_2$ under in vitro conditions

Under in vitro conditions, optimisation of the comet assay in haemocytes was performed following exposure to different concentrations of H$_2$O$_2$. Following exposure, cell viability was observed to be above 90% in all of the treatments (Figure 3.4), indicating that DNA strand breaks induced by H$_2$O$_2$ were in live cells. Tail DNA % demonstrated concentration dependent DNA strand breaks in haemocytes of *Mytilus* sp.. Statistical analyses showed a significant, concentration-dependent increase (p<0.05) for DNA strand breaks in exposed samples compared to the concurrent control (Figure 3.5). This was in line with an earlier validation study carried out in the haemocytes of bivalves under laboratory conditions following exposure to H$_2$O$_2$ (Cheung *et al.*, 2006). Thus, the comet assay with optimised conditions as detailed in the methods proved to be a sensitive technique for detecting DNA strand break in *Mytilus* sp. haemocytes.

![Figure 3.4](image.png)

Figure 3.4 Cell viability and DNA strand break (represented as % of Tail DNA) in mussel haemocytes following exposure to H$_2$O$_2$ under in vitro conditions. The dashed line represents cell viability and solid line represents %Tail DNA.
Figure 3.5 Images showing different degrees of DNA damage as observed in the comet assay. (A): control haemocytes after electrophoresis; (B) to (F): increased DNA damage as a function of H$_2$O$_2$ concentrations under *in vitro* exposure conditions. (C) and (F) showed detailed measurement of head and tail DNA.
3.3.2 Optimisation of acetone concentration as solvent control for in vivo exposure

Exposure to a range of acetone concentration was found not to affect the mussels significantly compared to control for clearance rate and DNA strand break induction for any of the treatments (Figure 3.6 and 3.7). The levels of DNA strand breaks in this batch of mussels were found to be quite high compared to published literature and historical control data from our laboratory, despite having no apparent evidence or sign of abnormalities or stress (e.g. spawning). This might be due to time of acclimation of the mussels in the laboratory after collection. The results however did not show any significant difference between control and the different treatment concentrations. This indicated that acetone does not induce any significant physiological and genotoxic stress in Mytilus sp. even at the highest concentration of 0.1% (v/v). Therefore, acetone as carrier solvent at the final concentration of 0.01% (v/v) was selected for the B(α)P exposure experiment.

![Clearance rates in Mytilus sp. (n=9) following 6 days exposure to acetone under in vivo condition.](image)

Figure 3.6 Clearance rates in Mytilus sp. (n=9) following 6 days exposure to acetone under in vivo condition.
Figure 3.7 Induction of DNA strand breaks (represented as % Tail DNA) in the haemocytes of *Mytilus sp.* (n=9) following 6 days exposure to acetone under *in vivo* condition.

### 3.3.3 Chemical analyses using GC-MS

Known amounts of B(α)P (dissolved in acetone) were injected into the seawater systems to provide nominal concentrations of 5.6, 56 and 100 μg/L. Samples of the seawater were collected immediately after the addition and after 6 & 12 days. They were extracted into DCM and analysed by GC-MS. Immediately after addition, concentrations of 49 ± 15 and 92 ± 3 μg/L were recorded for the 56 and 100 μg/L nominal concentrations. For all other samples, the levels were below the limit of detection of the selected analytical technique (< 0.3 μg/L).
3.3.4 Biomarker responses to B(α)P at different levels of biological organisation

Clearance rates as an indicator of physiological response to indicate feeding behaviour of the mussels remained unaffected by any of the exposure regimes and none of the exposed mussels fed differently to those in the untreated control mussels. This suggests that there was no significant physiological response, in term of feeding behaviour, in mussels induced by the range of sublethal B(α)P concentrations used in the experiment. The lowest clearance rates were obtained for the 56 µg/L B(α)P exposure (1.23 L/h and 0.86 L/h after 6 and 12 days exposure, respectively), suggesting individual mussels were reacting more promptly with the middle range B(α)P exposure concentration. Following 12 days exposure, 30% decline in clearance rate was detected compared to 6 days for all exposure conditions, including the untreated control (Figure 3.8).

Figure 3.8 Clearance rates of Isochrysis galbana in Mytilus sp., following 6 & 12 days B(α)P exposure under in vivo conditions. Each bar represents the means of 9 replicates examined (n=9) and S.E.M are indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p < 0.05.
Histological observations indicated that there were no histopathological signs of abnormalities in untreated specimens (e.g. haemocyte infiltration, necrosis or other injuries). In contrast, histopathological changes in B(α)P treated mussel tissues were identified (Figure 3.9). The digestive gland showed changes in digestive tubule structure, as featured by diffused nuclei and lack of distinction in some epithelial cells. Exposed gills had swollen filaments filled with haemocytes, with the front cilia of epithelial border absent, i.e. filament necrosis. The adductor muscle samples showed histological abnormalities with loss of muscle bundle structure, increased intracellular spaces and decreased extracellular spaces in connective tissue. Tissue damage was also identified in treated mantle which showed decreased adipogranular (ADG) and vesicular connective tissue (VCT) cells in the connective tissue, as well as loss of gonadal duct structure and breakage of epithelial lining of tubules. Histopathological abnormalities were not observed in all B(α)P treated samples (n=6) and no concentration-response relationship could be identified from the quantified results (Table 3.2). No occurrence of neoplasia was found in any sampled tissues.

Table 3.2 Percentage of histopathological changes in different tissues following in vivo B(α)P exposure (n=6) in mussels

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>5.6 µg/L</th>
<th>56 µg/L</th>
<th>100 µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 D</td>
<td>12 D</td>
<td>6 D</td>
<td>12 D</td>
</tr>
<tr>
<td>Adductor muscle</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>66.7</td>
</tr>
<tr>
<td>Digestive gland</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>33.3</td>
</tr>
<tr>
<td>Gill</td>
<td>0</td>
<td>0</td>
<td>16.7</td>
<td>0</td>
</tr>
<tr>
<td>Mantle</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 3.9 Light micrographs of sections through digestive gland, gill, adductor muscle and mantle of *Mytilus sp.* showing histological structure of control and B(α)P treated mussels stained with H & E at 5 μm thickness. A-E are untreated controls; a-e are exposed to B(α)P. A & a: digestive gland; B & b: gill; C & c: adductor muscle; D & d: mantle (male); E & e: mantle (female), where A-D are 400x magnification and E-F are 100x. dt=digestive tubule; ct=connective tissues; at=atrophy; fc=frontal cilia; lc=lateral cilia; gf=gill filaments; amb=adductor muscle block; mgt=male gonad tubule; fgt=female gonad tubule. Scale bar = 25 μm.
Single cell gel electrophoresis (comet assay) was used to measure the degree of DNA strand breaks in mussel haemocytes by monitoring the proportion of DNA in the tail (Figure 3.10). Induction of DNA strand breaks was maximum at the highest B(α)P concentration of 100 µg/L on day 6 with 67.86 ± 1.26% tail DNA. A significant concentration-dependent increase in DNA strand breaks was obtained in comparison to the untreated control (p<0.05). A similar result was obtained after 12 days B(α)P exposure. A significant decrease in DNA strand breaks was seen in the treated groups after 12 days exposure in comparison to 6 days at all three concentrations. On the contrary, no significant difference was observed in untreated control group for both the exposure periods.

Figure 3.10 Induction of DNA strand breaks (represented as % Tail DNA) in Mytilus sp. haemolymph following 6 & 12 days in vivo B(α)P exposure. * indicates significant increase of %Tail DNA in treated groups compared to control group (p<0.05). # indicates significant differences of %Tail DNA between day 6 and day 12 within each treated groups (p<0.05).
3.3.5 Optimisation of gene expression analyses

Total RNA was extracted from different tissues and the concentration was measured using the Agilent 6000 Nano assay. The electropherogram of the ladder and example of RNA samples are shown in Figure 3.11A and B, respectively. The successful ladder run showed the major feature of 7 well resolved peaks with correct peak size assignment in the electropherogram. Good quality RNA showed the major feature of 3 well resolved peaks, including 1 marker peak (time of 22.5 s) and 2 ribosomal peaks (time of 41.12 s and 46.67 s for 18S and 28S rRNA, respectively). The peak area of 18S rRNA was significantly greater than 28S rRNA in sample electropherograms (Figure 3.11B). Both 18S and 28S are the sub-units of rRNA which is classified according to their sedimentation coefficients (S). In mussels, 18S rRNA band was clearer than the 28S rRNA band in the gel profile of a successful run sample (Figure 3.11C). Both these results indicate breaks in 28S rRNA which is in agreement with previous findings on other mollusks and invertebrate, and was likely due to ‘conversion’ of the 28S into components of the size of 18S during the extraction procedure (Muttray et al., 2008). This ‘conversion’ is caused by the denaturation of 28S rRNA to release two similar sized fragments that both migrate closely with 18S rRNA (Winnebeck et al., 2010). The ranges of extracted RNA concentration were variable depending on the tissue type. RNA concentrations were in the range of 10-25 ng/µL from adductor muscles, 200-400 ng/µL from gill, 600-1100 ng/µL from digestive gland and 70-300 ng/µL from mantle.
Figure 3.11 Agilent 6000 Nano chip assay results. (A) the electropherogram of the ladder; (B) the electropherogram of RNA extracted from adductor muscle as an example; (C) the band locations of 18S and 28S rRNA in adductor muscle.
Following PCR analyses, selected PCR amplicons were electrophoresed and the sizes of the resulting single bands for both target and reference genes were as expected (Figure 3.12). Weaker $p53$ bands were yielded compared with the other three selected genes ($ras$, $actin$ and $18S rRNA$), indicating $p53$ had relatively less amplification compared to the other three genes after 35 cycles of PCR. Sequencing of the amplicons confirmed the amplification of the intended targets.

Figure 3.12 Gel images of amplified target genes. Lanes 1-4: $p53$ at size of 151 bp; lanes 5-8: $ras$ at size of 231 bp; lanes 1’-3’: $actin$ at size of 118 bp; lanes 5’-8’: $18S rRNA$ at size of 150 bp; lanes 9 and 9’: 50 bp ladder.
Real-time PCR using a dilution series of RNA was performed to help select a suitable starting RNA concentration for future applications. The amplification curves showed the amplification of expected genes with a lower Ct value when the initial RNA mass was higher which indicated early amplification (Figure 3.13). Melting curve analyses showed that $p53$, $ras$, $actin$ and $18S$ rRNA amplification always resulted in a single product with melting temperatures $™$ of 81, 82, 82.5 and 85 °C, respectively (Figure 3.14). No peaks were found at lower Tm indicating no primer-dimer was formed with all 4 pairs of selected primers. No peaks were found at gene specific melting temperatures in the reverse transcription negative controls and no template control samples indicating no contaminating DNA was present in the RNA used. The standard curves of 4 selected genes which were generated from the quantitation analyses indicated acceptable linear relationships between RNA amount (range from 0.1 ng to 100 ng) and the Ct values ($R^2$>0.98) (Figure 3.15). The linear relationship for $p53$ expression did not show a perfect fit ($R^2$=0.85) (Figure 3.15A) indicating relatively low amplification which may be caused by lower expression of $p53$ in tissues as observed in the gel electrophoresis results. In addition, amplification curves for $p53$ showed weak amplification at the starting concentrations of 0.10 ng and 1 ng being below the limits of detection for this primer set (Figure 3.13A). Therefore, 10 ng RNA was selected for the following gene expression analyses.
Figure 3.13 Examples of real-time PCR amplification curve of p53 (A), ras (B), actin (C) and 18S rRNA (D) using different initial quantities of RNA.
Figure 3.14 Real-time qPCR melting curves of target genes. A: \(p53\) melting curve indicating Tm of 81 °C; B: \(ras\) melting curve indicating Tm of 82 °C; C: \(actin\) melting curve indicating Tm of 82.5 °C and D: \(18S\) rRNA melting curve indicating Tm of 85 °C.
Figure 3.15 Example of standard curves of 4 target genes. A-D are standard curves plotted as dilutions of started RNA quality against the Ct values for p53, ras, actin and 18S rRNA respectively.
3.3.6 Analyses of $p53$ and $ras$ expression efficiency in gill tissue

Tissues from mussels exposed to 56 µg/L B(α)P were selected for gene expression analyses as this concentration induced the greatest feeding behaviour response based on clearance rate and significant DNA strand breaks at cellular level. The log of the input amount of cDNA (dilution series) vs. $\Delta$Ct (difference of Ct value between target gene, $p53/ras$ and reference gene, actin) for the RT-qPCR of $p53$ and $ras$ was plotted, and a linear regression was used to approximate a line through the data (Figure 3.16). The slopes of log input amount vs. $\Delta$Ct of $p53$ and $ras$ were 0.0998 and -0.0128, respectively, indicating similar reaction efficiency (slope < 0.1) of the target gene ($p53$ and $ras$) and control gene (actin) (Brzuzan et al., 2006). The efficiency curve also showed relatively stable expression of $p53$ and $ras$ in gill tissue after normalisation with the housekeeping gene actin (P<0.05). This indicated that actin is a suitable housekeeping gene for the use in normalisation of gene expression results in mussels.

![Figure 3.16](image_url)

Figure 3.16 A plot of the log of input amounts of cDNA against respective $\Delta$Ct value for $p53$ and $ras$ in control gill tissue as described in material and method section. Straight line presents $p53$ gene and dotted line presents $ras$ gene.
3.3.7 Analyses of p53 and ras expressions in different tissues under control condition

p53 and ras expression are presented as ΔCt (E 2.3) (Figure 3.17 ). A higher ΔCt value represents later amplification of the target gene and therefore lower gene expression. p53 transcription showed a tissue-specific expression pattern. The maximum expression was seen in gill tissue with the lowest ΔCt (2.02 ± 0.48 and 1.50 ± 0.56) after 6 and 12 days exposure, respectively, followed with the digestive gland, mantle and adductor muscle in all untreated samples for both the incubation periods. A significantly lower p53 expression level was seen in the adductor muscle compared to the other three tissues (p<0.05). Mantle tissue also showed significantly different p53 expression with higher levels compared to adductor muscle but less than the digestive gland and gill tissues. Gill tissue revealed the highest expression of p53 and was significantly different to other tissues apart from digestive gland in Figure 3.17A. p53 expression was not affected by different incubation time in gill, digestive gland and mantle under control condition. In contrast, significantly higher p53 expression in adductor muscle was found after 12 days incubation (ΔCt = 6.15 ± 1.69) compared to 6 days (ΔCt = 10.48 ± 0.75) (Figure 3.17A). A similar tissue-specific expression abundance was obtained for ras expression analyses with the trend of gill > digestive gland > mantle > adductor muscle. Significantly lower ras expression was detected in the adductor muscle and mantle. ras expression showed no significant difference in gill and digestive gland for both incubation times under control conditions. ras expression abundance was significantly increased in adductor muscle after 12 days incubation (Figure 3.17B).
Figure 3.17 Constitutive p53 (A) and ras (B) genes expression patterns in various tissues. The gene transcript levels were semi-quantified in adductor muscle, digestive gland, gill and mantle using real-time qPCR and are expressed relative to actin level. Each bar represents the means of 6 replicates examined (n=6) and S.E.M are indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p<0.05.
3.3.8 Relative quantification of p53 and ras expression induced by B(α)P

Relative quantification of p53 and ras expression was normalised in different tissues using the $2^{-\Delta\Delta Ct}$ method (E 2.4) (Livak and Schmittgen, 2001; Pfaffl et al., 2002) (Figure 3.18). Wide inter-individual variation of p53 expression was revealed in all the tissues. After 6 days exposure, a significant increase in p53 expression was detected in the adductor muscle, approximately $2.34 \pm 0.89$-fold higher than the control (relative expression level = 1). A small but insignificant decrease in p53 expression was found in the other three tissues. After 12 days of exposure, significantly increased p53 expression was detected in both the mantle and adductor muscle with $5.93 \pm 0.83$- and $3.28 \pm 1.23$-fold higher expression levels respectively. Low induced p53 expression in gill and digestive gland was identified but it was not significantly different compared to the control. p53 expression also increased with longer B(α)P exposure period. About 6-fold higher p53 expression was seen in the mantle after 12 days exposure compared to 6 days. The other three tissues also showed slightly increased p53 expression after longer exposure times but the differences were not statistically significant (Figure 3.18A).

There was no significantly induced or suppressed ras expression in gill, digestive gland and mantle after 6 days exposure (Figure 3.18B). A significant $2.29 \pm 1.01$-fold higher ras expression was seen in the adductor muscle after 6 days exposure. After 12 days exposure, the mantle showed significantly (2.18 ± 1.14-fold) higher ras expression but the adductor muscle and digestive gland showed no significant increase in expression. The gill tissue showed a slightly decreased ras expression but was not statistically significant. Increased ras expression was found in all the tissues apart from adductor muscle after longer B(α)P exposure. In contrast, no significant difference in ras expression was seen in the digestive gland and gill after 12 days compared to 6 days exposure. Slightly decreased expression was seen in the adductor muscle again.
indicating tissue specific expression.

Figure 3.18 Relative quantitative \( p53 \) (A) and \( ras \) (B) gene expression pattern in various tissues exposed to B(\( \alpha \))P at 56 µg/L for 6 and 12 days. * presents significantly induced gene expression.
3.3.9 Relative quantification of $p53$ and $ras$ expression in adductor muscle

Relative $p53$ expression changed together with exposure concentrations and exposure time to B(α)P. $p53$ expression in adductor muscle was significantly increased after B(α)P exposure at both 56 and 100 µg/L concentrations (Figure 3.19A); lower $p53$ relative expression was, however, only obtained at the higher concentration of B(α)P (100 µg/L) over the same exposure period. In contrast, an increased expression was found after longer (12 days) exposure compared to the shorter 6 days exposure at the same exposure concentration. Only the adductor muscle exposed to 56 µg/L B(α)P for 6 days showed significantly induced $ras$ expression, approximately $2.28 \pm 1.01$-fold higher (Figure 3.19B). No significant change in $ras$ expression was identified in any treatment conditions. As with $p53$ expression, the B(α)P exposure concentration and time also affected $ras$ relative expression. A significant decrease in $ras$ expression was seen at the higher exposure concentration (100 µg/L, about 2.63-fold less) after 6 days exposure compared to the lower exposure concentration. In contrast, the expression remained at similar levels for both the exposure concentrations after 12 days. Interestingly, expression levels decreased in the 56 µg/L exposure concentration after a longer time exposure, while no significant alteration in expression was seen after 12 days exposure compared to 6 days exposure at the dose of 100 µg/L. This suggests that exposure dose and time influence $ras$ expression differently. High inter-individual variations were again found for all the samples.
Figure 3.19 Relative quantitative p53 (A) and ras (B) genes expression pattern in adductor muscle exposed to B(α)P at 56 µg/L and 100 µg/L for 6 and 12 days. * represents significant increased genes expressions compared to control level. # presents significant decline of gene expression compared between different exposure doses.
3.4 DISCUSSION

3.4.1 H\textsubscript{2}O\textsubscript{2} exposure under \textit{in vitro} conditions

H\textsubscript{2}O\textsubscript{2} exposure under \textit{in vitro} conditions indicated that the comet assay was sensitive enough to detect DNA damage in \textit{Mytilus} sp. haemocytes. At the highest exposure concentration of 1000 µM, H\textsubscript{2}O\textsubscript{2} induced the most DNA damage in haemocytes but without causing cell death, with cell viability results showing greater than 90% of live cells. In this study, investigations of DNA damage at H\textsubscript{2}O\textsubscript{2} concentrations higher than 1000 µM were not included. However, the results showed there was no significant difference in tail DNA% between 500 µM and 1000 µM treated samples. A study by Cheung \textit{et al.} (2006) also showed similar results observing that tail DNA% in \textit{M. edulis} haemocytes was almost continuous from 500 to 5000 µM of H\textsubscript{2}O\textsubscript{2}. These results indicate that cell death will occur only when the damaged level reach a certain level, otherwise, DNA repair and cell cycle checking mechanisms will become involved to defend cells against DNA damage. As only short term exposure carried out in the optimisation, it is also possible that cells can be released from H\textsubscript{2}O\textsubscript{2} induced stress rather soon after the exposure to avoid more severe damage, i.e., cell death. The results also indicated that the comet assay is a sensitive tool to evaluate chemical-induced responses because it can detect genetic changes at low exposure levels.

3.4.2 Acetone exposure under \textit{in vivo} conditions

The acetone \textit{in vivo} exposure results indicated that acetone does not induce significant DNA damage and physiological changes in mussels at a concentration of 0.1% (v/v) because tail DNA% and feeding rate results showed no significant difference between control and treated mussels. The 0.01% (v/v) acetone concentration was selected to dissolve the desired concentrations of B(α)P for the \textit{in vivo} exposure experiment as this concentration was confirmed to be well within the concentrations where the
physiological and genetic changes were not seen to be induced in *Mytilus sp.*. However, the degree of DNA damage was considerably higher compared to the results for H₂O₂ *in vitro* exposure experiment, suggesting cells were already under stress but the stress was not induced by acetone as there was no difference between exposed and control groups. The detailed discussion on this high level of DNA damage in haemocytes under control condition was addressed in Chapter 7.

### 3.4.3 B(α)P exposure under *in vivo* conditions

The *Mytilus sp. in vivo* exposure experiments with B(α)P were set-up for 6 and 12 days. The concentrations selected for the experiments were those that have been previously shown to induce a response in mussels (Halldórsson *et al.*, 2008). They do, however, exceed the documented solubility of the compound (2.3 μg/L) (ToxProbe Inc., 1997). The presence of dissolved organic matter has been shown to enhance solubility (Doring and Marschner, 1998) and owing to the high K<sub>ow</sub> (6.04) (Readman *et al.*, 1982) B(α)P rapidly partitions onto particulates and the mussels themselves. This is consistent with the comparatively low GC-MS measured concentrations, their variability and the rapid depletion of the B(α)P from the aqueous phase.

In the higher B(α)P exposure experiments (56 and 100 μg/L), there was no observed difference in clearance rates between untreated control and exposed mussels. This suggests that *Mytilus sp.* is relatively tolerant to these types and concentration range of organic chemicals and can metabolise them without showing marked physiological changes in feeding behaviour (Canty *et al.*, 2009). Previous work has reported that blue mussels exposed to 1 μg/L C<sup>14</sup>-labeled B(α)P for 6 h, followed by 6 h in clean seawater, had tissue concentrations 70-80% of the initial B(α)P concentration introduced into the water (Durand *et al.*, 2002), suggesting the ability of mussels to accumulate B(α)P in the tissues. Furthermore, research on mussels exposed to radio-labeled B(α)P also suggested
that \( \text{B(}\alpha\text{)P} \) can easily bioaccumulate in tissues (Skarphéinsdóttir et al., 2003).

Clearance rates of mussels after 12 days exposure were significantly lower than 6 days, suggesting other conditions, for example starving stress, rather than direct \( \text{B(}\alpha\text{)P} \) -induced stress, affected the physiological condition of the mussels. Lower molecular weight PAHs have been reported to exert narcotic effects on the gill ciliary activity, leading to decreased clearance rates in blue mussels (Halldórsson et al., 2008). This does not apply to this study as \( \text{B(}\alpha\text{)P} \) has a higher molecular weight and has not been shown to exert narcotic effects on gill activity (Donkin et al., 1991). Only a minor decrease in clearance rates was observed at 56 \( \mu \text{g/L} \) \( \text{B(}\alpha\text{)P} \) exposure which was statistically insignificant compared to control. This effect may have been caused by the direct toxic effect of \( \text{B(}\alpha\text{)P} \) rather than narcotic gill ciliary activity as \( \text{B(}\alpha\text{)P} \) has been shown to induce blood cell lysosomal membrane damage in mussels (Okay et al., 2000).

Histopathological results indicated physiological changes in mussel tissues; however, these changes were not detected in a concentration-dependent manner. A total of 6 mussels was examined for histopathological analyses. This sample size was not large enough to confirm the presence of a concentration-dependent relationship (Aarab et al., 2008; Bignell et al., 2008). No tumours (neoplasia) were found in any of the tissues sampled. Only necrosis was observed in some of the treated tissues, indicative of the very early stage of tumour development (Vakkila and Lotze, 2004). However, in line with other aquatic invertebrates, attempts to induce neoplasia in vivo in mussels has as of yet been unsuccessful. This indicates that the etiology of neoplasia in invertebrates under laboratory conditions involves factors other than pollution alone (Barber, 2004). The complex and multistep of carcinogenesis is also an explanation of no neoplasia was found after short term in vivo \( \text{B(}\alpha\text{)P} \) exposure under applied conditions. Nonetheless,
the histopathological results provide convincing evidence of the physiological toxic effects of B(α)P that could lead to disease development such as neoplasia and suppression of immune function (Carlson et al., 2004; Depledge, 1998).

Significantly higher incidence of DNA strand breaks was also observed at elevated B(α)P concentrations, demonstrating the capability of B(α)P to induce a genotoxic response in *Mytilus sp.* Interestingly, the level of DNA damage, showed a significant decrease in DNA strand breaks after 12 days exposure compared to day 6. This decrease suggests that *Mytilus sp.* is responding to the exposure to B(α)P probably via the involvement of cell defences, e.g. DNA repair, DNA recombination and cell cycle checking mechanisms (Villela et al., 2006). The mechanism of decreased DNA damaged is likely to be via the replacement of damaged cells through apoptosis (Hook and Lee, 2004). Toxicant concentrations via dosage or exposure period are required to reach a threshold level before DNA repair systems are initiated (Ching et al., 2001). Since *in vivo* control values were relatively high (30% of Tail DNA) to begin with when compared to the *in vitro* study using H₂O₂ (less than 10% of Tail DNA) (Cheung et al., 2006), it is likely that the mussels used in our study were already stressed and therefore, were more sensitive to B(α)P thereby lowering their toxicity threshold. Although both DNA strand breaks and histopathological effects suggest that B(α)P can indeed cause stress in *Mytilus sp.*, gene expression studies on key neoplasia genes, which are highly conserved in structure and function in a range of natural biota, are required to indicate whether this PAH could introduce mutagenesis and carcinogenesis via similar tumour development pathways in all organisms ranging from bacteria to man as concluded by Mohn and Deraat (1993).

In normal conditions, without any induced stress, *p53* and *ras* expression showed a tissue-specific expression level pattern. The highest expression abundance was detected
in the gill tissue followed by the digestive gland, mantle and adductor muscle tissues. Of all four tissues, the gill and digestive gland of bivalve molluscs play the most important role in food collection, absorption and digestion (Aarab et al., 2008). The gill is the initial tissue to interact with genotoxic chemicals present in the water and the digestive gland is the main centre for metabolic regulation, participating in the mechanisms of immune defence and homeostatic regulation, as well as in the processing of detoxification and elimination of xenobiotics (Moore and Alen, 2002). Cells in these two tissues are likely to be faced with immediate stresses and therefore, are likely to be best adapted to respond to a wide range of stresses. As such it would be expected that any DNA repair, detoxification and neoplastic defence mechanisms such as p53 and ras would be most active in these tissues.

Enhanced expression of p53 has been shown in mammalian studies where brains were treated by adrenalectomy and excitotoxic treatment (Sakhi et al., 1994; Schreiber et al., 1994). Increased expression has also been shown with apoptosis of antral ovarian follicles in rats in response to exposure to sulfur dioxide and B(α)P (Qin and Meng, 2009). Gao et al. (2011) have also reported p53 overexpression in mouse cervix via DNA damage following B(α)P exposure. Interestingly, not all the tissues studied showed increased p53 and ras expression after exposure to B(α)P in this study. Only the adductor muscle, for both the exposure periods, and the mantle, after 12 days exposure, showed an obvious increase in expression for both the tumour-regulating genes (p53 and ras). The adductor muscle is the main tissue required for shell movement and the haemolymph can be easily extracted from it. It consists of less cell types compared to other tissue, e.g. digestive gland which is consisted with about 6 different cell types (Birmelin et al., 1999). Mantle is the main reproductive tissue for germ cell production and has previously been found with gonadal neoplasia (solid tumours) in mussels.
collected from polluted sites (Barber, 2004; Barber et al., 2002) while no evidence of neoplasia occurred in other tissues in the same individuals. The increase in \( p53 \) and \( ras \) expression in the mantle seen after 12 days and other studies support that mantle cells or germ cells during spermatogenesis process could be more sensitive to chemicals. Using fish as models for environmental carcinogenesis, it has also been reported that different tissues responded differently to carcinogens to generate different types of tumour (Bailey et al., 1996).

Average \( p53 \) and \( ras \) expression increased after longer exposure periods to B(\( \alpha \))P. The increase, however, was not significant. The high inter-individual variation within our sample set is likely to be caused by intrinsic and extrinsic factors experienced by the individual mussels. This could include gender, reproductive stage, or the variability of cell composition in each tissue type (Klaus and Keijer, 2004; Mitchelmore and Chipman, 1998a). It is worth noting that the accumulation of organic pollutants in mussels is determined by a dynamic balance between uptake and depuration processes that are, in turn, influenced by a dynamic equilibrium between pollutants in sediment, water, food particles and the organisms themselves (Venier and Canova, 1996). Other physiological factors such as feeding and reproduction also affect the pollutant uptake in an individual organism. Sexual status, which affects the lipid content of an individual, can also influence pollutant accumulation. As a consequence, the body burden of organic pollutants in individuals inhabiting the same site, as well as the resultant antioxidative responses, may vary considerably (Cheung et al., 2001). Therefore, high inter-individual variability was already exist in mussels after collection prior to further variability caused by B(\( \alpha \))P uptake and accumulation in different tissues during the experiment.

The adductor muscle tissue showed increased \( p53 \) and \( ras \) expression at the B(\( \alpha \))P
concentration applied which was at 56 μg/L. Consequently, a higher exposure level was examined to determine whether there was any concentration-dependent tumour-regulating gene expression. A lack of clear p53 gene induction was found at the higher B(α)P exposure concentration (100 μg/L) suggesting that expression was inhibited in the mussels exposed to higher concentrations of carcinogens. This down-regulation might further initiate tumour development as inhibited p53 expression has been found in several human cancers where the wild-type p53 was mutated (Shu et al., 2007; Troester et al., 2006). Interestingly, no clear concentration-response relationship for ras expression was observed, i.e. a significant decline of ras expression obtained at higher B(α)P concentration after 6 days exposure, but with no difference of expression seen after 12 days in this study. There is no existing evidence to explain B(α)P induced responses in ras expression and the mechanisms remain unknown. One explanation could be the relatively high threshold of chemical concentration to induce increased ras expression. In addition, it is believed the lack of increased ras expression at higher concentration of carcinogen (100 μg/L) probably involves a series of signaling pathways, as ras functions in cell differentiation and proliferation in humans (Patra, 2008; Rajalingam et al., 2007), and will require further study. The result of longer exposure times increased p53 expression which explains the finding of decreased DNA damage after 12 days exposure indicating the potential involvement of DNA repair mechanisms. This is further supported by the fact that both p53 and ras function in association with DNA repair and apoptosis processes (Patra, 2008; Rajalingam et al., 2007; Shu et al., 2007; Troester et al., 2006). The increased p53 expression suggests that at the concentration of 56 μg/L B(α)P can induce p53 expression to halt the cell in G0 phase for repair. ras, as a proto-oncogene, will be activated too when the cells undergo this stress. No neoplastic cells were found in the experimental animals suggesting the lack of increased ras expression did not cause the response to the ras
related pathways leading to uncontrolled cell growth.

Combining DNA strand break and gene expression results, B(α)P can significantly induce DNA damage in haemocytes and increases expression of $p53$, but this is not significant for all four different tissues, possibly due to tissue specific differential sensitivity to B(α)P. The degree of DNA damage measured in the haemocytes may be different in other cell types and therefore, as mixture of different cell types, tissues might have different responses to B(α)P. In addition, similar results regarding significant increases in DNA damage are not always necessarily related to an increased $p53$/$ras$ expression. This was found in human cells under in vitro conditions where increased micronuclei formation had no obvious relationship with $p53$ expression following exposure to certain chemicals using cell lines (Salazar et al., 2009). In addition, Banni et al. (2009) found up-regulation of $p53$ in the digestive gland tissue and a large down regulation in haemocytes following exposure of $Mytilus$ $sp$. to B(α)P and crude oil. In this study, no modulation for $p53$ expression was observed in gill, mantle and adductor muscle tissue. Moreover, a significant increase in DNA strand breaks was observed only in haemocytes. These results further suggest that gene expression in single cell types may give significantly different results in different tissues; bearing in mind that genotoxicity is a cell specific process.

In conclusion, whilst B(α)P induced tissue and DNA damage in exposed mussels confirms its function as genotoxicant, it also induces tumour-regulating genes (i.e. $p53$ and $ras$) expression following 12 days B(α)P exposure with high inter-individual variation. Normal expression of $p53$ and $ras$ occurred in a tissue-specific manner, likely being closely related to tissue function. B(α)P significantly induced $p53$ and $ras$ expression in the adductor muscle and mantle tissue only. Moreover, DNA damage has been confirmed in association with increased expression of tumour-regulating gene
expression, i.e. *p53* and *ras* genes (Halazonetis *et al.*, 2008), but there is no significant correlation between these biological responses. A potential reason could be that DNA damage analyses was only carried out on the haemocytes due to technical limitations while the gene expression analysis was performed in whole tissues. Therefore, the *p53* and *ras* expression in haemocytes are needed to fill this gap. DNA strand breaks can also been measured in the cells of individual tissues (Emmanouil *et al.*, 2007a; Mitchelmore and Chipman, 1998b). It is, however, difficult to be involved in the adopted approach due to the scare of samples. In addition, it has been documented that both *p53* and *ras* function is closely related to post-transcriptional modification in response to DNA damage (Appella and Anderson, 2001; Artandi and Attardi, 2005; Goodwin *et al.*, 2005; Patra, 2008). With each stress, the responses may show similarities, but there will also be differences essential for eliciting a unique molecular signalling outcome. It appears, therefore, that multiple sites targeted by an integrated network of signalling pathways highly sensitive to genotoxic stresses must be modified to yield a functional p53 (Appella and Anderson, 2001). Therefore, the work at translational levels of *p53* and *ras* is required to further elucidate the mechanism.
CHAPTER 4

Integrated biological responses and tissue-specific expression of $p53$ and $ras$ genes in marine mussels following exposure to benzo(α)pyrene and/or C$_{60}$ fullerenes
4.1 INTRODUCTION

Anthropogenic contaminants are discharged into aquatic environments at a large scale in line with increased industry development and human activities. A large proportion of these are potentially genotoxic and carcinogenic substances (Jha, 2004). Apart from PAHs, other contaminants, such as metals, endocrine disrupting chemicals (EDCs) and persistent organic pollutants (POPs) etc., due to their inherent chemical properties also cause environmental stress. Organisms in nature are generally exposed to mixtures of different substances which can interact in many ways (i.e. additively, synergistically, or antagonistically) to induce biological responses at different levels of biological organisation. The interaction between compounds potentially changes the toxic properties compared to when the single compound is present on its own, and in turn causes altered stress in the organisms (Holmstrup et al., 2010; Loureiro et al., 2009). Elucidating the response of organisms to a exposure of mixed chemicals is more environmentally realistic.

B(α)P has been proven to induce a series of responses in marine mussels, *Mytilus sp.*, at different levels of biological organisation in the previous study (Chapter 3). The expression pattern of *p53* and *ras* genes also presents tissue-specific changes after exposure. The successful application of the integrated biomarker approach to evaluate B(α)P induced biological responses in mussels makes it possible to include another type of environmentally realistic compound to study the interactive effects in mussels following combined exposures.

In the present study engineered nanoparticles (ENPs) were selected as another model chemical due to their increased use and production in a wide range of applications, meaning they are increasingly being released as pollutants in the environment to cause
potential damages in the biota (Hassellöv et al., 2008; Ju-Nam and Lead, 2008; Wilkinson et al., 2007).

Features of ENPs are a large surface area to volume ratio, small size, and high surface reactivity, which can potentially cause harmful effects to living organisms (Yang et al., 2010). C$_{60}$ fullerenes (C$_{60}$), a family of carbon allotropes in the shape of a hollow sphere, are one of the most ubiquitous ENPs; their unique properties make them ideal candidates for widespread applications in areas as diverse as drug delivery and energy conversion. Given their growing use, a number of studies have indicated that C$_{60}$ can potentially cause cell damage by inducing cellular oxidative stress as it has been shown to be able to cross (external) cellular membranes and be preferentially localised to the cellular organelles (e.g. mitochondria, lysosomes, nuclei) and the cytoplasm, (Baun et al., 2008). Studies have also indicated that C$_{60}$ can cause oxidative damage in rat liver microsomes following photosensitisation (Kamat et al., 1998). Another consideration concerning C$_{60}$ is the potential interactive effect between suspended C$_{60}$ and other aquatic pollutants. The toxicity of C$_{60}$ and associated contaminants is particularly interesting as different C$_{60}$-derivatives are being investigated as potential drug-carriers, intended for transporting pharmaceuticals towards specific organs and overcoming cell defences such as the blood-brain barrier (Levi et al., 2006). This potential vector-function of C$_{60}$ may be a significant factor, when considering environmental effects of C$_{60}$, due to possible interaction with other anthropogenic contaminants in the environment. After discharge, C$_{60}$ may interact with other xenobiotic compounds as demonstrated by sorption studies with phenanthrene carried out by Yang et al. (2006). This interaction may alter the availability of these compounds to aquatic organisms as they could potentially carry more low soluble compounds into cells. However, the lack
of information on the environmental levels of C₆₀ makes it difficult to estimate its ecologically relevant concentrations.

In this integrated study, biomarkers at different biological levels, as applied in Chapter 3, were adopted to evaluate the biological responses in \textit{Mytilus sp.} induced by B(α)P and C₆₀ either alone or in combination. In addition to tumour-related gene expression analysis, total glutathione (tGSH) level was also selected to indicate the potential oxidative stress induced by B(α)P and/or C₆₀. Oxidative stress is potentially experienced by all aerobic life when anti-oxidant defences are overcome by pro-oxidant forces. Oxidative damage results from an imbalance between the production and removal of oxidants (e.g. free radicals), and it has been shown that environmental contaminants may actually enhance oxidative stress in aquatic organisms (Winston, 1991). It has been reported that the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent metabolism of B(α)P in the digestive gland of \textit{Mytilus edulis} results in the production of hydroxyl and superoxide anion radicals, which are extremely potent oxidants and capable of reacting with critical cellular macromolecules, including DNA and proteins (Cheung \textit{et al.}, 2001; Livingstone \textit{et al.}, 1988). C₆₀, itself, has also been reported to induce oxidative stress in cells (Oberdörster, 2004). Therefore, biomarkers which can indicate oxidative stress are required for analysis of C₆₀-induced responses in organisms.

Over 100 years of biomedical research have documented innumerable situations in which glutathione (GSH) — a tripeptide consisting of glycine, cysteine and glutamic acid—participates in essential aspects of cellular homeostasis (Pompella, 2003). Cell injury induced by electrophiles was long believed to be the mere result of alkylation of cellular macromolecules by their reactive metabolites (Hayes and McLellam, 1999). Several studies, however, highlighted that, in some instances, most of the cell injury occurs after GSH depletion and may actually depend on the onset of extensive,
uncontrolled oxidative processes (Casini et al., 1982; Casini et al., 1985; Comporti, 1989). GSH has revealed different aspects either as the major endogenous antioxidant produced by cells, participating directly in the neutralization of free radicals and reactive oxygen compounds removing $\text{H}_2\text{O}_2$ by coupling the reduction to $\text{H}_2\text{O}$ with the oxidation of glutathione (Hannam et al., 2010). GSH is also among the most efficient tools that cells can exploit in detoxification of drugs and xenobiotics in general by direct conjugation. GSH in fact is both a nucleophile and a reducant, and can therefore react with electrophilic or oxidizing species before the latter interact with more critical cellular constituents such as nucleic acids and proteins.

It is hypothesised that the interaction between B(α)P and C$_{60}$ will change the responses of *Mytilus* sp. at different biological organisations when compared to individual chemical induced response. Biomarkers at different biological levels can show a variety sensitivity to B(α)P exposure either alone or in combination with C$_{60}$. A tissue-specific gene expression, as well as a chemical-specific expression pattern of *p53* and *ras*, following the exposure to B(α)P and/or C$_{60}$ is also hypothesised. The main aim of this study is to apply the established integrated approach (including clearance rate, histopathological abnormalities, tGSH level alteration, DNA strand breaks and transcriptional alteration of *p53* and *ras*) to assess impacts in *Mytilus* sp. following the exposure of model PAH (B(α)P) and ENP (C$_{60}$) to understand the potential risk caused by the interaction of two groups of chemicals in a marine bivalve.
4.2 MATERIAL AND METHODS

4.2.1 Experiment design

Mussels were collected at Trebarwith Stand in June 2010 and maintained as described in Chapter 2.2. The experimental design for the 3 days static exposure period adopted in this study is shown in Figure 4.1. The exposures were carried out at 15 °C and mussels were fed at the beginning of the exposure (day 0). The exposure vessels were 12 L glass tanks, each containing 10 L of seawater (filtered to 10 μm) and containing ten mussels. The tanks were individually aerated using Pasteur pipettes and were covered with glass.

Appropriate volumes of B(α)P dissolved in acetone were added to the seawater to yield nominal final concentration of 56 µg/L, and in each case, the final acetone concentration was 0.01% (v/v). The B(α)P concentration of 56 µg/L selected for the experiment has been previously studied to induce different levels of responses in mussels (Halldórsson et al., 2008; Skarphéinsdóttir et al., 2003). The stock suspension of C60 in seawater was ultra-sonicated (35 kHz frequency, Fisherbrand FB 11010) for 2 h prior to the start of the experiment to ensure C60 was well suspended and added to seawater to yield the final concentration of 1 mg/L. This concentration of C60 has been reported to induce a series of responses in mussels in a previous study in our laboratory (Al-Subiai et al., 2012). The same concentrations of chemicals were re-dosed 1 h after the seawater was changed and mussels were fed every day during the 3 days exposure time. After 3 days exposure, seawater was changed and mussels were fed daily for another 3 days without any chemical addition for mussel recovery. The tanks were checked for mortalities on a daily basis throughout the whole experiment. Seawater quality parameters were checked daily in each tank as described in Chapter 3.2.3.
4.2.2 Determination of B(α)P concentration in water samples by GC-MS

Water samples, taken at the beginning of experiment, were placed into glass bottles with dichloromethane (DCM) at a 1:100 ratio (10 mL DCM: 1 L seawater sample), shaken thoroughly and stored in the dark at 4 °C. Samples were spiked with 2 mL phenanthrene d10 (42 mg/L) as an internal standard. After spiking, samples were processed by routine GC-MS measurement as described in Chapter 3.2.4.
4.2.3 Determination of C\textsubscript{60} concentration in tissue samples by high-performance liquid chromatography (HPLC)

Adductor muscle, digestive gland and gill tissue were dissected from individual mussels exposed to C\textsubscript{60} only and were carefully washed with pure toluene to ensure that no C\textsubscript{60} particles were adsorbed to the surface of the organs. Tissues were then extracted in toluene at a 1:9 ratio (v/v) followed by ultra-sonication of the homogenates for 15 min and centrifugation at 9000 × g prior to HPLC analysis. The HPLC method was developed for C\textsubscript{60} analysis using a Hypersil (5µm) Elite C18 (250 × 4.6 mm I.D.) column. The mobile phase was composed of pure toluene. Mobile phase flow-rate was set at 1.0 mL/min. Sample injections were performed manually with volumes of 100 µL. The eluent was monitored at 330 nm using a Shimadzu SPD-6 AV UV-Vis spectrophotometer (Shimadzu, Germany). Integration was performed using a Shimadzu-C-R3A chromatopac data processor (Shimadzu, Germany). For HPLC calibration, a standard curve was generated for C\textsubscript{60} concentrations ranging from 0.125 to 2.0 mg/L. The concentration of C\textsubscript{60} in test samples was determined by comparison to the standard curve.

4.2.4 Determination of clearance rate, histopathological effects and DNA strand breaks

A total of 6 mussels were collected from each treatment at each sampling day. Clearance rate, histopathological effects and DNA strand breaks were analysed as described in Chapters 2.3, 2.4 and 2.7, respectively. It should be noted that histopathological analysis was only applied to tissue samples collected after B(α)P exposure either alone or in combination with C\textsubscript{60}. No such analysis carried out for tissue samples following C\textsubscript{60} alone exposure because the samples were used for C\textsubscript{60} bioconcentration analysis.
4.2.5 Determination of tGSH level in adductor muscle

The posterior adductor muscles from three mussels (0.2 g wet weight), collected after 3 days exposure and 3 days recovery, were dissected and were homogenized using the method as described by Al-Subiai et al. (2009). Briefly, the tissues were ground with acid-washed sand (0.5 g) using ice-cold extraction buffer (20 mM Tris-chloride, pH 7.6, containing 0.15 M KCl, 0.5 M sucrose and 1 mM EDTA, freshly supplemented with 1 mM DTT and 100 µL protease inhibitor cocktail (Sigma-P2714; reconstituted according to manufacturer’s instructions) using a ratio of 1:3 (w/v). The crude homogenate was centrifuged for 35 min (10,500 × g at 4 ºC) and then the supernatant was separated and stored at -80 ºC until use.

The tGSH (i.e. reduced: GSH, and oxidised: glutathione disulphide (GSSG)) content in adductor muscle extract was determined as described by Al-Subiai et al. (2009). Samples were treated with 5-5''-Dithio-bis(2-nitrobenzoic acid) (DTNB) by mixing at a 1:1 ratio (v/v) with buffered DTNB (10 mM DTNB in 100 mM potassium phosphate 235 µL, pH 7.5, containing 5 mM EDTA). Glutathione reductase (0.6 U, Sigma G-3664 from Saccharomyces cerevisiae) was mixed with DTNB-treated samples (40 µL). After equilibration for 1 min the reaction was started by the addition of 1 mM NADPH (60 µL). The rate of absorbance decrease at 412 nm was measured over 5 min. A 20 µM GSH standard and a blank were used to calibrate the results. tGSH contents were measured in triplicate in 96 well plates using a microplate reader (Optimax, Molecular Devices, Sunnyvale, CA).
4.2.6 Gene expression analyses

Haemolymph and tissues, including digestive gland, adductor muscle, mantle and gill, were collected from total of 6 mussels from each treatment at each sampling day. Total RNA was extracted, cleaned by DNase treatment and reverse transcribed (10 ng of RNA) to cDNA as described in Chapters 2.8 and 2.9. Real-time PCR for target genes: p53 and ras and reference gene: 18S rRNA (18S) was performed in triplicate for each sample as described in Chapter 2.12. The primer sequence for 18S is detailed in Table 3.2 (Chapter 3). It is important to note that the reference gene in this study was changed to 18S after the analysis of the stability of 18S and actin by geNorm qbasePLUS software (geNorm biology, USA) following manufacturer’s instruction. In general, the software has been written to automatically calculate the gene-stability measure $M$ for all control genes in a given set of samples. The average pairwise variation of a particular gene ($M$ value) is defined as the internal control gene-stability based on the determination of pairwise variation of every control gene with all other control genes as the standard deviation of the logarithmically transformed expression ratios. The calculation relies on the principle that the expression ratio of two ideal internal control genes is identical in all samples, regardless of the experimental condition or cell type. In this way, variation of the expression ratios of two real-life housekeeping genes reflects the fact that one (or both) of the genes is (are) not constantly expressed, with increasing variation in ratio corresponding to decreasing expression stability ($M > 1.5$). Genes with the lowest $M$ values have the most stable expression. The program enables elimination of the worst-scoring housekeeping gene (that is, the one with the highest $M$ value) and recalculation of new $M$ values for the remaining genes (Vandesompele et al., 2002).
4.3 RESULTS

4.3.1 Chemical analysis using GC-MS and HPLC

Known amounts of B(α)P (dissolved in acetone) were injected into the seawater systems to provide nominal concentrations of 56 µg/L. Samples of the seawater were collected immediately after the addition. They were extracted into DCM and analysed by GC-MS. Immediately after addition, concentrations of 47±15 µg/L were recorded for the 56 µg/L nominal concentrations.

Serially diluted C₆₀ was measured by HPLC to generate a standard curve for calibration (Figure 4.2), which showed good linear fit between C₆₀ concentration (range from 0.125 to 2.0 mg/L) and area of the peak (R²=0.996) (Figure 4.3). Concentration of test samples was determined by comparison to the standard curve. Adductor muscle, digestive gland and gill tissue were dissected from mussels sampled after 3 days C₆₀ (1 mg/L) exposure and after additional 3 days recovery from C₆₀ exposure in fresh seawater. The results showed significantly higher C₆₀ concentrations compared to controls in all three tissues after 3 days exposure, suggesting the ability of mussels to accumulate C₆₀ in organs (Figure 4.4). Significantly higher amounts of C₆₀ (14.267 ± 7.207 µg C₆₀/gww) was bioaccumulated in the digestive gland, followed by gill and adductor muscle (Figure 4.5). After 3 days recovery, C₆₀ concentrations in the three tissues dropped in comparison with after exposure for 3 days and similar to control levels (fresh seawater only), indicating that the C₆₀ that had accumulated in each tissue had been bio-transformed and/or excreted from the tissues after this time (Figure 4.6). The digestive gland showed higher bio-transformation ability as the C₆₀ concentration significantly decreased (0.630 ± 0.189 µg/gww) after recovery compared to 3 days exposure. The control levels of C₆₀ were consistent in all the samples after both exposure and recovery, indicating good performance of the HPLC.
Figure 4.2 Demonstration of peaks in chromatograph at different concentrations of \( \text{C}_{60} \) standard.

Figure 4.3 Standard curve of \( \text{C}_{60} \) concentration to peak area.
Figure 4.4 C\textsubscript{60} concentrations in tissues after 3 days exposure to C\textsubscript{60}. Star indicates significantly increased concentration in exposed mussel tissues in comparison to control maintained in fresh seawater only.

Figure 4.5 Demonstration of C\textsubscript{60} peak in chromatograph in tissues after exposure to C\textsubscript{60} alone for 3 days.
Figure 4.6 $C_{60}$ concentrations in different tissues of mussels after 3 days recovery in fresh seawater. Star indicates significantly decreased $C_{60}$ concentration after recovery in comparison with control.
4.3.2 Clearance rate

There was no significant difference in clearance rate after 1 day exposure to any chemicals compared to the fresh seawater control (Figure 4.7). Significant increase in clearance rate was found after exposure to the chemicals for 3 days. Mussels showed the most activated feeding behaviour after B(α)P exposure only (about 2-fold increase compared to fresh seawater control samples) followed by fresh C₆₀. The combination of B(α)P and C₆₀ did not change the physiological response of mussels in terms of feeding behaviour. After 3 days recovery from exposure, all sampled mussels showed a further increased clearance rate including the control (but not significantly) compared to samples collected after exposure. The increase was significant compared to 1 day exposure but not significant in comparison to 3 days exposure, except for samples recovered from B(α)P and C₆₀ combined exposure.

![Figure 4.7 Clearance rate in mussels sampled after 1 and 3 days exposure and 3 days recovery. Each bar represents the means of 6 replicates examined (n=6) and S.E.M are indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p < 0.05.](image-url)
4.3.3 Histopathological analysis

Histopathological analysis of the adductor muscle, digestive gland, gill and mantle tissues showed no pathological signs in control specimens such as haemocyte infiltration, necrosis or other injuries. However, there were pathological alterations in treated mussel tissues (Figures 4.8, 4.9).

4.3.3.1 Posterior adductor muscle

Transverse section of the posterior adductor muscle showed normal histology consisting of muscle blocks, each made of distinct bundles of muscle fibres. The bundles of muscle were surrounded with connective tissue. There was no evidence of haemocyte infiltration, necrosis or other injuries in the controls or untreated mussels (Figure 4.8A1). The adductor muscle showed histological abnormalities after B(α)P and B(α)P in combination with C\textsubscript{60} exposures, i.e. loss of muscle bundle structure, increase in intracellular spaces and decrease in extracellular spaces of connective tissue with an extreme example of complete breakdown of bundles of muscle fibres (Figure 4.8A2-A3).

4.3.3.2 Digestive gland

Transverse sections of the digestive gland in controls showed normal structures (several round/oval) lined by columnar epithelia. All the digestive tubules were connected to each other by connective tissue. There was no evidence of haemocyte infiltration, necrosis or other injuries in the digestive gland of control mussels (Figure 4.8B1). Most of the digestive tubules collected after B(α)P exposures showed reduced epithelial cell height and haemocyte infiltration inside the tubules and in surrounding connective tissue (Figure 4.8B2). The histological abnormalities after combined exposure showed different features, such as no clear distinction in some epithelial cells and destroyed...
architecture of digestive tubules. In some extreme cases, the complete breakdown of the epithelium was observed (Figure 4.8B3).

4.3.3.3 Gill
The histopathological analysis showed several abnormalities in gills of mussels in exposed or treated conditions. Gills from the control group showed well preserved structures including gill filaments covered with a ciliated epithelium on their external surface, simple frontal cilia, and lateral cilia. The frontal cilia emerge from the frontal epithelia, while the lateral cilia emerge from lateral cells (Figure 4.8C1). Most of the gills from B(α)P treated mussels exhibited injuries, i.e. swollen gill filaments filled with haemocytes, inflammation and filament necrosis (Figure 4.8C2). Most of gills from mussels collected after combined exposure showed abnormalities such as absence of the front epithelial border, hyperplasia in the frontal and lateral cilia, and hypoplasia in the lateral cilia. In addition, pore structures were only found in frontal epithelial of gills dissected from mussels after combined exposure (Figure 4.8C3).

4.3.3.4 Mantle
The histopathological analysis showed normal mantle tissues to contain gonads (testis for male and ovary for female) and connective tissues. Gonads consist of an organized network of branching tubules and appear as follicles. The tubules terminate into a short gonado-duct that opens into the mantle cavity (Figure 4.9). There were no significant histological abnormalities in mantle tissue after B(α)P exposure either alone or in combination with C_{60}.

Although, the histopathological alterations were observed in some tissue samples after chemical exposures, it is worth to noting that not all treated samples exhibited abnormalities. Figures 4.8 and 4.9 only showed the examples of histopathological profiles of tissues in control and exposed groups. A summary of percentage of tissues
that showed abnormalities is presented in Table 4.1. Increased occurrence of abnormalities was found in all tissues after exposures and a slightly decreased occurrence was also found after recovery in fresh seawater for 3 days compared to 3 days exposure. There was no difference in percentage abnormalities induced by the two types of exposure. No tissues showed more sensitivity to a particular exposure types.

Table 4.1 Percentage of histopathological abnormalities in different tissues following *in vivo* B(α)P exposure alone or in combination with C₆₀ (n=6).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>1 day exposure</th>
<th>3 days exposure</th>
<th>3 days recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B(α)P</td>
<td>B(α)P+C₆₀</td>
<td>B(α)P</td>
<td>B(α)P</td>
</tr>
<tr>
<td>Adductor muscle</td>
<td>0</td>
<td>33</td>
<td>17</td>
<td>83</td>
</tr>
<tr>
<td>Digestive gland</td>
<td>0</td>
<td>33</td>
<td>17</td>
<td>67</td>
</tr>
<tr>
<td>Gill</td>
<td>0</td>
<td>33</td>
<td>33</td>
<td>83</td>
</tr>
<tr>
<td>Mantle</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 4.8 Light micrographs of digestive gland, gill, adductor muscle of *Mytilus sp.* showing histological structures of control and treated mussels stained with H & E at 5 μm thickness. A1-C1: control; A2-C2: exposed to B(α)P; A3-C3: exposed to B(α)P with C₆₀. A: adductor muscle (×200 times); B: digestive gland (×400 times); C: gill (×400 times). dt=digestive tubule; ct=connective tissues; fc=frontal cilia; lc=lateral cilia; gf=gill filaments; amb=adductor muscle block. Black triangle indicates abnormalities. Scale bar=20 μm.

Figure 4.9 Light micrographs of mantle of *Mytilus sp.* showing histological structure of control mussels stained with H & E at 5 μm thickness. MF: female mantle; MM: male mantle; MC: mantle connective tissue. fgt=female gonad tubule; mgt=male gonad tubule; ct=connective tissue. Scale bar=20 μm.
4.3.4 DNA strand break analysis by comet assay

There was no significant increase in % tail DNA after 1 day exposure to B(α)P and/or C\textsubscript{60} (Figure 4.10). Significantly increased DNA strand breaks (statistical level p < 0.05) were found after 3 days exposure where the highest DNA damage (70% tail DNA) was induced by B(α)P exposure only followed by C\textsubscript{60} only (62%) and B(α)P in combination with C\textsubscript{60} (56%) exposure. After 3 days recovery, DNA damage was significantly decreased compared to the 3 days exposed samples. However, there was still a significantly increased DNA damage induced by chemicals compared to the control condition.

![Graph showing DNA strand break analysis](image)

Figure 4.10 Induction of DNA strand break (represented as % Tail DNA) in *Mytilus* sp. haemocytes following 1 & 3 days exposure to B(α)P and/or C\textsubscript{60}. * indicates significant increase of % Tail DNA in exposed groups compared with control group (p < 0.05). # indicates significant differences of % Tail DNA among different time treated samples (p < 0.05).
4.3.5 Total glutathione (tGSH) analysis

The tGSH level in adductor muscle tissue was measured in mussels sampled after 3 days of exposure to B(α)P and/or C60 (Figure 4.10). There was an increased level of tGSH after exposure to chemical treatments. The increase was significant for individual C60 or B(α)P exposed samples but not significant after combined exposure compared to control samples.

![Graph showing total glutathione levels in adductor muscle after 3 days exposure to chemicals, either alone or in combination. * indicate significant increase compared to control (p<0.05).]

Figure 4.11 Total glutathione levels in adductor muscle after 3 days exposure to chemicals, either alone or in combination. * indicate significant increase compared to control (p<0.05).
4.3.6 Gene expression analyses

4.3.6.1 \(p53\) and \(ras\) expression in different tissues after normalisation with 18S rRNA

Tissue samples (adductor muscle, digestive gland, gill and mantle) together with haemocyte samples were collected after 1 and 3 days exposure and 3 days recovery for gene expression analyses. The geNorm analysis was applied to compare the stability of 18S and \(actin\) expression in gill tissues of mussels under different conditions. An output \(M\)-value of less than 1.5 indicates the housekeeping gene is suitable as an internal standard for gene expression normalisation. Lower \(M\)-values indicate higher expression stability. The geNorm analysis generated a lower \(M\)-value for 18S compared to \(actin\), suggesting 18S is more stable, probably because its transcript is so much higher expressed and will not reflect regular mRNAs. It is important to note that the \(M\)-values of both 18S and \(actin\) were less than 1.5, which indicates that both the genes are suitable as housekeeping genes, however, as 18S was lower; this was selected for the study to make sure the normalised relative gene expression is more accurate. In order to evaluate if using 18S as housekeeping gene will significantly change the normalisation results of gene expression analyses, the expression abundance of \(p53\) and \(ras\) in different tissues were analysed using the \(\Delta Ct\) method (E 2.3) in control mussels (Figure 4.12). After normalisation, \(p53\) showed highest expression in the digestive gland, followed with gill, mantle, haemocytes and adductor muscle. The expression abundance of \(ras\) in all the tissue showed the following trend: haemocyte>digestive gland> adductor muscle \(\approx\) gill> mantle.
Figure 4.12 Constitutive p53 (A) and ras (B) genes expression pattern in various tissues. The gene transcript levels were semi-quantified in tissues using real-time qPCR and are expressed relative to 18S level. Each bar represents the means of 6 replicates (n=6) and S.E.M are indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p<0.05.
4.3.6.2 Relative quantification of $p53$ expression in different tissues following exposure to chemicals

The relative quantification of $p53$ and $ras$ expression was normalised in different tissues by $2^{-\Delta\Delta Ct}$ method (E 2.4) using $18S$ as housekeeping gene. Relative expression of 1 is defined as the control level after normalisation with housekeeping gene and control. High inter-individual variation was found in all the results.

In haemocytes (Figure 4.13A), induced $p53$ expression was only found after 3 days exposure to $B(\alpha)P$ alone ($1.81 \pm 0.33$-fold). After exposure to $C_{60}$ alone, significantly increased $p53$ relative expression was detected after 1 day of exposure and kept increasing after 3 days exposure, however, the increase was not significant compared to 1 day exposure. After 3 days recovery from exposure, $p53$ relative expression decreased dramatically, but was still significantly higher than control levels ($17.21 \pm 6.66$-fold). After the exposure to combined $B(\alpha)P$ and $C_{60}$, $p53$ expression was significantly induced by $17.88 \pm 5.62$-fold. The induction increased to $98.35 \pm 8.46$-fold after 3 days exposure. The induction of $p53$ expression after both exposure times was lower compared to $C_{60}$ exposure alone. The recovery from combined exposure of chemicals showed a decline in $p53$ relative expression, but was the same as the $C_{60}$ exposure; the level was still significantly higher than the control ($28.37 \pm 14.73$-fold).

$p53$ relative expression in the digestive gland showed a similar pattern as haemocytes but with a quicker response to $C_{60}$ exposure (Figure 4.13B). $B(\alpha)P$ only induced $p53$ expression after 3 days exposure. The combination of $B(\alpha)P$ and $C_{60}$ intended to induce more $p53$ expression compared to $B(\alpha)P$ alone, about $4.5 \pm 0.5$-fold of $p53$ expression induced after 1 day exposure and $6.28 \pm 1.7$-fold after 3 days exposure. $p53$ relative expression dropped to control levels after exposure to both $B(\alpha)P$ alone and in combination with $C_{60}$. $p53$ expression responding to $C_{60}$ exposure showed a different
pattern. Significantly increased $p53$ expression (over a thousand-fold) was found after 1 day exposure. This level dramatically decreased to $32 \pm 15$-fold after 3 days exposure. After recovery, the level continued decreasing but was still higher than control level.

Relative higher $p53$ expression was induced in mantle compared to the other tissues (Figure 4.13C). After $B(\alpha)P$ exposure alone, $6.27 \pm 5.17$-fold of $p53$ expression was induced after 1 day exposure. The induction increased to $283.03 \pm 157.80$-fold after 3 days exposure. No induction of $p53$ expression was detected after 3 days recovery. After $C_{60}$ exposure alone, significantly increased $p53$ expression was detected after 1 day exposure. The level was similar after a longer exposure time (3 days) but returned to control levels after recovery. Unlike haemocytes and the digestive gland, the combined exposure showed the ability to induce more $p53$ expression in mantle tissue. A significant induction about $3515 \pm 2491$-fold of $p53$ was detected after 1 day exposure. Further increased $p53$ expression was induced after 3 days exposure. After recovery, induced $p53$ expression decreased but was still significantly higher than the control level.

In the adductor muscle, a similar $p53$ expression pattern was found (Figure 4.13D) but responded quicker compared to the mantle. After $B(\alpha)P$ exposure alone, significant induction of $p53$ was found after 1 day of exposure. No induction of $p53$ expression was found after both 3 days exposure and 3 days recovery. After $C_{60}$ exposure alone, highest induction of $p53$ expression was shown after 1 day exposure, less but still significantly induced $p53$ expression was found after 3 days exposure. The level was similar after recovery compared to 3 days exposure. The combined exposure of $B(\alpha)P$ and $C_{60}$ induced significantly $p53$ expression after 1 day exposure. Decreased expression was found after longer exposure and no induction of $p53$ expression after 3 days recovery.
Similar to mantle tissue, combined chemical exposure induced more $p53$ expression compared to single chemical exposure in adductor muscle.

The relative expression pattern of $p53$ in gill tissue after exposure was similar to digestive gland and haemocytes (Figure 4.13E). There was no induction of $p53$ relative expression after B(α)P exposure alone at any sampling time. Induced expression was found after 1 day exposure to C$_{60}$ alone and the level increased after 3 days exposure. $p53$ expression decreased after recovery. However, there was no significant difference in $p53$ expression when comparing different time points after C$_{60}$ exposure due to high variability between replicates. The combined exposure induced $p53$ expression after 1 day exposure, the level dropped after 3 days exposure but increased slightly after recovery. The differences in $p53$ expression at different time points were still not significant.
Figure 4.13 Relative quantitative p53 expression pattern in haemocytes (A), digestive gland (B), mantle (C), adductor muscle (D) and gill (E) exposed to B(α)P at 56 µg/L and/or C_{60} at 1 mg/L for 1 and 3 days followed by 3 days recovery. Each bar represents the means of 6 replicates (n=6) and S.E.M are indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p<0.05. * indicated significant up-regulated genes expression compared to control. # indicated significant down-regulated genes expression compared to control.
4.3.6.3 Relative quantification of ras expression in different tissues following exposure to chemicals

The relative expression of ras in haemocytes (Figure 4.14A) showed a different pattern compared to p53 expression. There was down-regulation of ras expression at any sampling time after any treatment. Similar results were found in gill (Figure 4.14E), where ras relative expression remained at control levels after all the treatments, except for after 1 day exposure to B(α)P alone and after recovery from B(α)P exposure.

In the digestive gland (Figure 4.14B), no significant induction of ras expression was found after B(α)P exposure alone. But after C_{60} exposure alone, ras relative expression was significantly induced after 1 day of exposure (108 ± 18.7-fold). The level significantly decreased after 3 days exposure and returned back to close to control level after 3 days recovery. The combined exposure also induced ras expression after 1 day exposure; however, the level (3.09 ± 0.2-fold) was significantly lower than C_{60} exposure alone. A decline of ras expression also found after 3 days exposure and kept at a similar level after 3 days recovery, which was very close to control level.

Relative expression of ras in mantle (Figure 4.14C) showed a different pattern compared to digestive gland, but like p53 expression in the same tissue. There was no significant induction of expression after 1 day exposure to B(α)P exposure alone, but the level increased significantly (73.2 ± 40.21-fold) after exposure to B(α)P for 3 days. After 3 days recovery, no induction of ras expression was found in mantle. There was no induction of ras expression after C_{60} exposure only after all exposure periods. The combined exposure of B(α)P and C_{60} showed the ability to induce significant ras expression. After 1 day exposure, over 200-fold increase in ras expression was found. The level increased to over 4000-fold after 3 days exposure to the combined chemicals.
Unlike other tissues, the mean level of ras expression in mantle remained at a relatively higher level after recovery compared to control. This level was statistically significantly higher than the control level but with very high inter-individual variations.

The expression of ras gene in the adductor muscle showed a similar expression pattern as mantle tissue but with quicker response times (Figure 4.14D). Expression was induced after 1 day exposure to B(α)P alone and then the level dropped to the control level after 3 days exposure and stayed at a similar level after 3 days recovery. There was no induction of ras expression after C₆₀ exposure alone at any sampling time. The expression level remained slightly below the control level. The combined exposure significantly induced ras expression after 1 day exposure, approximately 647 ± 424-fold. After 3 days exposure, the induction decreased to 30.97 ± 28-fold higher than the control and decreased to similar to the control level after 3 days recovery in fresh seawater.
Figure 4.14 Relative quantitative ras gene expression pattern in haemocytes (A), digestive gland (B), mantle (C), adductor muscle (D) and gill (E) exposed to B(α)P at 56 µg/L and/or C₆₀ at 1 mg/L for 1 and 3 days followed by 3 days recovery. Each bar represents the means of 6 replicates (n=6) and S.E.M are indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p<0.05. * indicates significant up-regulated genes expression compared to control. # indicates significant down-regulated genes expression compared to control.
4.4 DISSUSSION

4.4.1 Determination of B(α)P concentration by GC-MS and C₆₀ concentration by HPLC

The measured concentrations of B(α)P in the water samples were lower than expected which is consistent with our previous results (Chapter 3), indicating its low solubility in seawater. The measurement of C₆₀ concentration in seawater was difficult as it tends to aggregate together after addition to a water system. Ultra-sonication of the sample water is required to re-suspend C₆₀, which is difficult to carry out in the setting of experimental tanks. Therefore, no confirmation of C₆₀ introduction to the seawater was able to be carried out. It was therefore assumed that the nominal C₆₀ concentration (1 mg/L) was introduced to the seawater in this study. However, a good fit standard curve (R²=0.996) of C₆₀ concentration was established between 0.125-2.0 mg/L. Although the standard curve was constructed using C₆₀ standards prepared in water with constant sonication followed by extraction to toluene before measurement by HPLC, it can still be used for the calculation of C₆₀ concentration in tissues based on the assumption that is the majority of bioaccumulated C₆₀ in tissues was extracted into toluene. The C₆₀ concentration in adductor muscle, digestive gland and gill were determined after 3 days exposure and 3 days recovery, and were calibrated according to the standard curve. The digestive gland was found to accumulate more C₆₀ after exposure compared to the other two tissues, which was not altogether surprising due to its main function of digesting absorbed compounds from water. C₆₀ tissue concentrations after 3 days recovery in seawater suggest all three tissues are able to metabolise or excrete C₆₀ completely within 3 days recovery in fresh seawater as the concentrations all decreased to control levels following recovery. This ability appears to have a tissue-specific pattern which is consistent with previous studies showing different concentrations of C₆₀ in rat tissues.
after tail vein administration (Kubota et al., 2011). However, the mechanisms of how each tissue in organisms performed generally at removing the C$_{60}$ are still unknown. Several studies to determine the interactions between nanoparticles and tissues have been performed by transmission electron microscopy (TEM) or confocal laser scanning microscopy (CLSM) and showed diffusion of selected nanoparticles (e.g.: TiO$_2$, poly (D, L-lactide-co-glycolide) nanoparticles) into cells and localized at different sites (Mühlfeld et al., 2007a; Mühlfeld et al., 2007b; Panyam et al., 2003). Unfortunately, measuring the interaction between absorbed C$_{60}$ and mussel tissues following exposure by scanning electron microscopy (SEM) or TEM was not undertaken in this study because of the technical difficulties associated with optimising suitable tissue fixation methods for different tissue types in mussels (Mühlfeld et al., 2007c), distinguishing introduced C$_{60}$ with the carbon naturally present in tissues (Glenn Harper, Electron microscopy unit, Plymouth University, personal communication) or with cell structures within the same size range as C$_{60}$ (Mühlfeld et al., 2007c). It is noteworthy that there is trace C$_{60}$ (less than 2.5 µg C$_{60}$/gww) found in control conditions. This is likely to be the background noise of measuring C$_{60}$ concentrations by HPLC.

4.4.2 Determination of clearance rate

Increased clearance rate was found in the “recovery group” compared to exposed groups and indicating decreased feeding activity in mussels induced by chemical exposure. Interestingly, the control group, in theory, should not have shown any significant difference in physiological response in term of feeding behavior but indeed exhibited higher clearance rate after a longer incubation time (6 days) in the experiment, suggesting that changes in ambient environment (e.g. mussels moved from bigger stock tank to smaller exposure tank) can also cause stress to mussels and it could take some time for mussels to acclimate (Dimitriadis and Koukouzika, 2003). There was no
depressed feeding behaviour in response to exposure at the same sampling day for both B(α)P and C₆₀ (alone or in combination). On the contrary, a significantly activated feeding behaviour was found in mussels exposed to chemicals (B(α)P and/or C₆₀) for 3 days which is not consistent with previous results showing decreased feeding rates caused by direct toxicity of chemicals in mussels (Halldórsson et al., 2008). The explanation for enhanced feeding activities in the treated group might be related to higher energy demands and metabolic activities required to deal with accumulated chemicals in mussels meaning that the mussels feed more to get this energy (Navarro and Winter, 1982). Mussels exposed to chemicals for 1 day did show lower feeding activities compared to longer exposures and after recovery, suggesting short-term exposure may cause direct toxicity to reduce the feeding activities, but the physiological response will switch to coping with the stress after long term exposure by increasing feeding activity in order to produce more energy by food metabolism. However, the changes in terms of feeding behaviour are in the range of normal for mussels (data was shown in Chapter 7 with detailed explanations), indicating that exposure cannot induce significant physiological change in mussels. In addition, mussels exposed to C₆₀ or B(α)P alone showed different feeding behavior compared to when exposed to the two chemicals together. This indicates that interactions between B(α)P and C₆₀ alters the property of chemicals, such as increased solubility of PAHs in association with C₆₀, and further influences the physiological response in the organisms (Yang and Xing, 2007; Yang et al., 2006).

4.4.3 Determination of histopathological alterations

Histopathological studies indicated physiological changes in the mussel tissues following exposure to contaminants. Mussels exposed to B(α)P with C₆₀ tended to exhibit more tissue damage compared to mussels exposed to B(α)P alone. A total of 6
mussels were examined for histopathological analysis due to laboratory limitations. Consistent results with Chapter 3 were found after all chemical exposures, such as no tumour occurrence (neoplasia) in any of the tissues sampled; only necrosis in some of the treated tissues perhaps indicative of a very early stage tumour development. All the results confirmed that attempts to induce neoplasia in vivo in mussels after acute exposure under laboratory condition are not realistic. The development of neoplasia in invertebrates under laboratory conditions is a time consuming process and might involve various factors other than introduced toxicant alone (Barber, 2004). The maintaining of cancerous cells isolated from invertebrate collected from contaminated sites is also difficult to perform. To our knowledge, the only successful lab maintaining invertebrates malignant cells has been conducted by Walker’s group in clam haemocytes by inoculating cancerous clam haemocytes into normal clams (Walker et al., 2009). Nonetheless, the histopathological results can still provide convincing evidence for the toxic effects of $B(\alpha)P$ and $C_{60}$ which can cause tissue abnormalities. These tissue abnormalities could then lead to disease development such as neoplasia and suppression of immune function over time (Carlson et al., 2004; Depledge, 1998). It is important to note that no samples after $C_{60}$ exposure alone were examined for histopathological analysis due to the tissues being specifically preserved for $C_{60}$ concentration analysis rendering them useless for histopathology. It has been reported previously that $C_{60}$ is able to cause tissue abnormalities but cannot cause any neoplasia when exposed at the applied concentration (1 mg/L) (Al-Subiai et al., 2011).

In addition, after exposure to both $B(\alpha)P$ and $C_{60}$, gill tissues showed different abnormalities in comparison to $B(\alpha)P$ alone with pore structures observed in gill front cilia. This particular structure has also been reported previously with the suggestion that
it could be either the structure of nanoparticles themselves after accumulated in tissues or generated when nanoparticles entering cells (Yang et al., 2010).

4.4.4 Determination of DNA strand breaks

Significant increases in DNA strand breaks were observed after 3 days exposure to the chemicals. The most pronounced DNA damage was induced by B(α)P alone followed by C_{60} alone and then the combined chemicals. The significantly induced DNA strand breaks after exposure to B(α)P alone is consistent with previous results observed in Chapter 3, confirming that the applied concentration of B(α)P can efficiently induce DNA damage in haemocytes of mussels. There was no significant DNA damage after 1 day of exposure indicating that DNA damage occurs during the metabolism of chemicals, and it may take time for organisms to switch on the essential machinery. After 3 days recovery, DNA damage was significantly less confirming the involvement of DNA repair, which is likely to be due replacement or renewal of damaged cells through apoptosis or DNA repair activity by arresting cells at cell-cycle check points (Hook and Lee, 2004). The replacement of damaged cells by newly generated cells could also dilute the observed responses. The results confirmed that, similar to B(α)P, C_{60} can induce DNA strand breaks, which is consistent with previous reports (Dhawan et al., 2006; Spohn et al., 2009). However, no additive DNA strand breaks were observed after the combined exposure which is conflict with the result reported by Al-Subiai et al. (2012) which showed enhanced DNA damage after the exposure to C_{60} and fluoranthene in combination. As B(α)P, composed of 5 benzene rings, is larger than fluoranthene (3 benzene rings), it is more difficult for it to enter the interspace between small aggregates of C_{60} which formed after its introduction into water (Yang and Xing, 2007). The rearrangement or break-down of larger aggregates after absorption into
organisms will release the entrapped molecules and potentially cause the enhanced DNA damage.

4.4.5 Determination of total glutathione levels

The results showed that C₆₀ or B(α)P alone can increase the glutathione level, suggesting that antioxidant defences have been switched on in response to pollutant exposure by generating more glutathione. The result is consistent with previous studies where up-regulation of glutathione has been found to correlate with increased burden in the bivalve *Perna viridis* (Cheung *et al.*, 2001). Although both C₆₀ and B(α)P are able to induce oxidative stress in mussels, the interaction between these two chemicals did not cause as high glutathione as single chemical induced oxidative stress because the enhanced level of total glutathione was not statistically significant compared to the control group.

Although information on DNA strand breaks, histopathological effects and total glutathione levels suggest that B(α)P and C₆₀ can indeed cause stress in *Mytilus sp.*, gene expression studies on key genes could indicate whether this PAH and the interaction with nanoparticles might proceed with common pathways for mutagenesis and neoplasia in phylogenetically different group of organisms.

4.4.6 Determination of gene expression

4.4.6.1 *p53* and *ras* genes expression in different tissues under control conditions

The expression abundance of *p53* and *ras* genes in different tissues under control conditions was analysed before the relative expression analysis was carried out, mainly because the housekeeping gene was changed to 18S in this study. As expected, the expression abundance trend of results for *p53* and *ras* after normalisation with 18S (excluding the expression in haemocytes which was not undertaken for *actin*
normalisation) was the same as the actin normalised results (Chapter 3). p53 tends to be more expressed in the digestive gland, followed by gill, mantle and adductor muscle tissues. ras gene was expressed at similar levels in the digestive gland, gill and adductor muscle and less in mantle tissue. These results confirmed that the change in housekeeping gene for normalisation did not affect the normalisation results and made the results comparable to the previous study. As 18S was found to be more stably expressed than actin in mussel gill tissue under different conditions using the geNorm program, using 18S as housekeeping gene can provide a more accurate picture on how much a gene expression altered solely depended on the responses to chemical induces stress rather than the variety of 18S expression itself.

4.4.6.2 Relative expression of p53 and ras genes in haemocytes following B(α)P and/or C₆₀ exposure

In bivalve molluscs, the haemocytes are responsible for cell-mediated immunity through phagocytosis and various cytotoxic reactions, such as the release of lysosomal enzymes and antimicrobial metabolites (Canesi et al., 2008). In the marine mussel Mytilus, haemocytes have been shown to represent a sensitive target for a number of environmental contaminants, including heavy metals and organic xenobiotics, with consequent immune-toxic effects or stimulation of immune parameters, leading to inflammation, depending on the compound and on the conditions of exposure (Canesi et al., 2005; Canesi et al., 2002). In particular, changes in lysosomal membrane stability and phagocytosis, and stimulation of lysosomal enzyme release and oxyradical production have been observed in response to different contaminants. Many of these effects are known to be due to interference with components of the signalling pathways involved in activation of the immune response (Canesi et al., 2007a; Canesi et al., 2007b). Therefore, analysis of the expression of key genes in haemocytes will provide
fundamental information about genetic response in mussels to environmental contaminants.

The increase of $p53$ expression in haemocytes after exposure to $B(\alpha)P$ and/or $C_{60}$ confirmed its function in DNA repair as more $p53$ has been expressed to halt the cells at cell cycle checkpoint for repair when cells are facing stress, e.g. DNA damage, oxidative stress, ionising radiation, caused by chemicals exposure (Arias-Lopez et al., 2006; Culmsee and Mattson, 2005). The reduced level of expression after recovery in all the treatments suggests that mussels are able to cope with the applied exposure concentration because there is no need for more $p53$ to be expressed. The damaged DNA has either been repaired and cells are allowed to pass through the cell cycle checkpoint, or the damage cannot be repaired and has led the cell to the apoptosis pathway. These results are closely related to the DNA strand break results, where less DNA damage has been found after recovery. Although $p53$ expression in haemocytes after 3 different treatments all presented the similar pattern, which is includes up-regulation after 1 day exposure, followed by a significantly increased expression after 3 days and finally a decline after recovery, the level of induced $p53$ expression was different in the different exposures. Higher $p53$ expression was expected after exposure to $B(\alpha)P$ in combination with $C_{60}$ because $C_{60}$ as a popular chemical carrier might contribute to delivery of more $B(\alpha)P$ into cells and interact with DNA to cause more damage (Baun et al., 2008). Surprisingly, even higher $p53$ was induced after $C_{60}$ exposure alone, which could be explained that haemocytes are either more sensitive to $C_{60}$ or the combination with $B(\alpha)P$ can actually protect cells from the toxic effects of $C_{60}$ alone, probably by changing the structure or occupying the free radicals to serve as radicals scavengers (Kubota et al., 2011; Yang et al., 2010). It is also possible that the $C_{60}$ is not served as carrier in the combined exposure with $B(\alpha)P$ but sequester $B(\alpha)P$ to
decrease the damage induced by B(α)P exposure alone under applied conditions. This assumption is not supported in the result which showed significantly higher $p53$ expression after combined exposure compared to exposure to B(α)P alone. However, information about the mechanisms of how organisms process nanoparticles after absorption is scarce. $p53$ expression in haemocytes collected from combined exposures and C$_{60}$ exposure showed very high level of expression even after recovery, indicating that haemocytes are probably still under stress and either need more time for recovery or cannot cope with the stress completely which will potentially cause the formation of neoplasia. As mentioned earlier, the only lab-maintained neoplasia has been found in haemocytes of clams suggesting that haemocytes of invertebrates are vulnerable to exposure (Walker et al., 2011).

$ras$ did not show any induced expression in haemocytes after all treatments. Down-regulation was found after 3 days exposure to chemicals suggesting $ras$ is still kept in the proto-oncogene form which is not closely involved in DNA repair. Ruiz et al. (2012) found no mutation of $ras$ at the traditional hotspots, i.e. codons 12, 13 and 61 in mussels after exposure to heavy fuel oil no. 6 and styrene, also suggesting $ras$ is resistant to keep proto-oncogene form under certain contaminations. $ras$ has been proven to function in cell differentiation and proliferation in mammalian cells (Fernandez-Medarde and Santos, 2011; Goodsell, 1999), but its function in invertebrates is still unclear. Our results suggest that $ras$, as a proto-oncogene, is involved in cell growth by some pathway other than DNA repair as no significant differences in expression between treated and recovered groups was detected. No induced $ras$ expression was expected as overexpression of $ras$ has only been reported in tumour cells (Jancik et al., 2010; Spandidos et al., 1992). Although occurrence of neoplasia has a long latency period, none of the haemocyte samples showed neoplasia which is consistent with the low level
of ras relative expression. The alteration in ras expression is still provide valuable information about whether the laboratory applied conditions are capable to activate Ras to its oncogenic form by altering the expression levels. As P53 and Ras are function in opposite way, the analysis of the ras expression can also elucidate the different performance of tumour suppressor gene and oncogene when the cells are under stress.

The change in gene expression of p53 and ras in haemocytes can be directly linked to DNA strand breaks as measured by the comet assay as both analyses were applied using the same samples. More DNA damage was found after 3 days exposure compared to 1 day exposure, which is consistent with the gene expression results where more p53 has been induced. There were no significant DNA strand breaks after 1 day exposure after all three exposures, but significantly increased p53 expression was detected after C_{60} exposure alone and B(α)P combined with C_{60} exposure after same period of exposure. As mentioned earlier, both B(α)P and C_{60} are able to cause oxidative stress. These results imply that p53 is involved in a common signalling pathway which can sense a wide range of stress, apart from DNA damage (Suh et al., 2011). It is also possible that DNA strand breaks did occur within 1 day exposure to chemicals. The induced p53 expression, however, is capable to repair the damage DNA coherently to avoid the accumulation of DNA damage. After 3 days recovery in fresh seawater, the level of DNA damage in haemocytes dramatically decreased but was still significantly higher than in the control, suggesting DNA repair is a time-consuming process. This is also confirmed by p53 expression analysis because a decline of p53 relative expression was found after recovery. When comparing the three different exposures, B(α)P was found to induce more DNA strand breaks compared to other two treatments, p53 expression was higher in B(α)P combined with C_{60} and C_{60} alone exposures, and total glutathione analysis showed highest GSH induction after C_{60} exposure alone. Taken together this
suggests that most oxidative stress was caused by C₆₀ with p53 expression being induced to react according to this stress as well.

4.4.6.3 Relative expression of p53 and ras genes in different tissues

Even though the expression of p53 and ras genes in haemocytes following treatments directly linked to DNA damage analysis, the expression patterns of these two genes in different tissues are of interest. In the previous study (Chapter 3), mussels exposed to B(α)P at the same concentration of 56 µg/L for 6 and 12 days showed significant increased expression for both the genes in adductor muscle and mantle tissues. However, no recovery analysis was included and no B(α)P was re-dosed on a daily basis in the previous study. After improving the limitations of the previous experiment design, both p53 and ras genes expression showed dramatic increase in adductor muscle after 1 compared to 3 days exposure to all the chemicals, suggesting this tissue is quicker in responding to B(α)P-induced stress to assist the cells to repair the damage or to undergo apoptosis. The significantly higher genes expression can also be caused by higher concentration of B(α)P in total being introduced by re-dose daily. The 3 days post-exposure period in fresh seawater allowed this tissue to recover from the stress as the level of expression for both genes dropped to the control level. However, p53 and ras expression in the adductor muscle responded differently in different treatments. The combination of B(α)P and C₆₀ exposure appears to be more toxic to adductor muscle cells since higher p53 expression was induced indicating there might be more DNA damage need to be repaired. A similar response has also been found in mantle tissue where significantly increased p53 and ras expression were found in the mussels exposed to the combined chemicals. However, unlike in adductor muscle, p53 and ras showed a slower response because higher p53 and ras expression were induced after 3 days exposure compared to 1 day. After recovery, p53 remained at a high level of
expression which suggests that cells in the mantle cannot cope with the combined exposure and this could potentially lead to the development of tumours in this tissue. This idea is supported by research on mussels collected from contaminated sites, where only leukaemia (haemocytes) and gonadal (mantle) neoplasia have been found in mussels (Ciocan and Sunila, 2005; Galimany and Sunila, 2008; Krishnakumar et al., 1999). No neoplasia has been reported in other tissues of mussels. Mantle is the main tissue to produce germ cells which requires faster development compared to other cells. Therefore, DNA abnormalities are under higher risk of passing from generation to generation without repair. Cells with un-repaired DNA damage will further develop into neoplasia. The gene expression results in the adductor muscle and mantle also suggest that the combined exposure is more toxic compared to individual chemical exposure. The interaction between B(α)P and C₆₀ has an additive effect in these two tissues based on the alteration of p53/ras expression (increased expression level) after combined exposure compared to exposure to individual chemicals. As mentioned earlier, C₆₀ can carry more B(α)P across cell membrane; therefore, more B(α)P can be released in the cells inducing more damage which in turn could induce p53 and ras expression.

In contrast to the mantle and adductor muscle, the combination of B(α)P and C₆₀ created an antagonistic effect rather than an additive effect in the digestive gland, similar to haemocytes. Significant increase in p53 and ras gene expression was detected after C₆₀ exposure only compared to other two types of exposure. This suggests that C₆₀ is more toxic to the digestive gland than B(α)P or B(α)P in combination with C₆₀. Considering the results from C₆₀ concentrations in different tissues after exposure, digestive gland was found to accumulate a lot more C₆₀ than the adductor muscle and gill. The more accumulated C₆₀ might be the main reason as to why a higher response level of gene expression was seen in this tissue. Because C₆₀ could act as the vehicle to transport more
B(α)P to the cells in the combined exposure, free available C$_{60}$ on its own will potentially be diluted in the digestive gland compared to the single C$_{60}$ exposure, and therefore could lead to less damage in the cell in the combined exposure. Digestive gland also showed a quick response because the highest induction of gene expression was detected after 1 day exposure compared to 3 days. This might be caused by its function as the main tissue to digest compounds absorbed from water, a quick response is required for further transport, including inter tissue translocation (Rothen-Rutishauser et al., 2007). Although, very high levels of $p53$ and $ras$ expression were induced after exposure, the level dropped to control level after recovery, suggesting the digestive gland is capable to cope with the chemical concentrations applied in this study or it is more resistant to induced stress.

Unlike other tissues and haemocytes, gill tissue does not appear to be under any stress after all the treatments. $p53$ was induced after exposure but not at as high a level as in other tissues. This suggests gill is more resistant to chemicals as less DNA damage was found in this tissue. Previous studies has conducted comet assay using cells isolated from gills of mussels (Lain et al., 2008; Rank and Jensen, 2003); such analysis has not been done due to the limitation of tissues samples which has been applied for both histopathology and gene expression analyses Yang et al. (2010) also reported that no increased lipid peroxidation was found in gill and liver of fish after exposure to C$_{60}$ but a significant increase was observed in brain tissue of the same fish samples, supporting the idea that gill is more resistant to C$_{60}$ exposure.

Both $p53$ and $ras$ showed significantly increased expression after exposure. The over-expression of $p53/ras$ in mammalian cells always relates to tumour formation (Cummings, 1996; Spandidos et al., 1992), however, no neoplasia has been seen in these two tissues following histopathological analysis in mussels, hence it is difficult to
attribute the up-regulation of these genes to this phenomenon, which is a multistage process. The over-expression of \( p53/ras \) therefore might indicate the very early stage of neoplasia, especially in haemocytes and the mantle as gene expression levels remain high level after recovery, and as mentioned earlier tumour formation takes a long time to develop in a multi stage process. In comparison to the previous results that the adductor muscle and mantle tend to induce more \( p53 \) expression compared to the digestive gland and gill after \( B(\alpha)P \) exposure, the same results were found in \( B(\alpha)P \) only exposed samples. A relatively higher expression level of \( p53 \) was detected in the adductor muscle (8.34 ± 5.24-fold after 1 day exposure) and mantle (6.27 ± 5.17-fold after 1 day exposure and 283.03 ± 157.8-fold after 3 days exposure), which further confirmed that the switch of housekeeping gene from \( \text{actin} \) to \( 18S \) does not affect the normalised gene expression results.

Average \( p53 \) and \( ras \) expression increased after exposure in different tissues. The increase was, however, not significant in some tissues. A high inter-individual variation was observed within our sample set and is likely caused by intrinsic and extrinsic factors experienced by the individual mussels. A detailed explanation was given in Chapter 3. Despite this, \( p53 \) and \( ras \) gene expression analysis has been found to be suitable to evaluate both eco-toxicological and nano-toxicological responses, as the expression profiles changed significantly according to the chemical exposures.

In conclusion, whilst \( B(\alpha)P \) and/or \( C_{60} \) induced tissue and DNA damage in exposed mussels confirms their function as genotoxicants, they also induced expression of tumour-regulating genes (i.e. \( p53 \) and \( ras \)) with high inter-individual variation suggesting they also act as carcinogens. \( B(\alpha)P \) and/or \( C_{60} \) induced \( p53 \) and \( ras \) expression in a tissue specific manner with the mantle and adductor muscle being more sensitive to the combined exposure and the digestive gland and haemocytes being more
sensitive to C$_{60}$ exposure alone. The adductor muscle and digestive gland were found to respond more quickly compared to the mantle and haemocytes. Gill was found to be tolerant to the chemical treatments it was exposed to and the $p53$ and $ras$ expressions in this tissue do not dramatically change. Both $p53$ and $ras$ gene expression profiles are changed in line with different chemical exposures, where C$_{60}$ either alone or in combination with B(α)P tends to induce higher levels of gene expression in comparison with B(α)P exposure alone.

DNA damage was confirmed to be associated with expression of tumour-regulating genes, and a significant correlation was identified between these biological responses and has been found by using haemocytes as target cell population. In addition, it has been documented that both $p53$ and $ras$ function is closely related to post-transcriptional modification in response to DNA damage (Appella and Anderson, 2001; Artandi and Attardi, 2005; Goodwin et al., 2005). With each stress, the responses may show similarities, but there will also be differences essential for eliciting a unique molecular signalling outcome. It appears, therefore, that multiple sites targeted by an integrated network of signalling pathways highly sensitive to genotoxic stresses must be modified to yield a functional $p53$ and $ras$. 


CHAPTER 5

Transcriptional effects of B(α)P and/or C_{60} fullerenes in *Mytilus sp.*: Modulation of *p53/ras* mediated signaling pathways
5.1 INTRODUCTION

Different molecular and cellular techniques are being used with an aim to provide early warnings of potential adverse alterations in organisms exposed to environmental stress/pollutants. In this field, a promising area of investigation is cell signalling, the whole mechanisms allowing the transduction of extracellular stimuli into modulatory processes of cellular activities. Based on the previous studies, two tumour related genes (i.e., p53 and ras) showed tissue-specific expression in Mytilus sp. following chemical-induced stress. However, it remained unclear whether the transcriptional alterations are regulated or will regulate in similar way or pattern found in other organisms in relation to carcinogenesis or tumour development process. Therefore, another five different groups of molecules, apart from the proto-oncogene ras and anti-oncogene p53, based on available sequencing information of Mytilus sp., have been screened and selected for p53/ras mediated signalling pathway analyses. These five groups of genes are briefly described below:

Group 1: cytochrome P450 family - CYP4Y1

Members of the cytochrome P450 (CYPs) super-family of enzymes form an essential part of the body’s ability to carry out both anabolic and catabolic metabolism. Indeed, the very fact that members of this super-family have been identified in all organisms, from Achaea to humans, underlines their importance for organism survival (Plant, 2007). Although all organisms possess CYP enzymes, the number of CYP genes per organism is not constant, with more ancient organisms having fewer CYP genes (Nebert and Gonzalez, 1987). CYP450 family enzymes, in general, mediate the first step in the metabolic elimination of lipophilic compounds; this will in some cases generate more toxic and even mutagenic and/or carcinogenic compounds. For example, the metabolites of B(α)P are crucial for the ultimate toxicity of the compound, since the position at
which B(α)P is attacked determines the carcinogenicity of the product (Livingston et al., 1989; Solhaug et al., 2005). Therefore, B(α)P metabolism by CYP enzymes is probably the first step to initiate the multistage carcinogenic responses in organisms.

The mechanisms underlying CYP regulation in marine invertebrates are currently unknown despite some studies available in the literature on different CYP families. The most studied members of CYP family in Mytilus sp. is the CYP4 sub-family which are also involved in fatty acid hydroxylation in mammals (Simpson, 1997). Compared to mammals, the invertebrate (e.g. insect) CYP4 sub-family is much diversified. For example, insects have more CYP4 genes than mammals, some of which are believed to be involved in the metabolism of xenobiotics (Plant, 2007). The expression of CYP4Y1, a partially sequenced CYP from the mussel Mytilus galloprovencialis, showed inhibited expression by crude oil (Snyder, 1998). However, no significant difference has been shown between control and mussels exposed to PCBs for the activation of CYP4Y family (Snyder, 1998). Moreover, reports have indicated that the CYP family (e.g. CYP2K, CYP2M) can metabolise C$_{60}$ fullerene (C$_{60}$) in vivo but the results are not consistent among different researchers (Oberdörster et al., 2006; Zhu et al., 2006) and hence the mechanism involved is still unclear. In this study, CYP4Y1, based on its available gene sequence, was selected as a representative member of the CYP enzyme family to analyse its role in metabolism of B(α)P and/or C$_{60}$ in marine mussels.

**Group 2: Murine Double Minute Clone 2 (MDM2)**

MDM2 after originally being cloned from purified acentric chromosomes harboured within a spontaneously transformed Balb/C3T3 cell line, have been shown to contribute to cellular proliferation and tumorigenesis (Cahilly-Snyder et al., 1987). The MDM2 family gene sequences have been divided into three regions of high identity: CR1, CR2 and CR3. CR1 is responsible for binding P53/P73, E2F1 and DP1, all of which are
proteins that modulate cell growth. CR2 codes for a putative zinc binding domain and partially overlaps a region required for binding the Retinoblastoma (Rb) tumour suppressor protein. CR3 encodes the RING finger domain, binds two Zn atoms, and contains a cysteine residue required for ubiquitin conjugation of P53 (Momand et al., 2000).

MDM2 is of particular interest since this gene is associated with P53, which, in turn is related to cancer and apoptosis (Momand et al., 2000). In response to DNA damage and other types of stress, such as heat shock, hypoxia and hyperoxia, P53 is responsible for either blocking cell cycle progression (allowing cells to repair the damage) or instigating apoptosis (if repair mechanisms fail) (Culmsee and Mattson, 2005; Gomez-Lazaro et al., 2004; Meek, 2004). In response to most stressors, the P53 protein level increases. The increased levels are due to a combination of an increase in P53 translation rate and a decrease in P53 degradation rate (Michael and Oren, 2003). The degradation rate is mainly controlled by MDM2 which can bind to P53 directly and process for ubiquitin conjugation by the 26S proteasome (Momand et al., 2000; Piette et al., 1997). Another important function of MDM2 is in transactivation block; P53 has a stretch of acidic amino acid residues near its N-terminus required for this function. MDM2 directly binds this region of P53 and shuts down P53-mediated transactivation by forming a complex with P53 (Culmsee and Mattson, 2005). MDM2 and P53 are involved in a negative feedback loop where P53 activates MDM2, which, in turn, down regulates P53 (Wu et al., 1993). MDM2 has been shown to increase the tumorigenic potential of cells when it is overexpressed (Totzke et al., 1996). Therefore, MDM2 received particular interest in this study to elucidate the interplay of p53 and mdm2 at transcriptional level.
**Group 3: Bcl-2 family - Bax**

Bcl-2 family members are central regulators of programmed cell death or apoptosis. They can integrate diverse survival and death signals generated out- and inside the cell and act like checkpoints through which these signals must pass before they determine the cell fate (Aouacheria, 2005). These proteins constitute an expanding and heterogeneous family that has been initially divided by function into either pro- or anti-apoptotic members. All of them are involved in the manipulation of cell survival outcomes after cytotoxic stress. Bcl-2 (anti-apoptotic members) plays an aetiological role in human follicular lymphoma, and is also expressed in other neoplasms including prostate, breast and ovarian cancers in a tissue-specific manner (Lu et al., 1996; Marx D Fau - Meden and Meden, 1997). The Bcl-2 interacting protein, Bax, is a pro-apoptotic member of the Bcl-2 family, and its expression is induced by gamma-radiation, chemotherapeutic drugs, and other forms of genotoxic stress (Gong et al., 1999; Guo et al., 2000).

It is now realised that the tumour suppression effect of P53 is related to the induction of irreversible cell-suicide processes or the imposition of reversible growth arrest phenomenon. Although numerous studies have implicated deregulated expression of the Bcl-2 family of genes or loss of p53 function in human neoplasia, these changes have rarely overlapped. Studies revealed that the pro-apoptotic bax gene is a direct transcriptional target of P53 (Basu and Haldar, 1998). In a reporter gene assay, P53 has been shown to strongly transactivate the bax gene promoter. Thus tumours with loss of P53 function are expected to contain relatively low levels of Bax protein. To date, among all Bcl-2 family members, only the bax sequence is available in *Mytilus sp.*. An attempt has therefore been made to link bax expression with p53 in both haemocytes
and other tissues, to establish the potential relationship between these two groups of genes in *Mytilus sp.*.

**Group 4: Mitogen-Activated Protein Kinase (MAPK) family – JNK**

MAPK is important amplifying molecule that can transduce stress signals into cellular response. Three subfamilies of the MAPKs have been clearly identified and extensively studied in mammalian experimental models: the extracellular regulated protein kinase (ERK), the c-Jun NH2-terminal Kinase (JNK) and the P38-MAPK (P38) (Châtel *et al.*, 2010). Several stresses have been reported to activate the MAPK signalling cascade to transform extracellular stress signals into intercellular responses in organisms. For example, oxyradicals can be highly toxic to animals, often resulting in lipid peroxidation of membranes, altered pyridine nucleotide redox status, and in oxidative DNA damage (Marnett, 1999). Among the signalling pathways involved in this adaptation, the MAPK signalling cascade has been shown to play a significant role. Indeed, Gaitanaki *et al.* (2004) showed that oxidative stress induced a time-dependent activation of P38 in *M. galloprovincialis* mantle. Oxidative stress induced by H2O2 has also been shown to induce an increase in MAPK phosphorylation in a range of organisms including marine bivalves, ascidian embryos and amphibians (Damiani *et al.*, 2009; Gaitanaki, 2003). In addition, activation of MAPK is crucial in the immune response of *Mytilus* haemocytes as a rapid, sustained, but transient activation/phosphorylation of both P38 and JNK MAPK has been shown to be associated with bacterial killing (Chatel *et al.*, 2011). JNK has also been reported to be involved in B(α)P-induced apoptosis, probably by trigger the AP-1 transactivation pathway, and this effect could be an important factor when trying to explain the tumour promoting effect of B(α)P (Betti *et al.*, 2006). In addition, JNK is reported to be critically required for Ras-induced transformation of mouse embryo fibroblasts (MEF)
in vitro and for Ras-induced lung tumour formation in vivo (Cellurale et al., 2011). The absence of JNK caused increased Ras-stimulated carcinogenesis with an increase in the number or the size of the lung tumour nodules, suggesting the potential regulation of JNK to Ras-induced tumour in mammalian cells (Kennedy, 2003). Furthermore, Papadakis et al. (2006) reported a JNK-dependent regulation of Bax to mediate the apoptotic release of cytochrome c based on the evidence of absent apoptotic death in JNK-deficient mouse embryonic fibroblasts.

All the existing studies have proved that JNK is a crucial factor mediating signalling transduction in organisms. Any abnormality in JNK could therefore be related to a series of dysfunctions in the cellular proliferation process. The role of JNK may however, be altered by the genetic background and tissue origin of the tumour. It should be pointed out that all the existing studies have focused on the protein levels due to lack of sequence information for the genes involved. Alone with the recently published jnk sequence in *Mytilus sp.*, the attempt to analyse jnk expression in different tissues is becoming increasingly possible which can provide valuable information for tissue-specific signalling pathways among the invertebrates in general and mussels in particular.

**Group 5: Rad51 family**

Rad51 is a protein involved in the repair of DNA double strand breaks (DSBs) by homologous recombination (HR) pathways. DSBs are induced in DNA by environmental factors including ionising radiations (IR), heat shock and hypoxia (Morrison et al., 2000). Rad51 attaches onto DNA break ends to form presynaptic filament, which seeks out a sequence homologous to (i.e. the same as) the damaged DNA on the neighbouring chromatid. The filament introduces itself into the intact strands and opens a D loop, which the broken strands then use as templates to repair
their sequence (Gerton and Hawley, 2005). In addition to the important role of Rad51 in DNA HR pathways, many researchers suggest that P53 can both physically and via signalling interact with Rad51 and then regulate its ability for DSB repair (Sturzbecher et al., 1999).

As the molecules and molecular events directly link to DNA repair pathways, *rad51* mRNA expression is used in vertebrates or mammalian systems as a biomarker to determine environmental stress (Bishay et al., 2001; Chinnaiyan et al., 2005). Studies using mouse, chicken and other mammalian cells have shown that inefficient repair or mis-repair of DNA damage can lead to genomic instability (Rollinson et al., 2007; Thompson and Schild, 1999; Zhao et al., 2007). This relationship between the DNA repair pathway and *rad51* mRNA expression can therefore potentially be adopted as a biomarker of contamination exposure in aquatic or any biota. Recently, *rad51* partial mRNA has been sequenced and applied for the assessment of environmental radionuclide-induced response in *Mytilus sp.* (AlAmri et al., 2012). Therefore, the study on *rad51* can not only provide the information pertaining to DSB repair in *Mytilus sp.* but also confirm if DNA repair can be generated in response to environmental contaminants or stressors such as B(α)P and C$_{60}$.

In summary, a common interplay among highly conserved genes exists in the organisms following exposure to environmental stress. This can be diagrammatically summarised in Figure 5.1. For example, B(α)P can induce DNA damage after initially metabolism to the carcinogenic form (BPDE) by the CYP enzyme family. The induced DNA damage stress, irrespective of the nature of the inducer, in turn activates the expression of p53 which can be regulated by MDM2 and can directly downstream regulate Bax expression. The extracellular stress can also directly induce Ras activation to process its role in cellular proliferation and the JNK related cascade to transduce the signal into several
intercellular responses. The activation of JNK can also inhibit Ras-stimulated tumourogenesis. In addition, as mentioned before, DNA damage can also activated the Rad51 related HR pathway to repair the damaged DNA.

Against the backdrop of the above information, in the present study, five candidate genes (i.e. *cyp4y1, mdm2, bax, jnk* and *rad51*) were selected as representative effectors which are involved in the *p53/ras* mediated signalling pathways analyses in marine mussels following exposure to B(α)P and/or *C₆₀*. The hypothesis was that “in common with higher organisms the classic signalling pathways also exist in lower level organism (i.e. marine mussels)”. As all these genes have been proved to be related to tumour development in different tissues, another hypothesis to be probed in this study was that “the signalling pathways comprising seven genes (including *p53* and *ras*) show tissue specific differences”. The main objective was to use real-time qPCR to analyses the changes in target genes expression and to provide fundamental conclusions about signal transduction at the transcriptional level.
5.1 Diagrammatic summary of classical signalling pathway involving selected gene groups following exposure to environmental stress. P53 is playing a central role in DNA repair and apoptosis and can be regulated directly by MDM2 and JNK. Both failure of cellular defence (e.g. DNA repair, apoptosis) and mutagenesis of proto-oncogene (e.g. ras) can result in necrosis, cell injury and tumour development.

5.2 MATERIALS AND METHODS

5.2.1 Mussel collection and experiment set-up

Mussels were collected and exposed to B(α)P (56 µg/L), C_{60} (1 mg/L) and the combination of B(α)P and C_{60} as described in Chapter 4.2.1 (same exposure conditions
were applied). The RNA samples isolated from haemocytes, digestive gland, mantle, adductor muscle and gill were reverse-transcripted for gene expression analyses as described in Chapter 4.2. The same cDNA were used for the analyses of gene expression participating in different pathways in this study.

5.2.2 Candidate genes screen, blast and alignment

Candidate genes were screened from two databases: (a) National Centre for Biotechnology Information (NCBI, US National library of medicine, USA, http://www.ncbi.nlm.nih.gov/) and (b) Mytibase (established by several European research institutes based on clusters of _M. galloprovincialis_ ESTs, http://mussel.cribi.unipd.it/) to select suitable genes which belong to the target gene family in _Mytilus sp._ The selected sequences were then run on Blastx against human gene using the NCBI provided Blastx tool to determine if the sequences were similar to the human sequences (e-value < 0.05) (Table 5.1). The expected value (e-value) represents the matching degree of sequences from two species. The e-value is close to zero indicating high degree of matching and the value less than 0.05 was defined as significant matching of the sequences (Madden, 2002). Bio-edit software was applied to generate alignment graphs of sequences in _Mytilus sp._ with the similar sequences in other organisms.

5.2.3 Primer design, PCR condition optimisation and sequencing

Primers for each candidate gene (i.e. _cyp4y1, mdm2, bax, jnk_ and _Rad51_) were designed using Primer Premier 5 as described in Chapter 2.10 (Table 5.2). The optimisation of PCR conditions for each gene was carried out by standard PCR as described in Chapter 2.10. The PCR amplicons showing a clear single band at the desired size after agarose electrophoresis were then sequenced as described in Chapter 2.11. Primers and PCR
conditions, which can yield desired amplicons after sequencing confirmation, were selected for real-time qPCR analyses.

5.2.4 Real-time qPCR and data analysis

Real-time qPCR was applied to analyse the expression of candidate genes using PCR conditions as described in Chapter 2.12. *18S rRNA* was used as the housekeeping gene to normalise the gene expression results. The expression abundance of each gene in different tissues was analysed by the ΔCt method and the relative expression was analysed by the $2^{-\Delta\Delta C_t}$ method as described in Chapter 3.2.6

5.2.5 Statistics analyses

Statistics analyses were carried out with the aid of Minitab V15 statistical package (Minitab Inc., USA) on the data normalised by $2^{-\Delta\Delta C_t}$ method. Significant differences of transcriptional alteration in individual gene between control (level of 1) and treated groups were studied using one-way analysis of variance (ANOVA) after testing for normality of the data and homogeneity of variances. Significant differences of expression change in individual gene among different treatments (i.e. different exposure scenarios and sampling times) were studied using ANOVA followed by Tukey’s test. All values are provided as means ± S.E.M. Significance was established at $P<0.05$. Correlation and regression analyses were also applied in the aid of Minitab V15 to investigate the relationship between paired genes which are expected to interplay with each other.
Table 5.1 Screened target genes in *Mytilus sp.* and respective match scores after Blastx against *Homo sapiens.*

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>cDNA/mRNA sequence</th>
<th>GeneBank Assessee (protein)</th>
<th>Blastx Result (Similar to <em>Homo sapiens</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Size (bp)</td>
<td>Status</td>
<td>Species</td>
</tr>
<tr>
<td>CYP4Y1</td>
<td>447</td>
<td>partial</td>
<td><em>Mytilus galloprovincialis</em></td>
</tr>
<tr>
<td>MDM2</td>
<td>2793</td>
<td>complete</td>
<td><em>Mytilus trossulus</em></td>
</tr>
<tr>
<td>Bax</td>
<td>1175</td>
<td>complete</td>
<td><em>Mytilus galloprovincialis</em></td>
</tr>
<tr>
<td>JNK</td>
<td>406</td>
<td>partial</td>
<td><em>Mytilus galloprovincialis</em></td>
</tr>
<tr>
<td>Rad51</td>
<td>1220</td>
<td>complete</td>
<td><em>Mytius edulis</em></td>
</tr>
</tbody>
</table>
Table 5.2 Primers used for real-time qPCR and relevant PCR conditions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Tested $T_A$ Range ($°C$)</th>
<th>Confirmed $T_A$($°C$)</th>
<th>Amplicon size (bp)</th>
<th>Gene bank access number (nuclides)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyp4y1</td>
<td>F 5’ CAAAGCACTTGAAAATAATC 3’</td>
<td>45-55</td>
<td>48</td>
<td>112</td>
<td>AF072855</td>
<td>Designed</td>
</tr>
<tr>
<td></td>
<td>R 5’ TAAGGGAACTGGTGATG 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mdm2</td>
<td>F 5’ CCTATGGTAAGACCAAGACGTGA 3’</td>
<td>50-60</td>
<td>55</td>
<td>60</td>
<td>HM004082</td>
<td>Muttray et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>R 5’ CCCATGATGACCTACTGTGT 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bax</td>
<td>F 5’ GAGAATACAAGCGACAGT 3’</td>
<td>50-60</td>
<td>55</td>
<td>128</td>
<td>MGC09152</td>
<td>Designed</td>
</tr>
<tr>
<td></td>
<td>R 5’ AAGTTCCTCCGATGAGATGG 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>jnk</td>
<td>F 5’ CCCGCCCTTCCAAAAAGT 3’</td>
<td>50-60</td>
<td>55</td>
<td>266</td>
<td>GQ454914</td>
<td>Designed</td>
</tr>
<tr>
<td></td>
<td>R 5’ ACCAGCAGAATGTAGATGGT 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rad51</td>
<td>F 5’ TGGGATGAGACTGGGTCAA 3’</td>
<td>45-55</td>
<td>50</td>
<td>120</td>
<td>FJ518826</td>
<td>Designed</td>
</tr>
<tr>
<td></td>
<td>R 5’ CCTTCACCTCCACCCATATC 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TA: Annealing temperature; F: forward primer; R: reverse primer.
5.3 RESULTS

5.3.1 Confirmation of amplified target genes by designed primers and selected PCR conditions

The designed primers for *cyp4yl*, *mdm2*, *bax*, *jnk* and *rad51* could amplify desired sequences using optimised PCR conditions. The gel electrophoresis image (Figure 5.2) shows clear single bands at the correct size for each gene, indicating no contamination or primer dimers formed. A weaker *mdm2* band was yielded compared with the other selected genes, indicating *mdm2* had relatively less amplification compared to the other genes after 35 cycles of PCR. The low nucleotide concentration can cause the fuzzy band when all the other electrophoresis conditions are the same (e.g., salt concentration of electrophoresis buffer.

![Figure 5.2 Gel electrophoresis image showing single band for each target gene. Lane 1 and 7: 25 bp ladder; lane 2: *cyp4yl* (112 bp); lane 3: *rad51* (120 bp); lane 4: *bax* (128 bp); lane 5: *jnk* (266 bp) and lane 6: *mdm2* (60 bp).]
5.3.2 Conserved sequence of target genes in different organisms

The blasting result of each target gene for different species showed highly conserved amino acid sequences among species. Rad51 protein in *Mytilus edulis* shares 85% identity with HUMRad51 in humans. JNK shares over 82% identity with human JNK. MDM2 and Bax showed relatively lower identity with the human sequences, 28 and 36% respectively, but the e-value indicated both sequences to be ‘significantly’ matched to the human sequence. CYP4Y1 showed over 52% identity with human CYP4F11 protein which could be considered as relatively high identity within the CYP450 family (Wereck-Reichhart and Feyereisen, 2000). The alignment for each target gene against different species is shown in Figure 5.3.
### Table 5.1: Comparison of APOBEC3G Sequences

<table>
<thead>
<tr>
<th>Species</th>
<th>APOBEC3G Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. trossulus</td>
<td>RFEDRKLKVS GSAQEYTVK FGRSYSTVYI</td>
</tr>
<tr>
<td>M. musculus</td>
<td>PLLKLKLKSV GAQKDITYMK EVLHYLQYI</td>
</tr>
<tr>
<td>O. cuniculus</td>
<td>PLLKLKLKSV GAQKDITYMK EVLHYLQYI</td>
</tr>
<tr>
<td>M. mulatta</td>
<td>PLLKLKLKSV GAQKDITYMK EVLHYLQYI</td>
</tr>
<tr>
<td>M. domestica</td>
<td>PLLKLKLKSV GAQKDITYMK EVLHYLQYI</td>
</tr>
<tr>
<td>C. jacchus</td>
<td>PLLKLKLKSV GAQKDITYMK EVLHYLQYI</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>PLLKLKLKSV GAQKDITYMK EVLHYLQYI</td>
</tr>
<tr>
<td>M. trossulus</td>
<td>CDRFLYND PSTVCKTP IGMTCYDPE</td>
</tr>
<tr>
<td>M. musculus</td>
<td>MTKRFLYDEQO QHVYCNDO LGDFE/QYI</td>
</tr>
<tr>
<td>O. cuniculus</td>
<td>MTKRFLYDEQO QHVYCNDO LGDFE/QYI</td>
</tr>
<tr>
<td>M. mulatta</td>
<td>MTKRFLYDEQO QHVYCNDO LGDFE/QYI</td>
</tr>
<tr>
<td>M. domestica</td>
<td>MTKRFLYDEQO QHVYCNDO LGDFE/QYI</td>
</tr>
<tr>
<td>C. jacchus</td>
<td>MTKRFLYDEQO QHVYCNDO LGDFE/QYI</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>MTKRFLYDEQO QHVYCNDO LGDFE/QYI</td>
</tr>
<tr>
<td>M. trossulus</td>
<td>TFTELKLLID ~~~~~~~~~~ ~~~~~~~~~A</td>
</tr>
<tr>
<td>M. gallo</td>
<td>VCVVILTVFL  VP*EHNLKDR PFCQVTHAKR  28</td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>YCAAYLTVTQ  QVVAIKKLSR PFCQVTHAKR  30</td>
</tr>
<tr>
<td>A. californica</td>
<td>YCAACVDTLD  TNVAIKKLSR PFCQVTHAKR  30</td>
</tr>
<tr>
<td>M. japonicus</td>
<td>YCAAYLTVTQ  QVVAIKKLSR PFCQVTHAKR  30</td>
</tr>
<tr>
<td>A. albopictus</td>
<td>YCAAYLTVTQ  QVVAIKKLSR PFCQVTHAKR  30</td>
</tr>
<tr>
<td>M. musculus</td>
<td>YCAAYLTVTQ  QVVAIKKLSR PFCQVTHAKR  30</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>YCAAYLTVTQ  QVVAIKKLSR PFCQVTHAKR  30</td>
</tr>
</tbody>
</table>

| M. gallo | AYREFVLMKL  VNHKNIIGLE DFTPOQKSL  58 |
| D. melanogaster | AYREFVLMKL  VNHKNIIGLE DFTPOQKSL  60 |
| A. californica | AYREFVLMKL  VNHKNIIGLE DFTPOQKSL  60 |
| M. japonicus | AYREFVLMKL  VNHKNIIGLE DFTPOQKSL  60 |
| A. albopictus | AYREFVLMKL  VNHKNIIGLE DFTPOQKSL  60 |
| M. musculus | AYREFVLMKL  VNHKNIIGLE DFTPOQKSL  60 |
| H. sapiens | AYREFVLMKL  VNHKNIIGLE DFTPOQKSL  60 |

| M. gallo | EFDGYLVMDE  LMDAYLQVTI AMDLDHERMS  88 |
| D. melanogaster | EFDGYLVMDE  LMDAYLQVTI AMDLDHERMS  90 |
| A. californica | EFDGYLVMDE  LMDAYLQVTI AMDLDHERMS  90 |
| M. japonicus | EFDGYLVMDE  LMDAYLQVTI AMDLDHERMS  90 |
| A. albopictus | EFDGYLVMDE  LMDAYLQVTI AMDLDHERMS  90 |
| M. musculus | EFDGYLVMDE  LMDAYLQVTI AMDLDHERMS  90 |
| H. sapiens | EFDGYLVMDE  LMDAYLQVTI AMDLDHERMS  90 |

| M. gallo | YLLY  93 |
| D. melanogaster | YLLY  95 |
| A. californica | YLLY  95 |
| M. japonicus | YLLY  95 |
| A. albopictus | YLLY  95 |
| M. musculus | YLLY  95 |
| H. sapiens | YLLY  95 |
Figure 5.3 Alignment graphs of the target genes in different species. (A) CYP4Y1; (B) MDM2; (C) Bax; (D) JNK and (E) Rad51. Black background indicates identical sequence and gray background indicates highly conserved sequence.
5.3.3 **Expression of target genes in different tissues under control conditions**

Expression abundance of all (selected) target genes showed tissue-specific patterns after normalisation with 18S rRNA by the ΔCt method (E 2.3). An example of gene (*mdm2*) amplification and melting profile in different tissues is shown in Figure 5.4. Only a single peak (83 °C for *mdm2*) is present in the melting curve. This confirmed that there was no fluorescent signal disturbance from contamination or primer dimers. Other genes showed similar profiles only with differences in the initial amplification time for each tissue and melting temperatures (77 °C for *cyp4y1*, 79 °C for *bax*, 78.7 °C for *jnk* and 79.5 °C for *rad51*). The normalised *cyp4y1* expression showed higher abundance in digestive gland, adductor muscle and mantle, but lower abundance in gill and haemocytes (Figure 5.5A). Highest expression of *mdm2* was in adductor muscle and least in gill tissues. Digestive gland, mantle and haemocytes showed similar expression level for *mdm2* under control conditions (Figure 5.5B). Adductor muscle and mantle showed higher *bax* expression compared to the other two tissues and haemocytes (Figure 5.5C). Similar results were found for *rad51* expression where higher abundance was in adductor muscle, mantle and haemocytes and lower in digestive gland and gill tissues (Figure 5.5D). In contrast, *jnk* expression showed a different pattern, more *jnk* was expressed in mantle and less in haemocytes (Figure 5.5E).
Figure 5.4 Example of real-time qPCR outputs showing amplification (A) and melting (B) characterisers of mdm2 gene in different tissues under control condition.
Figure 5.5 Expression abundance of genes in different tissues and haemocytes under control condition. A: cyp4y1, B: mdm2, C: bax, D: rad51 and E: jnk. Each bar represents the means of 6 replicates (n=6) and S.E.M were indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p<0.05.
5.3.4 Relative quantification of target genes expression in different tissues and haemocytes after B(α)P and/or C₆₀ exposure

Relative quantification of expression of all selected target genes (i.e. cyp4y1, mdm2, bax, jnk and rad51) was analysed using the $2^{-\Delta\Delta Ct}$ method (E 2.4). In order to link the expression profile of these genes induced by B(α)P and/or C₆₀ with the anti-oncogene p53 and the proto-oncogene ras under same stress, the quantified results of p53 and ras relative expression which have been presented in Chapter 4.3 are also briefly included in this Chapter for comparison. Similar to p53 and ras relative expression results, high inter-individual variation was found in all the results.

5.3.4.1 Relative expression of target genes in haemocytes

The relative expression of cyp4y1 was significantly induced after 3 days exposure to B(α)P alone (2.86 ± 1.43-fold) (Figure 5.6A). C₆₀ exposure alone significantly induced cyp4y1 expression after 1 day exposure. The induction level increased to over 700-fold after 3 days exposure. Similar results were found after the combined exposure. Significantly increased cyp4y1 expression (over 400-fold) was found after 1 day exposure, the level then significantly dropped after 3 days exposure (5.96 ± 0.42-fold).

After 3 days recovery from all three exposure scenarios, the relative expressions of cyp4y1 were still significantly higher than the control level.

There was significantly induced jnk expression following 1 day exposure to B(α)P alone (Figure 5.6B). The induction level increased to 11.61 ± 6.55-fold after 3 days exposure and then dropped below control level after 3 days recovery. Significantly increased jnk expression (over 100-fold) was also found after 1 day exposure to C₆₀ alone. The level significantly increased after 3 days exposure and reached over a thousand fold. After the recovery period, jnk expression level decreased but still was significantly higher than
the control level. A similar pattern of $jnk$ expression has shown after the exposure to combined chemicals. A significant induction of $jnk$ expression was shown after 1 day exposure, which was also higher than the level induced by exposure of two chemicals alone. After 3 days exposure, there was no significant difference of the induction level of $jnk$ compared to 1 day exposure, but significantly decreased after 3 days recovery in fresh seawater.

The relative expression of $mdm2$ and $bax$ showed a similar pattern (Figure 5.6C and 5.6D). After B(α)P exposure alone, increased expression level was observed after 1 day exposure, the level increased significantly after 3 days exposure and decreased to below the control level after 3 days recovery. After C$_{60}$ exposure alone, the highest induction of $mdm2$ and $bax$ relative expression was found after 1 day exposure (over 200- and 100-fold to $mdm2$ and $bax$, respectively). The induction level dropped after 3 days exposure. There was no significant change in $mdm2$ expression after 3 days recovery compared to after 3 days exposure. However, a significant decline of $bax$ expression was found after the recovery. Less inductions of $mdm2$ and $bax$ expression were found after combined exposure in comparison to C$_{60}$ exposure alone. Significantly increased $mdm2$ and $bax$ expressions were found after both 1 and 3 days exposure, and 3 days recovery.

The relative expression of $rad51$ showed different pattern compared to $mdm2$ and $bax$ genes expression (Figure 5.6E). There was a significant induction of $rad51$ expression (66.68 ± 24.53-fold) after 1 day exposure to B(α)P alone. The level significantly decreased (2.53 ± 0.77-fold) after 3 days exposure and returned back to control level after 3 days recovery. The significant induction of $rad51$ expression was only found after 3 days exposure to C$_{60}$ and then dropped to close to control level. Significant induction for $rad51$ expression was found after exposure to combined chemicals. A
401.49 ± 250.28-fold inductions was found after 1 day exposure and the level increased significantly to over two thousand-fold after 3 days exposure. The relative expression level of rad51 significantly decreased after 3 days recovery from exposure.

As mentioned in Chapter 4, p53 was significantly induced by C60 alone and the combined exposure of chemicals. Highest induction was found after 3 days exposure and the level significantly dropped after recovery in fresh seawater (Figure 5.6F). There was no significant ras induction after any exposure at any time (Figure 5.6G).

The overall transcriptional alteration of target genes in haemocytes of Mytilus sp. is summarised in Table 5.3 for comparison.
Figure 5.6 Relative expression of target genes (A-cyp4y1; B-jnk; C-mdm2; D-bax, E-rad51; F-p53 and G-ras) in haemocytes after exposure to B(α)P and/or C60. Level of 1 is the control level. Each bar represents the means of 6 replicates (n=6) and S.E.M are indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p<0.05. * indicates significant up-regulated genes expression compared to control. # indicates significant down-regulated genes expression compared to control.
Table 5.3 Summary of changes in expression of target genes in haemocytes of *Mytilus sp.* following different exposures.

<table>
<thead>
<tr>
<th>Time</th>
<th>Exposure</th>
<th>Target genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>cyp4y1</em></td>
</tr>
<tr>
<td>1 Day exposure</td>
<td>B(α)P</td>
<td>↑/—</td>
</tr>
<tr>
<td></td>
<td>C&lt;sub&gt;60&lt;/sub&gt;</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C&lt;sub&gt;60&lt;/sub&gt;</td>
<td>↑↑</td>
</tr>
<tr>
<td>3 Days exposure</td>
<td>B(α)P</td>
<td>↑*</td>
</tr>
<tr>
<td></td>
<td>C&lt;sub&gt;60&lt;/sub&gt;</td>
<td>↑↑</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C&lt;sub&gt;60&lt;/sub&gt;</td>
<td>↑</td>
</tr>
<tr>
<td>3 Days recovery</td>
<td>B(α)P</td>
<td>↑*</td>
</tr>
<tr>
<td></td>
<td>C&lt;sub&gt;60&lt;/sub&gt;</td>
<td>↑ (†)</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C&lt;sub&gt;60&lt;/sub&gt;</td>
<td>↑↑ (†)</td>
</tr>
</tbody>
</table>

↑: up-regulated gene expression, increased number of the arrow indicating increased level of induced gene expression after exposure;
—: no change in gene expression;
↓: down-regulated gene expression;
†: decreased expression level compared to the level after exposure;
*: decreased expression level compared to 1 day exposure.
5.3.4.2 Relative expression of target genes in digestive gland

There was significantly increased \( cyp4y1 \) expression found after both 1 and 3 days exposure to B(α)P alone, and after 3 days recovery in fresh seawater (Figure 5.7A). A significantly higher induction for \( cyp4y1 \) (10.56 ± 1.33-fold) was observed after 1 day exposure, the level dropped slightly after 3 days exposure and was maintained at similar level after 3 days recovery in fresh seawater. The combined exposure to B(α)P and C\(_{60}\) induced the highest \( cyp4y1 \) expression after 1 day exposure (over 2000-fold). The expression level significantly decreased after 3 days exposure and 3 days recovery, but the level was still significantly higher compared to control or untreated group.

After B(α)P exposure alone, \( jnk \) relative expression was significantly increased after 1 and 3 days exposure (Figure 5.7B). C\(_{60}\) exposure alone can also significantly induce \( jnk \) expression. The highest induction of \( jnk \) expression was found after the combined exposure of B(α)P and C\(_{60}\) for 1 day. The level significantly decreased after 3 days exposure and further decreased after recovery in fresh seawater. The induced expression level after recovery was, however, still statistically higher than the control level.

The only significant induction in \( mdm2 \) after B(α)P alone exposure was found after 3 days (Figure 5.7C). C\(_{60}\) exposure alone significantly induced expression of \( mdm2 \) in comparison with B(α)P. The highest \( mdm2 \) expression (over thousand-fold) was found after 1 day exposure to the combined chemicals. The level was significantly decreased after recovery, but still significantly higher than the control level.

The relative expression of \( bax \) in digestive gland was similar to \( mdm2 \) expression (Figure 5.7D). The expression was significantly induced by all three exposure scenarios. Similar to other genes expression profiles, a quicker response in \( bax \) expression was observed because the highest induction of \( bax \) expression was found after short period
exposure (i.e. 1 day). After recovery from different exposures, the expression level of bax decreased to the similar level but was still significantly higher than the control.

The expression of rad51 was significantly induced after B(α)P exposure for 3 days. A significant induction of rad51 expression was found after C₆₀ alone exposure for both 1 and 3 days, and after recovery (Figure 5.7E). The combined exposure of B(α)P and C₆₀ significantly induced rad51 expression after 1 day exposure (267 ± 13.81-fold). The level decreased after 3 days exposure and remained the similar level after recovery in fresh seawater.

Unlike rad51, significantly increased p53 expression was found after C₆₀ exposure alone compared to other two exposure types. A decline in expression was observed after 3 days exposure and recovery (Figure 5.7F). The relative expression of ras was also significantly induced by C₆₀ exposure alone after 1 day exposure (Figure 5.7G). The level decreased after 3 days exposure and then returned to control level after recovery. For all the exposures, the expression level or ras returned back to control or close to control level after the recovery.

The overall transcriptional alterations of target genes in digestive gland of Mytilus sp. in response to B(α)P and/or C₆₀ are summarised in Table 5.4 for comparison.
Figure 5.7 Relative expression of target genes (A-cyp4y1; B-jnk; C-mdm2; D-bax, E-rad51; F-p53 and G-ras) in digestive gland after exposure to B(α)P and/or C60. Level of 1 is the control level. Each bar represents the means of 6 replicates (n=6) and S.E.M are indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p<0.05. * indicates significant up-regulated genes expression compared to control. # indicates significant down-regulated genes expression compared to control.
Table 5.4 Summary of changes in expression of target genes in digestive gland of *Mytilus sp.* following different exposures

<table>
<thead>
<tr>
<th>Time</th>
<th>Exposure</th>
<th>cyp4y1</th>
<th>jnk</th>
<th>mdm</th>
<th>bax</th>
<th>rad51</th>
<th>p53</th>
<th>ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day exposure</td>
<td>B(α)P</td>
<td>↑</td>
<td>↑</td>
<td>↑/—</td>
<td>↑</td>
<td>—</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>C₆₀</td>
<td>↑↑</td>
<td>↑</td>
<td>↑</td>
<td>↑↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C₆₀</td>
<td>↑↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>3 Days exposure</td>
<td>B(α)P</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑/—</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>C₆₀</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C₆₀</td>
<td>↑↑</td>
<td>↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>3 Days recovery</td>
<td>B(α)P</td>
<td>↑</td>
<td>—</td>
<td>↑/—(†)</td>
<td>↑</td>
<td>—(†)</td>
<td>—(†)</td>
<td>—(†)</td>
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<tr>
<td></td>
<td>C₆₀</td>
<td>↑(†)</td>
<td>↑/—(†)</td>
<td>↑(†)</td>
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<td>↑/—(†)</td>
<td>—(†)</td>
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<tr>
<td></td>
<td>B(α)P and C₆₀</td>
<td>↑(†)</td>
<td>↑(†)</td>
<td>↑(†)</td>
<td>↑(†)</td>
<td>↑↑(†)</td>
<td>↑/—(†)</td>
<td>↑(†)</td>
</tr>
</tbody>
</table>

↑: up-regulated gene expression, increased number of the arrow indicating increased level of induced gene expression after exposure;  
—: no change in gene expression;  
↓: down-regulated gene expression;  
†: decreased expression level compared to the level after exposure.
5.3.4.3 Relative expression of target genes in mantle

The target gene expression in mantle showed different pattern compared to digestive gland and haemocytes where most of the genes were responding more to C\textsubscript{60} exposure alone. After B(α)P exposure alone, \textit{cyp4y1} expression was significantly induced after 3 days exposure (Figure 5.8A). The only significantly increased expression of \textit{cyp4y1} was found after 3 days exposure to C\textsubscript{60}. The combined exposure of two chemicals significantly induced \textit{cyp4y1} expression in mantle. Over thousand-fold increased expression was observed after 1 day exposure and the level remained increasing to over ten thousand-fold after 3 days exposure. A significant decline for \textit{cyp4y1} expression was found after recovery from all three exposure scenarios.

Similar to \textit{cyp4y1} expression, \textit{jnk} expression also showed enhanced response following combined chemicals exposure. After B(α)P exposure alone, significantly increased \textit{jnk} expression was observed after 1 and 3 days exposure (Figure 5.8B). A significant increase (14.02 ± 6.43-fold) of \textit{jnk} expression was observed after 3 days exposure to C\textsubscript{60} alone. The combined exposure of B(α)P and C\textsubscript{60} induced significantly increased \textit{jnk} expression (over 700-fold) after 1 day exposure. A further significant increase was found after 3 days exposure. All the levels of the \textit{jnk} relative expression exposed to three exposure scenarios were back to or below control level after recovery in fresh seawater for 3 days.

B(α)P exposure alone significantly induced \textit{mdm2} expression after 1 day exposure (106.39 ± 104.76-fold) (Figure 5.8C). The induction increased after longer (i.e. 3 days) exposure and reached to over thousand-fold and then significantly dropped to control level after 3 days recovery in fresh seawater. C\textsubscript{60} exposure alone induced expression of \textit{mdm2} after 3 days exposure only. The combined exposure of chemicals induced
significantly increased \textit{mdm2} expression after exposures. The induction was significantly decreased after recovery in fresh seawater.

A very similar expression pattern of \textit{mdm2} was found in \textit{bax} expression after 3 different exposure scenarios. \textit{B(α)P} only significantly induced the \textit{bax} expression after 3 days exposure and the same as \textit{C_{60}} exposure (Figure 5.8D). A significantly increased \textit{bax} expression (over 700-fold) was found after 1 day exposure to the combined exposure to chemicals. Significantly enhanced expression (over 30 thousand-fold) was also found after 3 days exposure. The increased expression level decreased to control level after recovery in fresh seawater.

All three exposures (i.e. two chemicals alone and then in combination) induced a significant \textit{rad51} expression after 3 days exposure with highest expression after the combined exposure followed by \textit{B(α)P} exposure and then \textit{C_{60}} exposure (Figure 5.8E). After 1 day exposure, the combined exposure and \textit{B(α)P} alone exposure significantly increased the \textit{rad51} expression. There was no induction in \textit{rad51} expression after recovery from any type of exposure.

As mentioned in Chapter 4, \textit{p53} and \textit{ras} expressions in mantle significantly increased after 3 days exposure to all 3 types of exposure (Figure 5.8F and 5.8G). The combined exposure also induced significantly increased \textit{p53} and \textit{ras} expression after 1 day exposure. However, unlike the gene expression pattern mentioned above, both \textit{p53} and \textit{ras} gene expressions after recovery from the exposures to the combined chemicals were dropped close to or below the control level.

The overall transcriptional alterations of target genes in mantle of \textit{Mytilus sp.} in response to \textit{B(α)P} and/or \textit{C_{60}} are summarised in Table 5.5 for comparison.
Figure 5.8 Relative expression of target genes (A-cyp4y1; B-jnk; C-mdm2; D-bax, E-rad51; F-p53 and G-ras) in mantle after exposure to B(α)P and/or C60. Level of 1 is the control level. Each bar represents the means of 6 replicates (n=6) and S.E.M are indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p<0.05. * indicates significant up-regulated genes expression compared to control. # indicates significant down-regulated genes expression compared to control.
Table 5.5 Summary of changes in expression of target genes in mantle of *Mytilus sp.* following different exposures.

<table>
<thead>
<tr>
<th>Time</th>
<th>Exposure</th>
<th>Target genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cyp4y1</td>
</tr>
<tr>
<td>1 Day</td>
<td>B(α)P</td>
<td>↑/—</td>
</tr>
<tr>
<td>exposure</td>
<td>C₆₀</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C₆₀</td>
<td>↑↑</td>
</tr>
<tr>
<td>3 Days</td>
<td>B(α)P</td>
<td>↑↑</td>
</tr>
<tr>
<td>exposure</td>
<td>C₆₀</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C₆₀</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>3 Days</td>
<td>B(α)P</td>
<td>↑/— (†)</td>
</tr>
<tr>
<td>recovery</td>
<td>C₆₀</td>
<td>↓ (†)</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C₆₀</td>
<td>↑/— (†)</td>
</tr>
</tbody>
</table>

↑: up-regulated gene expression, increased number of arrow indicating increased level of induced gene expression after exposure; 
—: no change in gene expression; 
↓: down-regulated gene expression; 
†: decreased expression level compared to the level after exposure.
5.3.4.4 Relative expression of target genes in adductor muscle

All five target genes (i.e. cyp4y1, jnk, mdm2, bax and rad51) showed similar expression pattern following exposure to the chemicals (Figure 5.9A to 5.9E). There was a significant induction of target genes after 1 day exposure to B(α)P. The level then decreased to control level after 3 days exposure and remained at the similar level after recovery in the fresh seawater. A significant induction of target genes was observed after 1 day combined exposure to chemicals. The level, however, increased further after longer exposure time (i.e. 3 days) but dropped back to control level after recovery under all conditions.

The combined exposure of B(α)P and C60 also induced significantly increased p53 and ras expression after 1 day exposure (Figure 5.9F and 5.9G). However, unlike other target genes, the increased level significantly decreased after longer exposure time and finally returned back to the control level. p53 showed different expression pattern compared to other genes since higher level of the p53 expression was induced by C60 alone compared to B(α)P exposure alone after 1 day exposure.

The overall transcriptional alterations of target genes in adductor muscle of Mytilus sp. in response to B(α)P and/or C60 are summarised in Table 5.6 for comparison.
Figure 5.9 Relative expression of target genes (A-cyp4y1; B-jnk; C-mdm2; D-bax, E-rad51; F-p53 and G-ras) in adductor muscle after exposure to B(α)P and/or C60. Level of 1 is the control level. Each bar represents the means of 6 replicates (n=6) and S.E.M are indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p<0.05. * indicates significant up-regulated genes expression compared to control. # indicates significant down-regulated genes expression compared to control.
Table 5.6 Summary of changes in expression of target genes in adductor muscle of *Mytilus sp.* following different exposures.

<table>
<thead>
<tr>
<th>Time</th>
<th>Exposure</th>
<th>Target genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>cytochrome p450</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>1 Day exposure</td>
<td>B(α)P</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>C&lt;sub&gt;60&lt;/sub&gt;</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C&lt;sub&gt;60&lt;/sub&gt;</td>
<td>↑↑</td>
</tr>
<tr>
<td>3 Days exposure</td>
<td>B(α)P</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>C&lt;sub&gt;60&lt;/sub&gt;</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C&lt;sub&gt;60&lt;/sub&gt;</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>3 Days recovery</td>
<td>B(α)P</td>
<td>↓/<em>↑</em>/(†)</td>
</tr>
<tr>
<td></td>
<td>C&lt;sub&gt;60&lt;/sub&gt;</td>
<td>—/<em>↑</em>/(†)</td>
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<tr>
<td></td>
<td>B(α)P and C&lt;sub&gt;60&lt;/sub&gt;</td>
<td>↓/<em>↑</em>/(†)</td>
</tr>
</tbody>
</table>

↑: up-regulated gene expression, increased number of arrow indicating increased level of induced gene expression after exposure;
—: no change in gene expression;
↓: down-regulated gene expression;
†: decreased expression level compared to the level after exposure.
5.3.4.5 Relative expression of target genes in gill tissue

The gene expression in gill tissue showed different pattern compared to other tissues. After B(α)P exposure alone, a significantly increased expression of cyp4y1 was observed after all three exposure scenarios (Figure 5.10A). The relative expression of jnk and bax showed similar pattern (Figure 5.10B and 5.10D). B(α)P exposure could only induce significantly increased jnk and bax expression after 3 days exposure. C₆₀ exposure alone induced the highest jnk and bax expression level compared to other two exposure scenarios. Significantly increased jnk and bax expression was observed after 1 day exposure; the level increased significantly after 3 days exposure and dropped close to the level of 1 day exposure after recovery, but was still significantly higher than the control level. The combined exposure induced jnk and bax expression starting from 1 day exposure but the level significantly lowered than C₆₀ exposure alone.

The B(α)P exposure alone induced significantly increased mdm2 expression after exposures (Figure 5.10C). The level decreased to control level after recovery. C₆₀ exposure also significantly increased the expression of mdm2 and the level was significantly higher than the level induced after exposure to B(α)P alone. This increased level of mdm2 expression remained at the similar level after exposure and recovery. Similar to B(α)P exposure, the combined exposure of chemicals induced the highest expression of mdm2 after 1 day exposure, the expression significantly decreased after longer exposure period and returned to close to the control level after recovery. However, there was still statistically significant induction for mdm2 expression after recovery when exposed to C₆₀ and combined exposure to chemicals.

A significantly enhanced induction for rad51 expression was only observed after 3 days exposure to B(α)P alone (Figure 5.10E). Similar to other genes, C₆₀ induced the highest rad51 expression in gill. A significantly increased expression was observed after 1 day
exposure. The expression level increased after longer exposure time. After the combined exposure, the highest increased in rad51 expression was induced after 1 day exposures. Longer exposure time did not cause enhanced induction and the expression level of rad51 decreased after recovery. However, rad51 expression did not return back to control level after recovery from both C\textsubscript{60} alone and the combined exposure of both chemicals.

C\textsubscript{60} exposure on its own induced significant p53 expression compared to other two exposure scenarios. But the induction level was not as high as in other genes (Figure 5.10F). There was no significant difference for expression levels among 3 different sampling days. There was no induction of ras expression under any exposure conditions observed (Figure 5.10G).

The overall transcriptional alterations of target genes in gill of *Mytilus* sp. in response to B(α)P and/or C\textsubscript{60} are summarised in Table 5.7 for comparison.
Figure 5.10 Relative expression of genes (A- cyp4yl; B-jnk; C-mdm2; D- bax, E-rad51; F-p53 and G-ras) in gill after exposure to B(α)P and/or C60. Level of 1 is the control level. Each bar represents the means of 6 replicates (n=6) and S.E.M are indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p<0.05. * indicates significant up-regulated genes expression compared to control. # indicates significant down-regulated genes expression compared to control.
Table 5.7 Summary of changes in expression of target genes in gill of *Mytilus* sp. following different exposures.

<table>
<thead>
<tr>
<th>Time</th>
<th>Exposure</th>
<th>Target genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cyp4y1</td>
<td>jnk</td>
</tr>
<tr>
<td>1 Day exposure</td>
<td>B(α)P</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>C₆₀</td>
<td>↑↑</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C₆₀</td>
<td>↑</td>
</tr>
<tr>
<td>3 Days exposure</td>
<td>B(α)P</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>C₆₀</td>
<td>↑↑↑</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C₆₀</td>
<td>↑</td>
</tr>
<tr>
<td>3 Days recovery</td>
<td>B(α)P</td>
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</tr>
<tr>
<td></td>
<td>C₆₀</td>
<td>↑ (†)</td>
</tr>
<tr>
<td></td>
<td>B(α)P and C₆₀</td>
<td>↑ (†)</td>
</tr>
</tbody>
</table>

↑: up-regulated gene expression, increased number of arrow indicating increased level of induced gene expression after exposure;  
—: no change in gene expression;  
↓: down-regulated gene expression;  
†: decreased expression level compared to the level after exposure.
5.3.5 Correlation of genes involved in different pathways

The correlation and regression analyses were carried out to investigate the interplay of paired genes in haemocytes and tissues of mussels. Selected genes were divided into 5 pathway groups in terms of their functions: (1) biotransformation pathway (cyp4yl and intercellular effectors of p53, ras, jnk and rad51); (2) apoptosis pathway (p53, jnk and bax); (3) DNA repair pathway (p53 and rad51); (4) Ras regulation related pathway (ras and jnk) and (5) p53 regulation related pathway (p53 and mdm2). Results which showed significant correlation (p<0.05) and well regression fit (R-Sq>60%) were selected to be present in this section. All the correlation analysis results were summarised in Appendix IV. The expressions of cyp4yl and jnk were found well related in haemocytes and all the tissues (Figure 5.11A). Mantle was the only tissue showed well related expression of cyp4yl with all selected downstream effectors (Figure 5.11C, F-H). The expression of bax showed closely related to p53 expression in haemocytes but to jnk expression in digestive gland, adductor muscle and gill (Figure 5.12). The expression of bax which was related to both p53 and jnk expression was observed only in mantle. Mantle was also the only tissue showing closely relationship between p53 and rad51 expression (Figure 5.13), and ras and jnk expression (Figure 5.14). It was noteworthy that relative higher R-Sq level might be generated by leverage points which are far away from the most data points (i.e., low level of change in transcription, <100 fold) in regression analysis. It is, however, non-realistic to ignor the leverage points during the regression analysis due to small sample size.
Figure 5.11 Regressions of paired genes in biotransformation related pathways: (A) cyp4y1 and jnk in haemocytes; (B) cyp4y1 and jnk in digestive gland; (C) cyp4y1 and jnk in mantle; (D) cyp4y1 and jnk in adductor muscle; (E) cyp4y1 and jnk in gill; (F) cyp4y1 and p53 in mantle; (G) cyp4y1 and ras in mantle and (H) cyp4y1 and rad51 in mantle. Black straight lines are illustrating the liner relationship and red dash lines are 95% Confidence Intervals (CI) regressions. Correlations between paired genes are at p<0.05.
Figure 5.12 Regressions of paired genes in apoptosis related pathways: (A) \(p53\) and \(bax\) in haemocytes; (B) \(bax\) and \(jnk\) in digestive gland; (C) \(bax\) and \(jnk\) in adductor muscle; (D) \(bax\) and \(jnk\) in gill; (E) \(p53\) and \(bax\) in mantle and (F) \(bax\) and \(jnk\) in mantle. Black straight lines are illustrating the liner relationship and red dash lines are 95% Confidence Intervals (CI) regressions. Correlations between paired genes are at \(p < 0.05\).
Figure 5.13 Regression of p53 and rad51 in mantle. Black straight lines are illustrating the liner relationship and red dash lines are 95% Confidence Intervals (CI) regressions. Correlations between paired genes are at p<0.05.

Figure 5.14 Regression of ras and jnk in mantle. Black straight lines are illustrating the liner relationship and red dash lines are 95% Confidence Intervals (CI) regressions. Correlations between paired genes are at p<0.05.
5.4 DISCUSSION

The experimental mussels were at risk of taking up high levels of chemicals into the body by filter-feeding behaviours following B(α)P and/or C_{60} introduction into seawater. Upon uptake, these extracellular molecules can then cross cell membrane via three pathways (Figure 5.11): (1) diffusion either directly across the cell membrane or through membrane channels; (2) general endocytosis and (3) receptor-mediated endocytosis (e.g. low density lipoprotein (LDL) through LDL-receptors) (Moore et al., 2004; Mukherjee et al., 1997). Subsequently, a series of metabolic transformations targeting these xenobiotics will be initiated and promoted for either detoxification to harmless derivatives, and then eventual excretion from the mussel’s body, or activation to toxic derivatives which may cause DNA damage directly by producing DNA adducts (e.g. B(α)P) or indirectly by promoting oxidative stress and inflammatory responses (e.g. C_{60}). Intercellular effectors, from the transcriptional to the translational level will then participate in related signal transduction to respond to the induced stresses. Signal transduction is a complex process in cells and can be influenced by many factors, including cellular origin and function, types of extracellular signal, persistence and frequency of signal (Cooper and Hausman, 2007). In this section, an attempt has been made to provide detailed interplay of selected genes.
Figure 5.15 Schematic to illustrate the possible mechanisms of cellular uptake of environmental pollutants/signals including diffusion, endocytosis and receptor-mediated endocytosis.

5.4.1 Determination of tissue-specific transcriptional profiles for target genes under control conditions

A total of 5 selected genes was successfully amplified using the designed primers and optimised PCR conditions. It should, however, be pointed out that gene sequences (i.e. cyp4y1, jnk, bax and mdm2) screened from GeneBank were not specific to single *Mytilus* sp.. It had been assumed that mussels found around most of the coast in the UK, including in Cornwall, are *M. edulis*, whereas those found in the Mediterranean are *M. galloprovincialis* and in Northern Pacific are *M. trossulus*, and all three are principal, closely related taxa (McDonald *et al.*, 1991). Recently, using a molecular probe for the Glu gene, the adhesion protein gene which has been shown to exhibit interspecies variation (Inoue *et al.*, 1995), it has been suggested that the species composition of
mussels at different sites in Devon and Cornwall (south west England) is quite variable and includes *M. edulis*, *M. galloprovincialis* and their hybrids (Hilbish *et al.*, 2002). At Trebarwith strand, where the samples were collected, 97% of the mussels have been reported to be *M. galloprovincialis* and 3% to be hybrids (Bignell *et al.*, 2011). This however needs further investigation to establish the species composition using markers for other genes. Since all the selected genes are highly conserved in different organisms, it is assumed that the sequences will be highly identical among *Mytilus sp.*. The sequencing results of amplified PCR fragments using designed primers confirmed that same amplified sequences exist among different *Mytilus sp.*. In order to be accurate, in the present context therefore it is appropriate to use the term *Mytilus sp.* for comparison with other studies (AlAmri *et al.*, 2012; Banni *et al.*, 2009; Ciocan *et al.*, 2005; Ciocan and Sunila, 2005).

All 5 target genes showed tissue-specific transcriptional profiles but with different patterns under control conditions. Digestive gland as the main tissue to metabolise xenobiotics exhibited higher abundance of *cyp4y1*, which belongs to the main metabolic enzyme family (CYPs) in most organisms. Mantle was another tissue which showed most expression abundance of all selected genes. Such a degree of expression is expected given that mantle is the main reproductive organ which requires rapid processing of external compounds and response to different stresses to guarantee the successful reproduction (Mikhailov *et al.*, 1995). Therefore, the selected genes, either involved in the metabolism or the signal transduction, were expressed at considerably higher levels in mantle under normal conditions. Haemocytes showed the least expression abundance of most of the genes, except for *rad51*, suggesting that the blood cells possess the necessary apparatus for the metabolism and stress response, but they
are not the main place for the metabolic function to take place as in other tissues or organs (Canesi et al., 2008; Carballal et al., 1997).

5.4.2 Biotransformation related pathways

B(α)P at a concentration of 56 µg/L was observed (Chapter 4) to induce a significant degree of DNA damage in haemocytes after 3 days exposure but not significantly so after 1 day exposure. The damage might be generated by the transformation or metabolic activation of B(α)P to carcinogenic B(α)P derivatives by CYP450 family enzymes. Extensive studies pertaining to the mutagenic and carcinogenic potential of B(α)P and its metabolites have identified the so-called bay- and fjord-regions as ultimate reactive species in a wide range of species (Dipple et al., 1984; Harvey, 1991). The first step in the metabolism to form these structures is catalysed by the cytochrome P450 isoenzyme CYP1A1 in mammals (e.g.: mice and rats) (Qin and Meng, 2009) and CYP3A in zebra fish (Tseng et al., 2005). Although it is unclear which CYP(s) are involved in this process in Mytilus sp., it seems that CYP4Y1 is not the main member responsible for the metabolic transformation of B(α)P in haemocytes (Figure 5.16). The expression of cyp4yl showed no correspondence with the level of DNA damage in the haemocytes, suggesting that CYP4Y1 is either involved in the late stage of B(α)P metabolism to generate non-carcinogenic products, or it is not the main enzyme involved in the transformation from B(α)P to carcinogenic derivatives. It is also possible that the DNA damage is induced through the generation of free radicals by B(α)P (Canova et al., 1998) but not in the CYPs catalysed reactions. It is also possible that the DNA damage induced following B(α)P exposure involves other CYP450 enzyme family members (e.g. CYP1A, CYP3A) in these cells (Boström et al., 2002; Haritash and Kaushik, 2009).
In common with haemocytes, B(α)P exposure alone induced only slightly increased cyp4y1 expression in digestive gland and gill tissues. This confirmed that CYP4Y1 is not the main enzyme for bio-transformation of B(α)P in Mytilus sp., especially taking into account of the functions of digestive gland (the main tissue to process contaminants) and gill (the first barrier to sort the contaminants). The bio-transformation of B(α)P in mantle appears to require CYP4Y1 as there was a significantly increased expression of cyp4y1 after exposure, and the same was observed in adductor muscle. It is assumed that there is difference of threshold of bio-accumulated B(α)P concentration which could trigger the synthesis of cyp4y1 for the metabolism. The lower levels of threshold might exist in cells of mantle and adductor muscle.

In contrast to B(α)P exposure, cyp4y1 showed time-dependent alteration of expression in haemocytes following C₆₀ (1 mg/L) exposure, suggesting that CYP4Y1 is involved in C₆₀ metabolism in haemocytes of Mytilus sp. (Figure 5.16). This is consistent with previous studies reporting the increased expression of CYPs members after C₆₀ exposure (Zhu et al., 2006). However, the detailed mechanisms of potential involvement of CYPs family members to metabolise C₆₀ and its metabolic products remain unclear. CYP4Y1 tends to participate more in C₆₀ bio-transformation compared to B(α)P in digestive gland and gill as investigated in haemocytes. Significantly increased cyp4y1 expression was observed from 1 day exposure which remained at a constant level until recovery, confirming that C₆₀ metabolism in those two tissues is a time-consuming procedure. However, there was no significant cyp4y1 induction in cells of mantle and adductor muscle observed. This further suggests that CYP4Y1 may be the CYP enzyme in B(α)P metabolism in mantle and adductor muscle but is more sensitive in C₆₀ metabolism in the same tissues.
As expected, the combined exposure of B(α)P and C₆₀ changed the signalling transduction via gene expression. There are at least 3 different types of particles expected in the water system after the introduction of combined chemicals: (1) suspended B(α)P particles, (2) suspended C₆₀ or aggregated C₆₀ particles, and (3) suspended or aggregated C₆₀ carrying B(α)P (Yang and Xing, 2007). However, the exact proportion of each particle in the water system is unclear. It is assumed that the response in mussels in terms of gene expression will result from a complex combination of these chemicals. There was significant induction of \textit{cyp4y1} following 1 day exposure to the combination of chemicals in haemocytes, followed by a significant decline after 3 days exposure. An assumption has been made that CYP4Y1 was activated in response to both C₆₀ alone and C₆₀ carrying B(α)P for their bio-transformation after short exposure period. The combination of C₆₀ and B(α)P will change the properties of both the chemicals compared to when they exist alone (Yang \textit{et al.}, 2006) and could, possibly, change the conformational structure of C₆₀ which could influence the recognition by metabolic enzymes. An enhanced recognition could lead to significantly higher expression of \textit{cyp4y1} compared to exposure of B(α)P or C₆₀ alone, as observed in the study. If the transcription and translation are going hand-to-hand or concurrently, it is possible that a feedback mechanism in which the C₆₀ and B(α)P together could influence the downstream regulation of CYP4Y1 enzyme. This hypothesis, however, needs to be probed at the protein level. Due to the technical difficulties or limitations, this assumption cannot be currently probed in the experiment. After a longer exposure period (e.g. 3 days), more B(α)P was released after transport and require other CYPs family member to participate in the metabolism. CYP4Y1, therefore, is not required which is consistent with the decreased expression of \textit{cyp4y1} in comparison to 1 day exposure in mussels. Same as the observation in haemocytes, following the combined exposure to B(α)P and C₆₀, \textit{cyp4y1} expression was significantly induced in tissues (i.e.
digestive gland, mantle, adductor muscle and gill) after exposure, suggesting the physicochemical structural change appears to favour metabolising capacity by induction of the cyp4y1 gene expression. It is also suggesting that the combined exposure appears to be the most toxic exposure scenario compared to the individual chemicals on their own.

Furthermore, the relative expression of cyp4y1 showed significant induction after recovery after all 3 types of exposure which indicates the metabolism of chemicals in mussels is a time consuming procedure and requires the participation of related enzymes for the entire exposure period. It appears that CYP4Y1 metabolism can transform absorbed B(α)P to genotoxic B(α)P derivatives which can in turn cause DNA adducts in the cells. Although in previous studies it was not possible to detect DNA adducts in mussel tissues exposed to fluoranthene, either alone or in combination with C_{60} (Al-Subiai et al., 2012). Diverse CYP sub-families were found in single organism and took different proportion in each organ (Simpson, 1997). This could explain the reason of altered cyp4y1 gene expressions corresponding to different chemicals in different tissues.

In association with the metabolism of chemicals (B(α)P and/or C_{60}) by CYP4Y1, the chemical induced stress activates the cell defence apparatus which is involving P53, Ras, Jnk and Rad51 as described in section 5.1. It is assumed that the products may not only cause direct DNA damage but is also able to induce other stresses, including oxidative stress (Magrez et al., 2006; Sayes et al., 2005). The correlation analyses confirmed that the activations of those intercellular effectors are close related to the cyp4y1 expression. In general, the extracellular stress signal induced by chemicals after CYP4Y1 catalyse reaction can trigger jnk expression in all sampled tissue of Mytilus sp.. It is likely because that JNK is involved in a wide range of signal transduction in cells (Weitzman, 2000). The other effectors are showing tissue-specific activations in response to cyp4y1 expression alteration and they might be involved in more specific cell defence pathway.
The decision of which intercellular responser is activated is also dependent on the sensitivity of cells to carcinogens and the distribution/combination of CYPs in tissues.

Figure 5.16 Diagrammatic representation illustrating potential biotransformation pathway mediated by CYP4Y1 in mussels. CYP4Y1 participated in the transformation of different chemicals in a tissue-specific pattern. The stress induced by the metabolised chemical derivatives can also initiate a series of intercellular effector response in a tissue-specific manner.
5.4.3 Apoptosis related pathways

The apoptosis related pathway is including P53, JNK and Bax in this study. Activated $p53$ expression, as described in detail in Chapter 4, resulted from B($\alpha$)P and/or $C_{60}$-induced DNA damage stress. Its product, P53, can regulate the transcription of a series of downstream factors related to either cell-cycle arrest or apoptosis. Bax, as a direct downstream molecule of P53 (Figure 5.17), can promote apoptosis by taking part in the opening of the mitochondrial permeability transition pore, release of cytochrome c, activation of caspases and degradation of survival proteins (Petros et al., 2004). Bax can also be directly regulated by JNK as reported by Papadakis et al. (2006) based on the observation that the JNK-deficient fibroblasts do not display increased caspase activity and DNA fragmentation, which correlates with a specific defect in activation of Bax.

The significantly increased $bax$ expression with increased $p53$ expression was found in haemocytes after exposure indicating that P53 can directly regulate Bax in the blood cells of Mytilus sp.. This regulation was also supported by the correlation analysis of $p53$ and $bax$ expressions. Their expressions showed significantly linear relationship in haemocytes. However, such regulation did not appear in digestive gland, adductor muscle and gill cells after exposure. An increased expression of $bax$ appeared to be more closely related to JNK rather than P53 in these tissues. It is assumed the Bax is directly regulated in a P53-independent way or indirectly regulated by a P53-dependent way in mussels as previous demonstrated using mammalian models (Leppa and Bohmann, 1999; Toshiyuki and Reed, 1995). The up-regulated expression of $bax$ was observed which could be induced by both JNK and P53 in mantle cells of mussels. However, it is difficult to identify which factor is the main upstream regulator for this observation. It is noteworthy that apoptosis related signal pathways is a complex network and can be regulated by diverse molecules. It is reported that P53-dependent
apoptosis in mammalian cells is induced by the changes in a series of factors, e.g., \textit{bcl-2}, \textit{puma} and \textit{noxa} (Culmsee and Mattson, 2005; Gross \textit{et al.}, 1999; Polyak \textit{et al.}, 1997). The inhibition of Bcl-2 by P53 can also contribute to the activation of Bax in the cells (Gross \textit{et al.}, 1999; Marx D Fau - Meden and Meden, 1997).

Figure 5.17 Diagrammatic representation illustrating apoptosis pathways regulated by P53, JNK and Bax in a cell of mussel.
5.4.4 DNA repair related pathways

Apart from P53’s function in DNA repair which has been discussed in detail in Chapter 4, Rad51 also participates dramatically in DNA repair by stimulating HR pathway. The high levels of induced rad51 expression were observed following 1 day exposure to B(α)P in haemocytes and tissues (apart from digestive gland), even though no significant DNA damage was found in haemocytes at this stage. It is possible that DNA damages are efficiently repaired in haemocytes which makes the damage is undetectable.

In addition, comet assay as the measurement of DNA strand breaks mainly measures the SSBs (Kumaravel et al., 2007) which makes the direct link between rad51 expressions with DSBs to be difficult. Rad51 as the main factor involved in HR repair would stabilise DNA (Richardson, 2005), which can explain the reason that rad51 expressed more after short term exposure (i.e. 1 day) to B(α)P to repair DSBs rapidly. As the DNA damage was promptly repaired by Rad51 mediated pathways, the amount of DNA damage was possibly not enough to induce p53 expression at this early stage. The DNA damage, however, accumulated after 3 days exposure and reached the threshold to induce p53 expression. Induction of p53 can activate different DNA repair pathways other than the Rad51 mediated HR pathway only to ensure the DNA damage is correctly repaired. However, there was no significant induction of rad51 after B(α)P exposure in digestive gland, suggesting that the accumulated DNA damage caused by B(α)P did not reach the threshold to active rad51 in HR pathways in this tissue. The less accumulated DNA damage could be the results of efficient DNA repair and/or efficient elimination of cells with un-repaired DNA.

In contrast to B(α)P exposure, there was only minor rad51 expression induced after 3 days exposure to C_{60} in haemocytes and tissues (apart from gill) which could be explained as C_{60} induced stresses leading to less DSBs in these cells. It is also possible
that \( C_{60} \) induced stresses in these cells are included not only the direct DNA damage but also other stresses, i.e. oxidative stress (Spohn et al., 2009), and HR is not the main defence mechanisms under such stresses. In contrast to \( p53 \), HR operated by Rad51 showed participation in \( C_{60} \) induced DNA DSBs.

Unlike other genes, \( rad51 \) showed higher levels of expression in haemocytes and all the tissues after the combined exposure of chemicals. This could be explained by more induction of DSBs by the combined exposure; therefore, more induction of \( rad51 \) is required for its mediated HR pathway. The increased DSBs could be generated by higher efficiency of B(α)P bioavailability transported by \( C_{60} \). Higher relative concentration of B(α)P in cells could therefore lead to induction of more DNA damage. The significantly induced \( rad51 \) expression after the combined exposure is also suggesting that the combination of chemicals is more toxic to mussels compared to single chemical.

Apart from the direct participate of P53 in HR repair pathway; it can also regulate Rad51 activation (Figure 5.18). The highly expressed \( p53 \) could inhibit the expression of \( rad51 \) to reduce the stabilisation of mis-repaired DNA (Arias-Lopez et al., 2006). This could explain higher level expression of \( rad51 \) in haemocytes and tissues is associated with low level expression of \( p53 \). An exception was found in mantle which showed significantly positive correlation between the expression of \( p53 \) and \( rad51 \). It is assumed that a relative high level of DNA damage were induced in mantle cells which would require both \( p53 \) and \( rad51 \) to participate in the DNA repair.
Figure 5.18 Diagrammatic representation illustrating DNA repair related pathway which is regulated by P53 and Rad51 in mussels. Green line is indicating B(α)P related signal transduction, yellow line is C$_{60}$ related and red is B(α)P in combination with C$_{60}$ related signal transduction. Solid line is indicating significant induction of gene expression and dashed line is non-significant induction.
5.4.5 P53-regulation related pathways

Both JNK and MDM2 are supposed to be the upstream regulator of p53 expression (Fuchs et al., 1998a; Momand et al., 2000). JNK is the positive regulator to induce the expression of p53 by transducing the extracellular signal into intercellular responses (Leppa and Bohmann, 1999; Wu, 2004), and MDM2 is the negative regulator which will suppress the expression of p53 by increasing the degradation rate of P53 (Momand et al., 2000; Piette et al., 1997) (Figure 5.19). A feedback loop of P53 activating MDM2 is also suggested by previous studies (Wu et al., 1993). The up-regulation of JNK to P53 was observed in haemocytes and tissues, except for digestive gland, supported by the result that increased p53 expression is concurrent with increased jnk expression after exposure. However, this correlation between JNK and P53 is not significant in the statistical analysis. The suppression of MDM2 to P53 was evidenced in the results of p53 and mdm2 expression in haemocyte, digestive gland and gill, but not in mantle and adductor muscle. The feedback loop of P53 activating MDM2 was observed in haemocyte, mantle and adductor muscle but not the other two tissues. It is assumed that there is a higher threshold level to overcome in order to trigger the degradation of P53 by MDM2 to keep a stable level of P53 in mantle and adductor muscle. Also, it is possible that the DNA damage induced by chemicals in these two tissues is requiring high level of p53 expression to participate in DNA repair. It is noteworthy that the interplay between MDM2 and P53 is crucial to keep the stable level of P53 to function in a series of downstream pathways (Harvey and Parry, 1998; Momand et al., 2000).
5.4.6 Ras-regulation related pathways

No ras expression was induced in haemocytes after exposure, suggesting that ras as proto-oncogene, without overexpression or mutation, will only function in signal transduction which can control cellular growth (Anderson et al., 1992). JNK can suppress the expression of ras as absence of JNK has been found in Ras-mediated tumour development (Kennedy, 2003). The result in this study showing increased jnk expression associated with inhibited ras expression in haemocytes after exposure, which further supports the relationship between JNK and Ras-mediated cell dysfunction (Figure 5.19).

The lack of induction of jnk was found in digestive gland and gill which can cause the dysfunction in its inhibition of Ras, and lead to the significantly increased expression of ras. The increase of ras expression would result into increased proliferation of cells which could develop neoplasia if the accumulated amount crossed the threshold level (Bos, 1988, 1989). However, the expression of ras recovered to control level at the end of the experiment which suggested that digestive gland cells are capable of coping with chemical-induced stress.

The inhibition of JNK to Ras was not observed in the mantle and adductor muscle as there were significantly induced ras expressions after exposure even though high jnk was expressed at the same stage. This increased ras expression could potentially induce necrosis or cellular injury, or even early stage of neoplasia (Buday and Downward, 2008).
Figure 5.19 Diagrammatic representation illustrating P53 and Ras regulation related pathways. Black arrow is indicating the up-regulation between two genes and red line is the down-regulation.
5.4.7 Tissue-specific, chemical-specific and time-specific signalling pathways mediated by p53/ras gene (overlap and differences in different tissues)

Signal transduction is a highly precise procedure to ensure cells respond correctly to extracellular signals. Any changes occurring in the process can cause significant diversity for different endpoints. In this study, the relative expression of selected genes showed tissue-specific, chemical-specific and time-specific interplays among different target genes involved in the stress related processes (Figure 5.20). Both B(α)P and C₆₀ proved to induce stresses to cells, probably, after bio-transformed by CYPs to induce modulation of series of transcriptional alterations of genes.

DNA damage, the main stress induced by B(α)P, stimulated significant increase for p53 and jnk expression in haemocytes, mantle and adductor muscle, but not in digestive gland and gill tissues. Similarly, the relative expression of ras was not stimulated in these two tissues (i.e. digestive gland and gills) under the same conditions. Gill tissue as the first tissue to face and sort xenobiotic compounds after filter-feeding from aqueous environment showed high resistance to stress (Silverman et al., 1996). It appears that both gills and the digestive gland (the main organ involved in the metabolising the xenobiotics) inherently require a high threshold to activate or induce transcription of genes following exposure to xenobiotics in order to use the energy for other metabolic processes. Oberdörster (2004) found no increase of lipid peroxidation which is the indicator to oxidative stress in cells, in gill and liver tissue of juvenile largemouth bass but no other tissues. The explanation of better antioxidant defences in these organs was suggested. This could be also the case in this study.

C₆₀ can induce cell stress, including directly DNA damage (Xu et al., 2012; Zhao et al., 2005), oxidative stress and other stresses which can eventually induce DNA damage (Kamat et al., 1998; Shinohara et al., 2009). The stresses are more complex and toxic to
cells compared to B(α)P induced stress, as \(p53\), \(jnk\) and \(ras\) expression were stimulated in all sampled tissues and haemocytes after exposure. It is reported that \(C_{60}\) can cause oxidative stress because it is redox active (Kamat et al., 1998; Sayes et al., 2005; Yang et al., 2010).

The combined exposure of these two chemicals can induce both direct DNA damage and oxidative stresses in cells, therefore can introduce enhanced toxicity by stimulating \(jnk\), \(p53\) and \(ras\) expression. The combination of two chemicals contributes not only to induce complex stresses compared to individual chemicals but the combination of these chemicals possibly also facilitates to carry more B(α)P to cross the cell membrane which in turn cause more DNA damage and other stress (Yang et al., 2006).

Mantle is more vulnerable to stress to ensure quick responses to extracellular stress by stimulating a series of intercellular signal and, in turn, pass error-free genetic information to germ cells. Also, mantle appeared to be affected more by B(α)P induced stress rather than \(C_{60}\). Adductor muscle in mussels is the tissue to extract haemolymph and takes in charge of mussle’s physical activities (e.g. opening of shells) (Al-Subiai et al., 2009; Bayne, 2009). It is not the main tissue of defence, therefore, have lower threshold to tolerate extracellular stress. Haemolymph is circulating in different organs and transports nutrients, oxygen and xenobiotic compounds (metabolised or not) to other tissues (Betti et al., 2006; Canesi et al., 2002). The response in haemocytes represents response to stress for majority of cells.

Whilst the tissues-specific and chemical-specific phenomenon of \(p53/ras\) related signal transduction was found in the present study, the time-specific character was also detected. Cells in digestive gland and adductor muscle appeared to have quicker response compared to other tissues and haemocytes, as there were more induced pathway genes expressions after 1 day exposure. Apart from the requirement of quicker
processing of stress in these two tissues, the time-specific phenomenon is probably also
in relation to cell-cycle status of the cells from different tissues. Cells in G1/S of G2/M
will need less time to activate P53 related downstream response, either by activating
DNA repair or apoptosis (Attene-ramos et al., 2010). There is also DNA/RNA and
RNA/protein differences in different tissues therefore the synthesis of functional
proteins will consume different time (Gao et al., 2012).

Whilst all selected genes showed induced expression after stress signals, only
haemocytes and mantle showed the potential to produce necrosis or cellular injury
which is consistent with the samples collected from field where only leukaemia and
gonadal neoplasia were found at contaminated sites in the bivalves (Ciocan and Sunila,
2005; Galimany and Sunila, 2008). In haemocytes, ras was not induced but there is a
possibility of failure of P53 related DNA repair and apoptosis which can lead to tumour
development. In addition to the similar failure potential in mantle, ras could also be
induced at a relative high level after recovery in fresh seawater which can lead to
tumour or carcinogenesis. However, whether this high level can be classified as
‘overexpression’ is not confirmed as no neoplasia has been reported in any of mantle
tissues collected after laboratory exposure.
Figure 5.20 Diagrammatic representation illustrating potential pathways of signal transduction in different tissues involving different target genes. Colour framed squares highlight the differences of pathways in each tissue in comparison to classic pathway: red-haemocytes; blue-digestive gland; green-mantle; purple-adductor muscle and yellow-gill. For example, B(α)P transformation by CYP4Y1 were absence in haemocytes, digestive and gill tissue; the suppression of Rad51 by P53 was absence in mantle and adductor; the potential of failed DNA repair or apoptosis was present in haemocytes and mantle tissue, etc.
In conclusion, the classic pathways which relate $p53$ and $ras$ indeed exist in *Mytilus sp.* Although there is a tissue-specific signal transduction, 3 types of pathways can be classified in this present study based on the similarity to the classic pathway as mentioned in introduction section of this Chapter (Figure 5.1). Firstly, the signal transduction pathway in haemocytes appears to be highly similar to the classic one with existence of interplay among most genes (Table 5.8). Secondly, the pathway in digestive gland and gill shares majority identical signal transduction way with classic one. The difference between them is the absence of direct up-regulation of Bax by P53 in the pathway in digestive gland and gill. The third pathway is belonging to mantle and adductor muscle. Differences to classic pathway are the absence of suppression of (1) P53 by MDM2, (2) Ras by JNK and (3) Rad51 by P53 in this third group.
Table 5.8 Summary of main signal transduction mediated by target genes in different tissues and haemocytes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Tissues/cells</th>
<th>P53→Bax</th>
<th>JNK→Bax</th>
<th>JNK→P53</th>
<th>MDM2→P53</th>
<th>P53→MDM2</th>
<th>P53→Rad51</th>
<th>JNK→Ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Haemocytes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Digestive gland</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Gill</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>3</td>
<td>Mantle</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Adductor muscle</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</tr>
</tbody>
</table>

→: up-regulation between two molecules;

←: suppression between two molecules;

+: presence of suggested regulation;

-: absence of suggested regulation.
It is important to note that there are over 200 genes involved in DNA repair, cell-cycle check point, apoptosis, senescence and tumour formation in organisms (Wood et al., 2001). The pathway genes are selected based on currently available genome information in *Mytilus sp.* and representative of their own families. The selected genes indeed interplay with each other and can be influenced by chemical induced stress. However, other genes can also regulate their expressions; the transcriptional alteration found in this present study could be the results of the complex net-work involved. It is noteworthy that no genome wide expression arrays were performed to look at the global transcriptional alteration in *Mytilus sp.* in response to applied exposure scenarios and the mRNA expression changes are not necessary related to the activity of protein. The direct link between actual protein expression and gene expression cannot be established solely based on this study. To date, the lack of antibody specific to mussels makes it difficult to analyse alteration induced by such chemicals at protein level (e.g. western blotting). This should be a crucial and attracting area deserve further scientific pursue. In this present study, the focus of transcriptional alteration of selected genes involving in general intercellular signal transduction provides the fundamental information. In common with classic pathway found in mammalian systems, marine bivalves indeed share a large portion of similarity in signal transductions in response to chemical induced stress. *Mytilus.sp.* therefore can serve as an ideal whole organism model for human cancer related signal pathway research.
CHAPTER 6

Implication of transcriptional change in $p53$, $ras$ and $rad51$ genes to assess the response of marine mussels following exposure to low doses of tritium
6.1 INTRODUCTION

Natural radioactivity or artificial radionuclides generated by human activities (e.g., nuclear medicine, biological research, nuclear industry, military applications etc.) are released in the environment, and ultimately reach to aquatic ecosystems. Consequently, elevated concentrations of radionuclides have been found in many estuaries and coastal environments. For example, about $1.21 \times 10^9$ Bq of liquid $\beta$-particle and $\gamma$-ray emitters and $5.99 \times 10^{13}$ Bq of liquid tritium have been discharged into the sea from the Flamanville nuclear power plant (NPP) in France in 2003 (Commission, 2005). It is therefore not surprising that in recent years there has been growing scientific, regulatory and public concern to protect the natural biota from the impact of ionising radiations (IRs) (Dallas et al., 2012; Pentreath, 2009).

In common with other contaminants, aquatic organisms have the ability to accumulate a variety of radionuclides in the body either through food or water (McDonald et al., 1993). Accumulation of these radionuclides, often with long half-lives, chronically exposes the organisms internally to different levels or doses of ionising radiation. These exposures pose high health risks to organisms through acute and chronic damage. Acute damage has a direct identifiable impact on the health of the organisms, and may range from mild tissue irritation or immune suppression to an increase in the formation of carcinogenic cells or mortality (Cardis et al., 2006). Chronic damage is considered as perturbations in the delicate balance of the biological food web, detectable only over long periods, potentially impacting environmental sustainability (Parache et al., 2011; Yamada et al., 1999). One of the fundamental differences between the action of chemicals and radiations is that the action of IRs on biological systems depends on their energy levels. This energy could directly affect the biomolecules (e.g. DNA, proteins, lipids) in cells by imparting the energy or could indirectly affect them by generating free
radicals. In this context, DNA is considered to be the most important target for the action of IRs (UNSCEAR, 1969) and diverse damage ranging from cellular to individual levels biological organisation is initiated following DNA aberrations.

Despite growing concern, there has been only a limited number of studies pertaining to the assessment of impact of ionizing radiation on natural biota. In the last 50 years, there have been only approximately 50 papers to assess the impact of ionizing radiations on aquatic invertebrates, which constitute more than 90% of extant species (Dallas et al., 2012). The effects of radionuclides on DNA integrity, reproductive ability and teratogenic effects in different life stages of aquatic organisms (both vertebrate and invertebrate) following exposure to a wide range of radionuclides and external radiation sources have been reported (Etoh and Hyodo-Taguchi, 1983; Gudkov and Kipnis, 1996; Hagger et al., 2005; Hyodo-Taguchi and Etoh, 1993; Knowles, 1999). Despite the facts that (a) DNA is the most important target for the action of ionizing radiation and (b) induction of genetic damage is considered to be one of the important parameters to assess its impact, as recommended by the International Commission on Radiological Protection (ICRP) (ICRP, 2008), there is scarcity of information on the potential impacts of environmentally realistic radiation exposures on the genetic systems of natural biota. In particular, information at the molecular level (e.g. transcriptional level) remains rare. This could be due to a lack of genomic information for natural biota including marine organisms.

In the previous studies, adopting an integrated approach, tissue-specific transcriptional alteration of p53, ras and rad51 genes in Mytilus sp. had been successfully applied to elucidate the impact of different environmentally relevant contaminants (i.e. a PAH and/or an ENP). The optimised technique makes it possible to assess radionuclide-induced impacts at the molecular level in marine mussels.
Amongst possible radionuclides, tritium was selected as a representative or model radionuclide because it is released from different nuclear sectors in large quantities. Tritium ($^3$H) is a radioactive isotope of hydrogen, decaying by beta emission. It emits an electron with a range of energies up to 18.6 keV (mean energy of 5.7 keV), and has a physical half-life of 12.3 years (AGIR, 2007). Tritium can be formed by the action of cosmic rays in the atmosphere, in nuclear reactors and in accelerators. It is discharged in the environment (both aerial and aquatic discharges) from nuclear reactors (both fission and fusion), nuclear fuel reprocessing plants, and other processing plants, such as those concerned with the manufacture of nuclear weapons. In addition, it is used in medicine and research as well as in some luminous products. In the UK, the greatest discharge of tritium arises from the nuclear fuel reprocessing plant and associated facilities at Sellafield, which discharged about 1600 TBq in liquid and 90 TBq in gaseous forms of tritium in 2005 (EA et al., 2006). In the same year the combined discharges from UK nuclear power stations were about 2300 TBq (99% of which was in liquid form), while the tritium production plant at Chapelcross discharged about 300 TBq (almost entirely gaseous) and the GE healthcare laboratories at Cardiff discharged 330 TBq (90% gaseous) (EC, 1996). The Cardiff liquid discharges are of particular interest since they include various tritium-labelled organic compounds resulting from the production of such compounds for use in pharmaceutical and life sciences research and development. In addition, the Royal Navy submarine flotilla now consists exclusively of vessels powered by pressurised water reactor (PWRs) which gives rise to activation products, including tritium, within the primary coolant circuit. The routine maintenance, servicing and refitting of submarine also produce a range of radioactive waste, all of which may be contaminated by tritium (EA et al., 2006).
Tritium mainly exists in the environment as tritiated water (HTO) or in organic molecules (organically bound tritium, OBT) and has been suggested to behave chemically like hydrogen, forming water molecules, dihydrogen gas, or biomolecules (Adam-Guillermin et al., 2012). Some other reports suggest that having two neutrons as opposed to none in hydrogen, tritium in the environment would behave differently (Baumgartner and Kim, 2000). This makes tritium a paradoxical radionuclide. While there have been some reports with respect to bioconcentration and biomagnification of OBT at different trophic levels in the marine-coastal food chain (McCubbin et al., 2001), there are some indications that biota and sediments near the vicinity of inorganic $^3$H discharges, mainly as HTO, might also have enhanced accumulation of $^3$H (Staume and Carsten, 1993). As such a large amount of discharge of tritium can be taken up freely by the biosphere and could be ingested by both humans and natural biota through food chain (Parache et al., 2011; Tomasallo et al., 2010). Several experiments have focused on administration of tritium to animals at a relative higher dose in order to demonstrate the impact of IR exposure (Gratman et al., 1984; Johnson et al., 1995; Revina et al., 1984; Seyama et al., 1991). Carcinogenesis (e.g. breast cancer in rats, myeloid leukaemia and ovary cancer in mice) has been observed in these experiments. Laboratory investigations (Hagger et al., 2005; Jha et al., 2005) have also demonstrated significant DNA and chromosomal damage responses at different life stages of marine mussels, Mytilus sp., following exposure to low HTO doses, lower than internationally-recommended exposure limits of 400 µGy/h (International Atomic Energy Agency, 1992). These studies have used acute exposure scenarios (embryos exposed upto 72 h (Hagger et al., 2005) and adults upto 96 h (Jha et al., 2005)). The biological or biomarker impact of HTO following long-term or chronic exposure, which is more environmentally realistic in the biota is however lacking in the literature.
The major DNA damage response (DDR) caused by IR has been reported as DNA double strand breaks (DSB) (Hartlerode and Scully, 2009). The related repair pathways in eukaryotic cells are error-prone non-homologous end-joining (NHEJ) and a high fidelity process based on homologous recombination repair (HRR) between sister chromatids (Hiom, 2010). Both the p53 and rad51 genes are closely involved in DSB repair pathways and the changes in expression of these two genes are reported after IR (AlAmri et al., 2012; MacCallum et al., 1996). In addition, the accumulation of DSB induced by IR in organisms is a potent inducer of tumour development. The transcriptional response of ras can be the early-stage warning of carcinogenesis in organisms. Furthermore, as the major tool in cancer therapy, the success of IR relies on its ability to selectively kill tumour cells while minimising the detrimental effect to normal surrounding tissues (Snyder, 2004). IR induced responses in organisms must express in a tissue-specific manner and, subsequently, the transcriptional alterations of p53, rad51 and ras gene are assumed to exhibit diverse expression profiles in different tissues of organisms.

Against the backdrop of the above information, in this study, the aim was to probe the hypothesis that HTO, as an environmentally relevant radionuclide, can induce biological effects in marine mussels, Mytilus sp.. Given that in common with other radionuclides, tritium is capable of inducing DNA strand breaks, adopting an integrated approach, it was aimed to assess the transcriptional response of key genes, including tumour suppressor gene p53, proto-oncogene ras and homologous repair related gene rad51 in a tissue-specific manner. The study of expression of key genes, which are highly conserved, especially in various tissues, can demonstrate the early response of genotoxic impacts induced by tritium to shed light on the conserved mechanism of IR induced responses (especially at low dose) in a range of organisms, including human.
The objective of this study was therefore to apply as optimised real-time qPCR technique to analyse the changes in expression of \( p53 \), \( rad51 \) and \( ras \) in haemocytes and in different tissues, including digestive gland, gill and mantles of \( Mytilus sp. \) following chronic exposure to low doses of HTO.

### 6.2 MATERIAL AND METHODS

#### 6.2.1 Experimental design

Mussels were collected in June 2011 at Trebarwith strand, Cornwall, a relatively clean site, and maintained for 14 days under standard conditions as described in Chapter 2.2 for depuration. HTO was obtained from Perkin Elmer (Cambridge, UK) with a nominal activity concentration of 185 MBq/mL and a purity of 99.96%. Stock solutions of HTO were prepared by appropriate dilution in filtered seawater (filtered < 10 µm).

The experiments were designed in collaboration with a separate PhD project carried by Lorna Dallas (Plymouth University, UK) for an integrated study and to complement the biological responses whilst minimising the resources used (Figure 6.1). Briefly, the mussels were placed in 2 L beakers (9 mussels in each beaker) after depuration and left to acclimatise in filtered seawater for 48 h. After this period, HTO dilutions with concentration of 1, 5 and 15 MBq/L were added to individual beakers (3 beakers for each concentration) resulting in the dose-rates of \( \beta \)-radiation as calculated in section 6.2.3. The beakers (in triplicate) without HTO served as controls. Mussels were exposed to HTO at 15 °C for 14 days. During the exposure, mussels were fed every 3 days, with a 100% water change 2 h after feeding. After the exposure period, a total of 9 mussels was sampled from each treatment. Haemolymph samples were extracted from the posterior adductor muscle and digestive gland, gill, mantle, foot, adductor muscle and byssus were dissected from each sampled mussel. Haemolymph, gill, mantle and
digestive gland samples were preserved in liquid nitrogen immediately after collection and then stored at -80 °C for RNA extraction in this study. The remaining haemolymph and tissue samples were preserved for histopathological, DNA and chromosomal damage and radioactivity measurements in the complementary PhD project.

Figure 6.1 Overall experimental design to determine the biological impacts of HTO in mussels, *Mytilus sp*. Red framed procedures were specified for this study and the black framed procedures for another project to establish impact of chronic exposure of HTO.

### 6.2.2 Water quality parameters and radioactivity in the water samples

Water quality parameters, including temperature, pH, salinity and dissolved oxygen, were monitored on daily basis throughout the exposure period. The activity concentrations in the seawater used for experiments with HTO were measured by liquid scintillation counting (LS 6500, Beckman, USA) before and after each water change. Samples of 100 µL of the seawater were added to 5 mL of liquid scintillation cocktail, mixed thoroughly and incubated for 2 h (at least) in the dark prior to counting. The
activity in the samples was determined after scintillation counting for 2 h or to 5% precision, whichever was the lesser.

6.2.3 Dosimetric calculations

Doses received by the mussels, \( D_\beta \) (Gy/h), were estimated assuming that the tritium spiked into the seawater rapidly attains 1:1 equilibrium within the tissues as tissue free water tritium (tritium in water trapped within tissues) and that the tritium is dispersed homogeneously within the tissues (Strand et al., 1977). The calculation was for follows (equation E 6.1):

\[
D_\beta = \frac{2.13\varepsilon_\beta c}{3.7\times10^6}
\]

where \( \varepsilon_\beta \) is the mean beta energy of \(^3\)H (0.00569 MeV), \( C \) is the activity concentration of \(^3\)H in Bq/mL, and 2.13 and 3.7 \( \times \) 10\(^6\) are unit conversion factors. Doses in the experiments were 3.9, 17.2 and 51.4 \( \mu \)Gy/h for 1, 5 and 15 MBq/L HTO, respectively. Relatively low dose rates were selected in this study based on previously research (Dimitriadis and Koukouzika, 2003) which demonstrated significant DNA integrity alteration in haemocytes of mussels after exposure to HTO at a dose of as low as 12 \( \mu \)Gy/h for 96 h.

6.2.4 Gene expression analyses

Haemolymph and tissues samples collected (as mentioned in section 6.2.1) were processed for RNA extraction. cDNAs were reverse transcribed from 10 ng DNase digested RNA sample as described in Chapters 2.8 and 2.9. Real-time PCR for target genes (\( p53 \), \( ras \) and \( rad51 \)) and reference gene (\( 18S \)) were performed in triplicate for each sample as described in Chapter 2.11. The relative expression of each target gene was analysed by the \( 2^{-\Delta\Delta Ct} \) method as described in Chapter 2.12.
6.3 RESULTS

6.3.1 Characteristics of water samples

Water quality parameters were within the normal range throughout the entire experiment period (DO > 90% at all times, salinity=34.54 ± 0.14‰, pH=7.92 ± 0.14, temperature=15.13 °C ± 0.20). The actual radioactivity in diluted HTO was in the acceptable range to yield expected tritium concentrations. The dose ranged from 94.3 to 1234 µGy/day and the total dose received by mussels after 14 days exposure as calculated according to E 6.1 is presented in Table 6.1.

Table 6.1 Estimated doses to mussels, *Mytilus sp.*, following exposure to different concentrations of tritiated water.

<table>
<thead>
<tr>
<th>Water activity concentration (MBq/L)</th>
<th>Dose</th>
<th>Total dose (14 days) (mGy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal</td>
<td>Actual</td>
<td>µGy/h</td>
</tr>
<tr>
<td>0 (control)</td>
<td>0.00 ± 0.01</td>
<td>0.004</td>
</tr>
<tr>
<td>1</td>
<td>1.20 ± 0.45</td>
<td>3.90</td>
</tr>
<tr>
<td>5</td>
<td>5.25 ± 1.76</td>
<td>17.20</td>
</tr>
<tr>
<td>15</td>
<td>15.70 ± 3.60</td>
<td>51.43</td>
</tr>
</tbody>
</table>

6.3.2 Comparison of relative expression of individual genes in haemocytes and tissues

The relative expression of *p53*, *rad51* and *ras* genes were plotted to show transcriptional alteration of individual genes in haemocytes and different tissues following the exposure to a range of doses of tritium.

The relative expression of *p53* in haemocytes of mussels showed no induced expression at the lowest dose-rate of tritium exposure (Figure 6.2A). Expression was induced
significantly (1.17 ± 0.11 and 19.91 ± 11.80-fold, respectively) at the medium and highest dose (17.20 and 51.43 µGy/h, respectively) after 14 days exposure. In contrast to haemocytes, significantly induced $p53$ expression in digestive gland was observed at all 3 applied dose of tritium exposure in this study. Similar to haemocytes, the highest tritium dose induced highest level of increased $p53$ expression in digestive gland after 14 days exposure. The relative expression of $p53$ in mantle showed similar alterations as in the digestive gland after exposure. A down-regulation of $p53$ expression, however, was observed at the medium dose of tritium exposure. Although there was a slightly induced $p53$ expression (21.23 ± 20.0-fold) at the lowest dose, no induction of $p53$ expression was found at either of other two tritium exposures in gill tissue. The highest $p53$ induction was found in digestive gland after exposure to the highest dose of tritium.

There was no significant induction of $rad51$ expressions in haemocytes and digestive glands after exposure to any dose of tritium (Figure 6.2B). In mantle, significantly increased relative expression of $rad51$ showed after the lowest and highest tritium exposure (18.34 ± 14.61 and 53.27 ± 40.88-fold, respectively), but not after exposure to the medium dose. Only significant induction of expression of $rad51$ was observed in gill after the lowest dose (63.27 ± 61.85-fold). The highest induction of $rad51$ was observed in mantle after the exposure of tritium at the highest dose received (i.e. 51.43 µGy/h).

There was no significant induction of $ras$ expression in haemocytes and tissues after any treatment (Figure 6.2C), apart from gill at the lowest tritium dose after 14 days exposure (10.23 ± 8.21-fold). In mantle, increased $ras$ expression (48.44 ± 47.51-fold) was observed at the highest dose of tritium but the increase was not statistically significant due to the high inter-individual variation.
Figure 6.2 Relative expression of *p53* (A), *rad51* (B) and *ras* (C) in haemocytes and different tissues following tritium exposure (Total dose received by the organisms over the exposure period is summarised in Table 6.1). Level of 1 is the control level. Each bar represents the means of 6 replicates (n=6) and S.E.M are indicated by error-bars. Means with the same letter indicate no significant difference based on ANOVA followed by Tukey’s test at p<0.05. * indicates significant up-regulated genes expression compared to control. # indicates significant down-regulated genes expression compared to control.
In comparison of relative expression of \textit{p53}, \textit{rad51} and \textit{ras} in haemocytes and digestive gland after exposure to applied tritium doses, significant induction of \textit{p53} was observed following exposure to the highest dose of tritium. No induction of both \textit{ras} and \textit{rad51} were found after any exposures in this tissue/cell type. In comparison of the relative expression of all 3 genes in gill, it showed an opposite trend compared to digestive gland and haemocyte. The mean levels of gene expression increased following exposure to lowest dose of tritium. These levels dropped to control level after exposure to the medium and highest doses. The transcriptional profile of 3 genes showed complex in mantle. The expression of both \textit{p53} and \textit{rad51} was significantly induced following the exposure to the lowest (3.9 \(\mu\)Gy/h) and highest (51.43 \(\mu\)Gy/h) dose of tritium. There was no induction of \textit{p53} or \textit{rad51} after the exposure at medium dose. High mean expression level of \textit{ras} was observed after the highest dose exposure in mantle. The high inter-individual variation made this result not statistically significantly different to the control level.
6.4 DISCUSSION

It is suggested that the low energy beta particles produced by tritium decays are more biologically effective than hard X-rays and gamma rays per unit absorbed dose based on previous experimental data (AGIR, 2007). β-particles from HTO have been reported to be more effective than $^{90}$Sr and $^{90}$Y γ-rays and external $^{60}$Co γ- and X-rays in inducing chromosomal aberrations in the embryo larval stages of fish, leading to an increased level of developmental abnormalities and mortality (Suyama et al., 1981). High radiosensitivities for HTO compared with X- or γ- radiation have also been reported in sea urchin embryo larvae (International Atomic Energy Agency, 1976). This potent biological effect of β-particles is probably due to the fact that tritium can induce 10-30 times more ionisations per unit of tissue volume compared to X- or γ-rays (AGIR, 2007).

As the dose in exposed organisms is usually very low, the direct measurement is not realistic. The standard calculation was applied in this study to make the results comparable to existing information. However, dose could be underestimated or inadequate by this method as the dose was calculated based on the assumption of homogenous distribution of tritium in the organisms (Strand et al., 1977). It has however been shown that HTO accumulates differentially in the mussel tissues (Jaeschke et al., 2011; Jha et al., 2005). This suggests that tritium is differentially incorporated into proteins, DNA and other biomolecules in the tissues, as these origin and metabolic functions are different. Therefore, the reliability of the concept of average dose to organisms must be questioned and addressed.

A new Environmental Risk of Ionising Contaminants Assessment tool (ERICA) has been, developed and applied to assess the radiological risk to biota. Based on a mathematical approach, the ERICA tool can provide more precise dose estimation by taking into account specific radionuclides and inter-individual differences for the
organism (Brown et al., 2008). ERICA has also been used in the separate PhD project simultaneously. In comparison to the equation approach used in this study to tritium dose calculation determined by ERICA tool suggested approximately 2-times higher dose in this study (Lorna Dallas, Plymouth University, personal communication). This indicates that the ERICA tool is more sensitive at determining radionuclide doses in the organisms and should be applied for accurate assessment of dose rate of radionuclides in future studies. More relative comparison between the two approaches however should be applied using different radionuclides and organisms for a robust comparison and their adoption.

The lowest dose of tritium (0.094 mGy/day) applied in this study is less than the previous level used in different studies (Gudkov and Kipnis, 1996; Jaeschke et al., 2011; Knowles, 1999) and is also much less than the recommended dose to protect aquatic ecosystems from external γ irradiation (0.24 mGy/day) (Garnier-Laplace et al., 2006). The other two doses used in the study were also below the level of 400 µGy/h, which is recommended to protect invertebrates from the potential detrimental impact of IR by IAEA (International Atomic Energy Agency, 1992). Although applied doses were below the recommended dose and should not cause risk to aquatic ecosystems in theory, the fact is that there is currently no internationally agreed standard recommended dose or threshold for IR which can protect ecosystems. It is therefore difficult to define the threshold levels of IR for the protection of the natural biota. In the separate project, significantly induced DNA integrity responses (% tail DNA in comet assay and induction of micronuclei per 1000 cells in micronucleus assay) in haemocytes of Mytilus, sp. were detected at the lowest dose of 0.094 mGy/day. A dose-dependent response was observed, where the DNA damages increased with increased tritium dose (Lorna Dallas, Plymouth University, UK, personal communication). It indicates that
tritium is a potent exogenous inducer of DNA alterations at very low doses. DNA alterations at the egg stage of blue mussels, determined by using randomly amplified polymorphic DNA (RAPD) profiles and the comet assay, were observed at a low dose rate of 0.3 mGy/day (Hagger et al., 2005). Similar results were obtained for adult mussels in which significant genotoxicity was detected at even the lowest dose tested (0.3 mGy/day) by micronucleus and comet assays (Jha et al., 2005). All these observations indicate that low-doses of tritium can induce DNA alterations. DNA damage, if not correctly repaired, may induce mutations, teratogenesis, and reproduction failure. In this way, damage at the molecular level can precipitate at higher levels of biological organisation and can have consequences at the population level (Adam-Guillermin et al., 2012).

Exposure of cells to IR results in DNA damage, including base damage, single-strand breaks (SSBs) and double-strand breaks (DSBs) (Wang et al., 1998). Damaged bases, the predominate from of which is 8-oxodeoxyguanosine, are repaired via the base excision repair (BER) pathway. In this pathway, the initial step is the recognition and removal of the damaged base, followed by the formation of an apurinic/apyrimidinic site, repair synthesis, and the sealing of the newly synthesized DNA by ligase activity (Sekiguchi and Tsuzuki, 2002). SSBs, formed either directly by IR exposure or as an intermediate during the repair of damaged bases (Beckman and Ames, 1997), are also repaired by a multi-step pathway (Caldecott, 2007). DSBs arise either directly from IR exposure or as a result of the overlap of the repair intermediates formed as a result of base excision during SSB repair (Budworth et al., 2002). The overlap of repair intermediates occurs frequently due to the tendency for radiation-induced damage to form in clusters on the DNA (Sutherland et al., 2000). In IR exposed cells, pathways of cellular defense mechanisms in addition to DNA damage include the cell cycle and cell-
death pathways. It is becoming increasingly clear that the defense pathways not only involve multiple steps and proteins, but operate under an exquisitely sensitive control system. Many of the defense responses are regulated by the tumor suppressor protein, P53, which serves as a transcriptional regulator in addition to its interactions with other proteins important in these processes (Gudkov and Komarova, 2003).

In addition, cells can also take advantage of a homologous sequence to repair DSBs by HR processes. IRs, and more specifically DSBs, stimulate HR in mammalian cells in a RAD51-dependent manner (Saintigny et al., 2008). In most cells, HR is essential to maintaining genome stability. However, HR should be precisely regulated, because an excess in HR can also result in chromosomal instability and disease (Purandare and Patel, 1997). Overexpression of RAD51, which stimulates HR (Vispe et al., 1998), has been reported in tumour cells (Maacke et al., 2000). Excess HR also has been reported in different precancerous situations, such as defects in p53 (Bertrand et al., 2004) ataxia telangiectasia (Meyn, 1993) and expression of the oncogenic kinase BCR-ABL (Philadelphia chromosome) involved in acute myeloid leukaemia (Slupianek et al., 2001). These results emphasize the potential dangers of excess HR. Therefore, the RAD51 dependent HR must be precisely regulated in cells.

In general, when the cells accumulate DNA damage, cells can undergo DNA repair processes to cope with the stress by initiating DNA repair regulating molecules, such as P53 and RAD51. Alternatively, they can pass the genetic information with error during replication when there is a loss of the function of these regulators. The accumulation of incorrect genetic information will then cause the development of tumour which can be indicated by overexpressed ras at the early stage (Bos, 1988). Therefore, when cells are exposed to a DNA-damaging agent, such as IR, the RNA expression profile of p53,
rad51 and ras provides not only a snapshot of the cellular response to the mutagen but also insight into the mechanisms of the damage response.

In the present study, waterborne exposure is clearly the most relevant to aquatic invertebrates and can make them particularly vulnerable to internal exposure because their body surface, respiratory apparatus (especially gills), and internal organs (in organisms with open vascular system) are in close contact with the surrounding water (Dallas et al., 2012). In haemocytes and tissues, apart from gill, up-regulated p53 and rad51 expressions were observed at the lowest and highest dose exposure, confirming that both these genes function in DNA repair. Also, no ras induction was found suggesting that mussels are able to cope with the applied tritium exposure by inhibiting the accumulation of DNA damage in cells. It also suggests that the induced rad51 expression did not exceed the threshold to cause excess HR. IR has been previously shown to induce P53 protein expression in the gut and other tissues of rat (MacCallum et al., 1996). It has also been reported that rad51 was up-regulated only in human TK6 cells after exposure to 5 Gy tritium (Akerman et al., 2005). AlAmri et al. (2012) reported increased rad51 expression in haemocytes of mussels collected from radionuclides contaminated site compared to control. One noteworthy pattern of altered gene expression in the present study was no dose-dependent pattern when comparing individual gene expression in different tissues (Figure 6.2). Jin et al. (2008) suggested mRNA transcripts are highly expressed in a human mesenchymal stem cell line in response to both low-dose (0.01-0.2 Gy) and high-dose (1 Gy) of IR exposure. Diang et al. (2005) suggested that both qualitative and quantitative differences exist between gene expression profiles in human fibroblasts after the induction of low-dose (2 cGy) and high-dose (4 Gy). Low-dose radiation may possess characteristics that distinguish it from its high-dose counterpart. The predominant functional groups responding to low-
dose radiation are those involved in cell-cell signaling, signal transduction, development and DNA damage responses, such as $p53$ and $rad51$. Thus it is plausible that the predominant response at high doses, where DNA damage is excessive and/or unrepairable, would involve inhibition of cell proliferation or programmed cell death (e.g. $p53$). On the other hand, at low doses where the extent and nature of DNA damage may make them more manageable, the primary response of the cells might be to activate DNA damage responses and signal transduction pathways that respond quickly to induction of the damage to repair them (Diang et al., 2005). Considering the multifunctions of P53, the induction of $p53$ at lowest dose of tritium might be related to initiation of cell cycle arrest and DNA repair and at highest dose to the function of apoptosis. The relative expression of $p53$ is suppressed at medium dose after 14 days exposure might be because the DNA damage level at this stage is high enough for dysfunction of P53 in DNA repair but not high enough to induce apoptosis, therefore, less p53 is required. In addition, the lack of multiple sampling times also limited the comprehensive explanation for actual transcriptional alterations of $p53$ at different doses of tritium exposure. It is possible $p53$ relative expression is induced after shorter or longer exposure time at the medium exposure dose but showed no induction after 14 days exposure. However, these assumptions need to be proved by involving more medium level doses, multiple sampling time and analysis of apoptosis. Although, no clear dose-response was observed in the comparison of single gene expression in different tissues, another angle is to compare different gene expression in same tissue/cell gave dose-dependent alteration for $p53$ gene expression, particularly in haemocytes, which was consistent with the highest degree of DNA damage and chromosomal aberration results observed in the same sample. This indicates that P53 indeed participates in DNA repair and its expression level is initiated by a certain degree of DNA damage (e.g. the level induced by highest dose tritium exposure). Dose-
dependent \( p53 \) expression was also clearly observed in digestive gland suggesting similar level of DNA damage or similar mechanisms in DNA repair processed as in haemocytes. There was no significant induction of \( p53 \) expression in gill after any dose exposure but significantly induced \( rad51 \) expression was observed after the lowest dose exposure. It is assumed that Rad51 participates, particularly, in DSB repair, this lowest dose may cause DSBs in gill cells but the level is not over the threshold to initiate P53 function. After exposure at other two doses (medium and highest doses), complex form of DNA damage produced but the level might not have been high enough to initiate \( p53 \) expression, or the damaged DNA is un-repairable, therefore, \( rad51 \) expression level dropped. It is difficult to explain the reason of induced \( p53 \) and \( rad51 \) expression at lowest and highest dose of tritium exposure in mantle. Considering the sensitivity of mantle to produce gonadal neoplasia, it is assumed that the cell defense mechanism was initiated by induction of DNA repair related gene expression (e.g., \( p53 \) and \( rad51 \)) at the lowest dose of tritium exposure. Along with the increased dose (medium dose at 17.2 \( \mu \)Gy/h), these defenses were suppressed probably due to high level of accumulated DNA damage which will suppress DNA repair function but was not high enough to induce apoptosis. At the highest dose, the relative expression of \( p53 \) and \( rad51 \) increased again. This increase could be essential in the regulation of effectors involved in cell death. However, this assumption will need experimental data to support it. For example, actual DNA damage level of mantle cells needs to be analysed to determine if dose-dependent response of DNA strand breaks occurred after exposure of tritium.

Although there are many studies with respect to gene expression in mammalian cells, there are only limited studies to evaluate expression of genes in aquatic organisms following exposure to IRs (Farcy et al., 2007). Limited studies has been carried (most of them use heat-shock gene \( hsp70 \) and metallothionein (MT) genes) (Farcy et al., 2011;
Mezentsev and Amundson, 2011) have shown no effect at the transcription level of genes. This study therefore indicates that selection of genes is very important while determining the effects of IR at transcriptional level. It is crucial to analyse global gene expression in response to IRs in invertebrate in order to understand its detrimental impact to the health of marine animal and environment.

Furthermore, apart from the ras gene, other two genes showed tissues specific differential expression patterns. One explanation is cells in different tissues are facing different level of stress induced by variable bioconcentrated tritium. Jaeschke et al. (2011) has reported tissue-specific bio-accumulation and depuration of tritium in mussels. Also, MacCallum et al. (1996) has found p53 expression in different tissues of murine following IR and extended these observations to generate the concept to divide three classes of cell population that differ with regard to their ability to induce P53 protein and apoptosis, indicating the existing of tissue-specific P53 protein expression. Although the P53 protein level is thought to be determined mainly by protein stabilisation and not by new transcription, presence of increased gene expression does not necessarily mean high levels of P53 protein. The tissue-specific transcriptional change could partially explain the tissue-specific protein expression.

The results obtained with HTO suggest that marine mussels as model invertebrates are sensitive to low doses of exposure to IR, although this conclusion is in contrast to the old paradigm that invertebrates are more radio-resistant (at the population level), because their cells have a protective response (i.e. can undergo programmed cell death before permanent damage occurs) (Dallas et al., 2012). A few studies about IR induced detrimental effects were only carried out in limited invertebrate species. The conclusion of invertebrates are radio-resistant cannot represent the entire response of invertebrates to environmental IR. The transcriptional alteration of selected genes indicates *Mytilus sp.*
is radio-sensitive at least at the molecular level. The possible explanation of sensitivity of invertebrates to IR is based on their developmental characteristics. Indeed, invertebrates generally undergo determinate development or mosaic development, in which cell lineage is determined primarily by the genome and cytoplasm of each individual cell (Brusca and Brusca, 2003). As a consequence, if a cell dies during development, then none of these tissues that would have formed from the progeny of that cell can develop. In contrast, vertebrates undergo indeterminate or regulated development, and cell lineage is strongly determined by the interaction of the genome with epigenetic (nongenetic) factors extrinsic to the individual cell, such as molecules released by neighbouring cells. Epigenetic factors interact with the cell’s genome to determine its fate, through a process known as induction. Because any cell’s fate is the consequence of induction, removal of a few cells will not adversely affect development since other cells will be simply induced to take over for the missing ones (Adam-Guillermin et al., 2012).

In conclusion, low doses of tritium exposure are capable of inducing DNA integrity alteration and this change can, in turn, trigger the expression of DNA repair pathway related genes \( p53 \) and \( rad51 \). The alteration at the transcriptional level showed tissue-specific patterns and appeared to be in the normal range for DNA repair. Based on the expression of these genes (i.e. \( p53 \) and \( rad51 \)), it could be assumed that cells did not undergo apoptosis or that HR is maintained within the normal range. Because the expression of \( ras \) showed no induction in haemocytes and tissues, it could be assumed that no carcinogenic event even at a very early stage under applied experimental condition is triggered. In addition, as a collaborating work, the gene expression results observed in this study can be further co-related or complemented with another project to provide an integrated and comprehensive response in \textit{Mytilus sp.} following exposure of
low doses of tritium. As mentioned earlier, the repair of IR induced DNA damage involves a series of signaling pathways, related signaling molecules should be involved in future study to understand the mechanisms of low dose tritium exposure leading cells to genomic instability, dysregulated proliferation or tumour development.
CHAPTER 7

General discussions and future perspectives
7.1 Reflection on overall experimental strategy

One of the main objectives of the present study was to establish the adoption of an integrated approach using biomarkers at different levels of biological organisation to assess the impact of environmental contaminants in a representative marine invertebrate species. In particular it was aimed to apply robust molecular biomarkers using transcriptional expression of key genes potentially activated during the processing of DNA damage, which could be a tissue specific phenomenon. Pollutants in the marine environment can induce diverse stresses in the marine organisms. Excessive stresses, if not repaired or processed effectively may induce biological responses (biomarkers) including genetic aberrations, either directly (e.g. DNA adducts) or indirectly (i.e. through generation of reactive oxygen species). Such responses may include induction of mutations and alteration in the expression pattern of key genes (Barzilai and Yamamoto, 2004; Valko et al., 2006; Waters and Fostel, 2004). Key biomarkers which measure DNA damage and indicate potential detrimental consequences, such as disease and tumour development, were the first consideration in this project. Carcinogens are able to interact with DNA to excessively induce either single or double DNA strand breaks, although such breaks are also formed during repair of DNA strand breaks using homologous recombination (HR) or non-homologous end joining (NHEJ) processes (Mladenov and Iliakis, 2011). Failure to repair, including mis-repair and inefficient repair, such excessive breaks in the DNA can lead to production of chromosomal aberrations (Natarajan et al., 1993). DNA repair is a complicated process, involving over 200 genes (Wood et al., 2001). Several DNA repair pathways, processing different types of damage, can recognize the damage and initiate the machinery to process either error-free or error-prone repair. Mis-repaired DNA damage can potentially lead to mutations which can cause loss of the normal function of cells and/or uncontrolled cellular proliferation. These induced mutations could also subsequently induce
secondary mutations (e.g. mutation of one of the mutated alleles) leading to back-mutations to wild-type, compensatory mutations in cis, intragenic crossovers, gene conversion and genomic instability, the hallmark of cancer (Hirschhorn, 2003; Sakai et al., 2008). Given its importance mentioned above, detection of DNA strand breaks at cellular level was the first biomarker selected in the study.

There are three main types of genes involved in the induction of tumours or malignancy. They are anti-oncogenes (tumour suppressor genes), oncogenes and mutator genes (i.e. genes involved in DNA replication, repair and nucleotide metabolism). Following on from induction of DNA strand breaks, in this study, the highly conserved tumour suppressor gene (anti-oncogene) p53 and proto-oncogene ras were selected as the key genes to investigate carcinogen-induced responses at the molecular level, as the alteration of these genes (either through mutation or change in expression) have been observed following exposure to genotoxic carcinogenic agents (Brzuzan et al., 2006; Lee et al., 2008a). These genes have been sequenced in the selected model organism, Mytilus sp.. Functions or roles of these highly conserved genes in DNA repair, apoptosis and cell proliferation have been well-studied in a variety of organisms and show functional similarity among species (Buday and Downward, 2008; Gomez-Lazaro et al., 2004). Although both the selected biomarkers can show responses at the cellular level, the aberration in a single cell will not cause immediate detrimental consequences at organism level. This is probably due to the fact that malignancy arises as a result of accumulation of damages in cells over a long period of time. Accumulation of aberrations or damage, which depends on the exposure scenarios (e.g. acute vs. chronic, repair efficiency of cells) along with interaction and communication among the cells, could, however, lead to failure of normal function or even death. It is, however, to be remembered that toxicants could manifest their toxicity in a variety of ways and could impact simultaneously different processes or systems in the body (Jha, 2008).
In order to provide a comprehensive concept about how organisms react to environmental contaminant induced stress, biomarkers at the tissue and individual levels were also considered in this project. Therefore, feeding behaviour indicating physiological health at individual level; histopathological abnormalities at tissue level; DNA strand breaks at cellular level and transcriptional alterations of key genes (p53 and ras) at molecular level were selected to constitute the integrated biomarker analyses.

Initially, B(α)P was selected as the representative environmental contaminant (i.e. PAHs) to evaluate sub-lethal biomarkers or biological responses in mussels. This environmentally relevant agent has been proved to be carcinogenic, can directly interact with DNA after biotransformation from B(α)P to BPDE by CYPs and leads to formation of DNA adducts (Harvey, 1985). The well-established effects of B(α)P in a variety of organisms therefore should help to establish the integrated approach as adopted in this study, to determine biological responses after exposure to environmentally relevant or higher concentrations at different levels of biological organisation for other environmental contaminants. Before B(α)P was actually used in the experiments, there were a few techniques which needed to be optimised in order to eliminate potential ‘false positive results’ as a result of technical or procedural errors. The comet assay as a reliable tool to analyse DNA strand breaks has been applied in a variety of studies (Attene-ramos et al., 2010; Buschini et al., 2003; Mitchelmore and Chipman, 1998a; Nogueira et al., 2006) using different parameters involving different procedural steps (Jha, 2008). The parameters for the comet assay, therefore, needed to be optimised to be specific to the haemocytes extracted via the posterior adductor muscle of Mytilus sp.. H$_2$O$_2$ exposure under in vitro conditions, therefore, was included for the optimisation because of its strong ability to induce DNA strand breaks. This agent has been used for the haemocytes of marine bivalves to determine DNA strand breaks using the comet assay (Cheung et al., 2006). A series of comet assay parameters,
including lysis time (30-60 min), lysis temperature (room temperature or 4 °C), unwinding time (15-60 min), electrophoresis time (15-45 min), constant electrophoresis voltage (15-25 V) were tested in haemocytes following exposure to a range of concentrations (10-1000 µM) of H$_2$O$_2$. These optimisation steps confirmed the optimum comet assay conditions which included a lysis time of 30 min at 4 °C followed with 30 min unwinding and another 30 min electrophoresis at 25 V. Furthermore, extraction of haemolymph samples was also optimised and a relatively high amount of haemolymph (> 300 µL) could be easily extracted from mussels within a short period of time without disturbing surrounding tissues. Following optimisation and validation of the assay, an apparent concentration-dependent increase in DNA strand breaks was observed, indicating a good performance of selected parameters.

The first application of the optimised comet assay condition was to evaluate the potential toxicity of acetone which is used as a solvent. B(α)P as a lipophilic compound has relatively low solubility in water. Solvents (i.e. delivery vehicle) are necessary to provide nominated amount of reference chemicals or contaminants [e.g. Benzo(α)pyrene] in the exposure systems containing fresh or seawater (Hutchinson et al., 2006). Acetone, a commonly used delivery vehicle, was selected and the applied concentration range was used to determine potential genotoxicity in haemocytes of mussels. The concentration range from 0.005-0.10 % (v/v; i.e. 50-1000 ppt) of acetone was tested, and clearance rate and DNA strand breaks were selected as biomarkers to determine whether applied acetone concentration range was capable of inducing any significant increase for DNA strand breaks in comparison to control (i.e. seawater alone). A concentration of 0.01% (v/v) was finally selected which was consistent with the recommendation from OECD (OECD, 2000).
Following these initial investigations, *in vivo* experiments involving exposure to B(α)P (range from 5.6 to 100 µg/L) were designed and mussels were sampled after 6 and 12 days of exposure for integrated biomarkers analyses. The results, as discussed in detail in Chapter 3, showed diverse sensitivities of biomarker at different biological levels. DNA strand breaks was the most sensitive biomarker as it showed obviously concentration-dependent response to B(α)P exposure, in contrast, there were either no significantly physiological responses (i.e. clearance rate), or no statistically analysed tissue abnormalities (i.e. histopathological alterations) after exposure. The transcriptional alteration of *p53* and *ras* genes showed a tissue-specific pattern, mantle and adductor muscle showing enhanced sensitivity to B(α)P exposure. Overall, the integrated biomarker approach, especially the molecular biomarkers involving transcriptional activation of *p53* and *ras* genes, was successfully applied in the experimental design. Adoption of this approach provided a comprehensive biological response in mussels following exposure to chemical stress.

Following on from biological responses studies with exposure to a single contaminant, combinations of contaminations were considered in the next set of experiments given that in natural environment contaminants occur as complex mixtures and they can interact in different ways to induce biological responses. Their effects on organisms could change dramatically compared to exposure to a single chemical due to the alteration in physico-chemical properties of the chemicals when present in combinations. A representative nanoparticle C\(_{60}\) was selected together with B(α)P to test the impact in *Mytilus sp.* with the expectation of the combination of C\(_{60}\) and B(α)P can induce altered impacts at different levels of biological organisation, a combined exposure of C\(_{60}\) and B(α)P was designed and the established integrated approach was applied. This experimental approach also maximised the use of biological samples in one set of experiment design. It is also noteworthy that the experimental design has been
complemented compared to B(α)P exposure by (1) involving 3 days recovery in fresh seawater after exposure; (2) feeding and re-dosing daily to confront the potential stress caused by starving; and (3) collecting haemolymph for both DNA strand break and gene expression analyses. There was a slightly altered physiological response in mussels after all three types of exposure and consistent histopathological abnormalities after exposure to B(α)P alone and in combination with C_{60}. As expected, DNA strand breaks were found after exposure and the damage level was significantly decreased after recovery confirming the initiation and processing of DNA repair. The significant increase for p53 expression after all the exposures further confirmed the role of P53 in DNA repair and showed no specificity to a particular chemical-induced stress. However, the induction level of p53 and ras showed tissue- and chemical-specific patterns as discussed in detail in Chapter 4. Relatively high levels of p53 and ras (over 100-fold) induction were found after C_{60} exposure either alone or in combination with B(α)P, suggesting that there is a relatively significant response in Mytilus sp. at the transcriptional level which provided advantage (i.e. significant alterations in genes expression are expected) to analyse other genes which are participating in p53/ras mediated signalling pathways to pursue the mechanism by which extracellular stress is transduced into intracellular response.

The curiosity about tissue-specific signal transduction in response to chemical-induced stress drove the study to focus on p53/ras mediated pathways in the next step. The important functions of P53 and Ras are dependent on the interaction with other function effectors. Genes belonging to five different groups were screened based on available genetic sequences and reported evidence about their roles in interacting with P53 and/or Ras (Chang and Karin, 2001; Gross et al., 1999; Piette et al., 1997; Simpson, 1997). The initial step in mussels after filtering chemicals from water is the metabolism of xenobiotics by CYPs (representative as cyp4y1). Following biotransformation, B(α)P and/or C_{60} induced DNA damage can activate p53 which is involved in different DNA
repair pathways (including HR, NER and BER) or rad51 which is mainly involved in the HR pathway. The stress signal (e.g., DNA damage) can also influence the transcriptional alteration of ras and jnk which could eventually lead to either normal cellular function for the control of cellular-proliferation (Ras) or transduce signal to a series of downstream regulators (JNK). Upstream of P53 activity can be regulated by MDM2/JNK which controls the level of P53 and downstream to regulate Bax which functions in the determination of apoptosis. There is interplay between selected genes, such as the inhabitation of Rad51 by activated P53 and Ras by activated JNK. All the functions mentioned above and interactions were only proposed based on research in mammalian models in different studies. Whether these mechanisms exist or perform in a similar way in Mytilus sp. was unknown. The optimised PCR conditions and real-time qPCR technique provided the opportunity to elucidate and explore the puzzle. To my knowledge, this study is the first to involve seven different gene families which play important roles in intercellular signal transduction, DNA repair, apoptosis and cell proliferation, in the analysis of intercellular interplay in different tissues of Mytilus sp.. As expected, the interplay among selected genes was similar to the classic model as discussed in detail in Chapter 5, with tissue-specific and chemical-specific alterations. The signal pathways can be classified into three groups based on the similarity of signal transduction: (1) haemocytes, (2) digestive gland and gill, and (3) mantle and adductor muscle. The combination of C_{60} and B(α)P can induce enhanced effects in mantle and adductor muscle but inhibited effects in haemocyte and digestive gland; no significant difference of transcriptional alteration was found in the gill. Although DNA damage induced signal transduction is a very profound net-work involving a range of effectors, the results suggested a common mechanisms indeed exists in Mytilus sp. and showed tissue-specific patterns in line with their particular metabolic functions. In addition,
Mytilus sp. was found to be a sensitive and ideal organism to explore this complex mechanism in relation to carcinogenesis research at the whole organism level.

The successful application of p53, ras and rad51 gene expression analyses confirmed that they are sensitive molecular biomarkers and can be indicator of the detrimental impact induced by a range of chemicals. In order to examine their ability in response to different chemicals, tritium, a β-emitter, was selected due to its environmental relevance and ability to induce damage in DNA, and compared to B(α)P and C₆₀ (Dallas et al., 2012). Relatively low exposure doses were selected because there are conflicting opinions about whether low dose IRs can damage cells (Pentreath, 2009; Staume and Carsten, 1993). Another reason to select low dose tritium was to test the sensitivity of p53, ras and rad51 expression alterations in response to IRs. Similar results were obtained after tritium exposure in comparison to B(α)P and/or C₆₀ exposure, both p53 and rad51 showed induction after the lowest and highest dose exposures, however, the induction, in most cases, was not statistically significant. No enhanced ras expression was found apart from mantle, suggesting the selected dose of tritium still has the potential to induce severe impact in organisms (especially in mantle). The results further confirmed that transcriptional alteration as important and that sensitive biomarkers can assess the response in organisms and provide early warning for disease development.

The overall experimental strategy has been shown in Figure 7.1 and the main objectives were successfully achieved at each step. It is noteworthy that this project is the first to analyse transcriptional alteration induced by diverse groups of chemicals in different tissues and to investigate the interplay of key genes involved in cellular functions in marine bivalve, Mytilus sp.. Understanding the causes of DNA damage and correlating them with related exposure mechanisms is a vitally important and virtually unexplored aspect in marine or aquatic species. This study suggested that exposure and transmission
pathways are similar between the bivalve *Mytilus sp.* and mammals. The incidence of tumours in marine mussels would be contraindicative for cancer research. Analyses of the effects of genotoxic and non-genotoxic compounds on malignancy are currently limited to vertebrate cancer models where tumours are induced (e.g., the mouse model). However, the use of vertebrates is subject to strict regulations under ethical guidelines. The mussel cancer model is more similar to an outbreeding, human clinical population than are those generated from inbred mouse strains intentionally exposed to known tumour viruses (Kovalchuk *et al.*, 2000). Marine mussels, *Mytilus sp.*, therefore, provide an ideal whole organism model for human and environmental health research.
Figure 7.1 Diagrammatic illustration of overall experimental strategy.

- **H₂O₂ optimisation**: Optimisation of suitable comet assay conditions for haemocytes from mussels.
- **Acetone validation**: Determination of suitable concentration of acetone as vehicle.
- **B(α)P exposure**: Establishment of integrated biomarkers at different biological levels to assess responses in *Mytilus sp.* following *in vivo* B(α)P exposure.
- **B(α)P and/or C₆₀ exposure**: Determination of suitable conditions (e.g., primers, PCR conditions, initial RNA concentration) to analyse p53 and ras gene expression.
- **HTO exposure**: Investigation of tissue-specific p53 and ras gene expression.
- **B(α)P and/or C₆₀ exposure**: Application of integrated approach to assess B(α)P and/or C₆₀ induced responses in *Mytilus sp.* *in vivo*.
- **B(α)P and/or C₆₀ exposure**: Complementation of experimental design by involving recovery period after exposure and haemocytes in gene expression analysis.
- **B(α)P and/or C₆₀ exposure**: Investigation of tissue-specific and chemical-specific transcriptional alteration of key genes involved in p53/ras mediated signal transduction.
- **B(α)P and/or C₆₀ exposure**: Prediction of stress-induced signal transduction pathways in tissues of *Mytilus sp.*
- **B(α)P and/or C₆₀ exposure**: Investigation of tissue-specific p53, ras and rad51 expression alteration following HTO exposure at low dose-rates.
7.2 Overall comparisons of biomarkers or biological responses following exposure to different contaminants

While the sensitivity and reproducibility of different sub-lethal biomarkers or biological responses was successfully demonstrated following exposure to a range of contaminants, the study also provides the opportunity to compare these biomarkers to establish the baseline level in mussels.

7.2.1 Analysis of ‘clearance rate’

Clearance rate analyses were applied to detect the physiological health of mussels. Although there was no significant difference in ‘clearance rate’ or feeding behaviour under the designed experimental conditions, the slight difference following exposure to different contaminants were still apparent. There was a conflicting result in two experimental settings ((1) B(α)P exposure for 6 and 12 days and (2) B(α)P and/or C_{60} exposure for 1 and 3 days followed by 3 days recovery in fresh seawater). Significantly decreased feeding activities were found after 12 days exposure to all selected B(α)P concentrations. On the contrary, increased feeding activities were observed after 3 days exposure to B(α)P and/or C_{60} in the second experiment. Although chemicals induced different physiological responses in two experiments, a consistent result, which was that mussels have adapted their feeding activities depending on ambient environment (maintenance tanks, food availability, temperature), was found in all control samples. In B(α)P exposure, no addition of food was, probably, responsible for the decreased feeding rates after 12 days compared to 6 days. It has been reported that threshold of algal concentration in ambient water exists for mussels to change their feeding activity (e.g. reduced feeding activity) (MacDonald and Ward, 2009; Maire et al., 2007). The decreased clearance rate could be the result of lower threshold of algae concentration.
for mussel to achieve steady feeding rate. This lower threshold might be caused by no 
addition of algae during the experiment. Because mussels were fed daily in B(α)P 
and/or C₆₀ exposure, changed ambient environment was probably involved in the 
increased feeding activities after 6 days acclimatisation in 12 L tank (after recovery) 
compared to 1 and 3 days (after exposure). In order to compare whether control mussels 
used in different experiments were in the similar physiological conditions, the clearance 
rate of all applied control mussels has been summarised in Table 7.1. There was no 
significant difference in the feeding behaviour among different control mussels used in 
different experiments. The clearance rates in control mussels ranged from 1.12 – 2.55 
L/h. The majority of observed clearance rates were in this range and was in line with 
earlier published work (Riisgård et al., 2011), suggesting that they were in 
physiologically healthy condition, even after exposure. The exception was found in 
mussels recovered from exposure in the second experiment (i.e. exposure to B(α)P 
and/or C₆₀), which showed increased feeding activity. This could be the additive effect 
of acclimatisation in the new ambient environment and release from exposure stress. 
The overall comparison of clearance rate in control mussels indicated the collection 
season did not change the general physiological condition in mussels. While seasonal 
variation has been shown to influence other parameters (Shaw et al., 2000), the 
influence of season on feeding rate of mussels have not been reported. Up to date, 
feeding behaviour in mussels is still a complex procedure and there is still no general 
agreement regarding the physiological control of water pumping by suspension-feeding 
bivalves in response to changes in ambient water (Riisgård et al., 2011). The argument 
was mainly focused on whether the feeding behaviour is physiologically regulated 
(Bayne et al., 1993) or whether as a basic autonomous process (Maire et al., 2007). This 
study observed the feeding ability was not significantly affected by chemicals at applied 
concentrations.
Table 7.1 Summary of clearance rate in *Mytilus sp.* collected for each experiment.

<table>
<thead>
<tr>
<th>Group</th>
<th>H$_2$O$_2$ optimisation</th>
<th>Acetone validation</th>
<th>B(α)P exposure</th>
<th>B(α)P and/or C$_{60}$ exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before exposure</td>
<td>After exposure</td>
<td>6 days exposure</td>
</tr>
<tr>
<td>Clearance rate in control (L/h)</td>
<td>1.22 ± 0.10</td>
<td>2.17 ± 0.17</td>
<td>1.81 ± 0.12</td>
<td>1.61 ± 0.27</td>
</tr>
<tr>
<td>Clearance rate range in experiment (L/h)</td>
<td>1.17 ~ 1.42</td>
<td>1.75 ~ 2.44</td>
<td>1.81 ~ 2.23</td>
<td>1.61 ~ 1.75</td>
</tr>
<tr>
<td>Mussels collection time</td>
<td>January 2009</td>
<td>January 2009</td>
<td>March 2009</td>
<td></td>
</tr>
<tr>
<td>Experimental container</td>
<td>50 L</td>
<td>50 L</td>
<td>2 L</td>
<td>12 L</td>
</tr>
</tbody>
</table>
7.2.2 Histopathological analyses

The histopathological analyses applied in this project can only provide evidence that chemical exposures are capable to induce tissue damage. Tissue abnormalities were found in some of sampled tissues after exposure but no such abnormalities were found in controls. However, large amount of samples needed in order to provide statistically significant results using the applied techniques. Total of 6 samples were analysed at each treatment due to the limitations of the experimental condition and no statistical analysis were carried for histopathological analysis. There are some new techniques established which allows to analyse histopathological alteration statistically in digestive gland with limited mussels samples. Dimitriadis and Koukouzika (2003) reported to measure the height of digestive tubules to assess the effect of sampling procedures, transportations stress and laboratory maintenance on the structure and function of the digestive gland epithelium in mussels. The height can be measured in each section (Figure 7.2) and comparison of average digestive tubules height from control and treated samples can be the statistical indictor for tissue histopathological alterations in response to treatment. However, no similar statistical measurement was reported in other tissues, such as gill, adductor muscle, and mantles. Large amount of sampling is still required in order to gain statistically significant or meaningful results.

Figure 7.2 Example of digestive tubules measurements. DGH1 and DGH2 indicate the variability of digestive tubules height. Black scale bar = 20 µm.
7.2.3 DNA strand break analysis

Comet assay was successfully performed, after proper optimisation and validation, as sensitive biomarker to detect DNA damage. Significantly increased DNA damages were found after all selected chemical exposures and showed clear concentration-dependent response. DNA strand break is a naturally occurring event and therefore a background level exists for different cell types. The DNA strand break level in all controlled samples used in different experiments has been summarised in Table 7.2, and surprisingly, the range showed significant difference for each experimental group. High background DNA strand break level were found in validation experiments using acetone and B(α)P exposure experiment. After comparing experimental conditions, long time acclimatisation in laboratory after collection showed significant impact on DNA strand breaks. The acclimation time in the present study (i.e. 14 days) induced background DNA strand break around 10% (Tail DNA%), longer acclimation time (i.e. 2 months) induced more background DNA damage (up to 58%; Table 7.2). Several studies have reported quantitative changes in digestive gland structure, gill muco-polysaccharides and haemocyte glycoproteins after long term laboratory maintenance of mussels (Bettencourt et al., 2008; Dimitriadis and Koukouzika, 2003), suggesting laboratory acclimation can be a potential factor to alter biological responses in mussels and should be considered as confounding factor in the experimental design while evaluating contaminants-induced biological responses in the natural biota. Furthermore, mussels collected during winter (January) showed lowest background DNA strand break level compared to other seasons; this is consistent with previously reported result which showed lower level of nuclear DNA damage in samples collected in December compared to August (Shaw et al., 2000). The transfer within two hours after collection showed no significant impact on the change of the background level. The high background DNA damage levels detected in the study are assumed to be independent of
chemical induced stress. Therefore, same conclusion could be made by comparing DNA strand break level after exposure to control mussels for the determination of chemical-induced DNA damage.

One of the applications of the comet assay, which is being recommended for mammalian in vivo regulatory studies (i.e. OECD), is its use for the determination of oxidative DNA damage using bacterial specific enzymes (e.g. FPG and Endo-III), which specifically recognises oxidised purine and pyrimidine bases. This modified comet assay could have been used to determine oxidative DNA damage as carried out in different aquatic species (Aniagu et al., 2006; Emmanouil et al., 2007b; Mustafa et al., 2011; Mustafa et al., 2012). Measurement of oxidised DNA bases (e.g. 8-OHdG) could also be determined by GC-MS and HPLC but it has been recognized by the European Standards Committee on Oxidative DNA Damage (ESCODD) group that DNA is prone to oxidation during sample preparation for these methodologies giving spuriously high estimates of damage (Azqueta et al., 2009) and this would have been a preferred technique to apply here to distinguish between direct damage to the DNA or through the generation of free radicals. The limited amount of haemolymph samples obtained and the number of samples used for other assays (i.e. gene expression and normal comet assay) however posed restriction to apply this modified assay in the present study.
Table 7.2 Summary of DNA strand break level (Tail DNA %) in control *Mytilus sp.* sampled in each experiment.

<table>
<thead>
<tr>
<th>Group</th>
<th>(\text{H}_2\text{O}_2) optimisation</th>
<th>Acetone validation</th>
<th>(\text{B(\text{a})P}) exposure</th>
<th>(\text{B(\text{a})P}) and/or (\text{C}_{60}) exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 days exposure</td>
<td>12 days exposure</td>
</tr>
<tr>
<td>Tail DNA %</td>
<td>4.46 ± 0.99</td>
<td>58.73 ± 0.26</td>
<td>32.39 ± 1.37</td>
<td>27.39 ± 5.99</td>
</tr>
<tr>
<td>Mussels collection time</td>
<td>January 2009</td>
<td>January 2009</td>
<td>March 2009</td>
<td></td>
</tr>
<tr>
<td>Experimental container</td>
<td>50 L</td>
<td>2 L</td>
<td>12 L</td>
<td></td>
</tr>
<tr>
<td>Acclimatisation period in stock</td>
<td>14 days</td>
<td>2 months</td>
<td>25 days</td>
<td></td>
</tr>
<tr>
<td>Measurement time after transfer</td>
<td>No transfer</td>
<td>6 days</td>
<td>6 days</td>
<td>12 days</td>
</tr>
<tr>
<td>from stock</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
7.2.4 Real-time qPCR for gene expression analysis

A consistent results for the transcriptional alteration of $p53$ and $ras$ was detected using real-time qPCR in both the experiments with respect to B(α)P exposure alone, suggesting it to be a reliable tool for gene expression analysis. It is noteworthy that the optimisation before actual qPCR analysis is essential in order to gain stable results. As mentioned in detail in Chapter 3, 10 ng initial RNA amount was selected based on the results which showed cDNA reverse transcribed from this amount will yield Ct value between 10 to 20 (reliable range for qPCR detection) under control condition. Both $actin$ and $18S$ are widely used housekeeping genes in many organisms due to their stable expression. These two housekeeping genes were also selected in this study and showed no change in the final normalised results. However, the geNorm analysis showed that $18S$ was more stably expressed than $actin$ in mussel. This indicates that more accurate normalised results can be obtained by using $18S$ as the reference gene (Vandesompele et al., 2002). All the gene expression results were based on the expression level relative to $18S$ and control condition. Real-time qPCR was also applied to identify $p53/ras$ mediated signal pathway in response to chemical induced stress. The main interplay among these genes showed similarities to reported mammalian pathway as discussed in detail in Chapter 5, which confirmed the technique is sensitive and accurate for such analysis. However, the pathways proposed in Chapter 5 were only based on the alteration at transcriptional level, which cannot cover the complex system involving interaction of molecule at different levels in organism. Analysis at translational level is required to identify the actual functional change of each molecule in response to chemical induced stresses because most interactions among these effectors are at protein level. Although the selected genes were closely related to signal pathways which can relate to carcinogenesis if they dysfunction, the transcriptional
change cannot directly indicate tumour formation in mussels under experimental conditions because there was no neoplasia found in any of the samples.

7.3 Future perspectives

7.3.1 Improvements of experimental design

While the selected biomarkers provided comprehensive analyses of response in *Mytilus sp.* at different biological levels, some improvements can be made in future studies to make them more robust. Firstly, mussels used in the final experiment should not be maintained in laboratory for a long time after collection to reduce negative influence. Previous research suggests 14-18 days acclimation is enough for mussels to get used to new ambient environment (Dimitriadis and Koukouzika, 2003) and the result of background DNA strand break level also showed 14 days acclimation induced the background level around 10%. Additional acclimation period should also be included after experimental mussels transferring from stock to experimental tank to eliminate the influence caused by changing of ambient water. Secondly, sufficient food should be provided during acclimation and experiment to make sure the feeding activity change will purely reflect the response to experimentally-induced stress (e.g. DNA strand break, oxidative stress) rather than change in algae concentration induced impacts. Thirdly, new technique to analyse histopathological results could be involved to provide statistical results for the analysis of tissue abnormality, at least in digestive gland, as mentioned in 7.2. Finally, new techniques such as energy-filtered transmission electron microscopy (EFTEM) should be involved in the analysis of interaction between NPs and tissues or cells (Porter *et al.*, 2006). These techniques make it possible to track the uptake and localisation of *C*<sub>60</sub> in cells after exposure.
7.3.2 Signal genes expression analyses

Genes belonging to 5 different groups were selected in p53/ras mediated signal pathway analysis and showed to be an easy and reliable way to understand complex signal transduction net-work in cells. More key genes should be involved in this type of analysis to provide relative comprehensive information. Some candidates have already been screened and the qPCR conditions and sequences were confirmed in the additional experiments (Appendix III) and can be used in the future:

1. **p53/p63/p73 family**

The family consists of p53, and the more recently discovered homologues p63 and p73. Unlike p53, both p63 and p73 give rise to differentially spliced mRNAs, which are translated into several different proteins. While all family members contain the highly conserved DNA binding domain (DBD), P63 and P73 are characterized by an additional C-terminal region, the sterile alpha motif (SAM), which is implicated in protein-protein interactions in developmental processes (Levrero et al., 2000). P63 and 73 have been implicated in stem cell identity, neurogenesis, epithelial development, natural immunity and homeostatic control. In some tumour cell lines, P73 is induced in response to DNA damage, mediating a P53-independent cell death pathway (Siah et al., 2008). Therefore, it is important to analyse other P53 family members rather than P53 alone to understand the potential of tumour development in response to environmental exposure.

2. **CYPs family**

As CYP4Y1 did not show clear evidence to involve in B(α)P metabolism in mussels, there should be other CYPs family members to participate in this process. The CYP1A family has received considerable attention due to its significance in the metabolism of PAHs in vertebrates (Chaty et al., 2004; Haritash and Kaushik, 2009; Snyder, 1998). In this context, CYP3A sub-family represents the most significant group due to two facts:
firstly, CYP3A enzymes are the most abundant CYPs in human liver, comprising between 30 to 50% of total CYP content, and hence represent the bulk of the CYP enzymes that a chemical is likely to be exposed to (Wilkinson, 1996). Secondly, a large active site results in substrate promiscuity, meaning that up 60% of therapeutics in use today that are subject to metabolism are substrates for CYP3A sub-family members (Plant, 2007).

3. CHK gene

Cell cycle checkpoints are control mechanisms that ensure the fidelity of cell division in eukaryotic cells. Cell cycle checkpoint kinase 1 (Chk1) acts downstream of ATM/ATR kinase to play an important role in DNA damage checkpoint control, embryonic development and tumour suppression (Liu et al., 2000; Sorensen et al., 2005). Activation of Chk1 involves phosphorylation of Ser 317 and Ser 345 and occurs in response to blocked DNA replication and certain forms of genotoxic stress (Zhao and Piwnica-Worms, 2001). Chk1 exerts its checkpoint mechanism on the cell cycle by regulating the Cdc25 family of phosphatases (Chen et al., 2003). Activated Chk1 can inactivate Cdc25C via phosphorylation at Ser 216, blocking the activation of Cdc2 and transition into mitosis (Zeng et al., 1998). Chk1 has been recently isolated and identified in Mytilus sp. (GenBank Accession No: GU812861) and can be used to analyse in vivo DNA repair mechanisms.

7.3.3 Cell culture based gene expression analysis

In this project, particular attention was paid on tumour related genes and their mediated signal pathways in different tissues of mussel in response to environmental genotoxicants/carcinogens induced stress. The expectation was to understand the mechanism of potential tumour development in Mytilus sp. to help the investigation of similar mechanism in more complex organisms. Although tumour is a naturally
occurring event in many marine bivalves, including *Mytilus sp.*, the effort to induce the disease into individual mussels which were maintained under laboratory conditions has not been successful, suggesting it to be time-consuming and complex processes (Barber, 2004). As there was no indication of neoplasia formation in tissues after selected exposures, it is difficult to directly link investigated transcriptional alteration of *p53/ras* and other interacted genes with tumour development in relation to selected exposures. Only preliminary or early warning can be provided from these observations, in line with studies carried out in human populations (Smith et al., 1998; Smith et al., 1996). Successful primary cell/tissue cultures have been reported using *Mytilus sp.* (Birmelin et al., 1999; Cornet, 2006; Faucet et al., 2004), making it possible to induce acute exposure by selected chemicals to primary cultured cells isolated from different tissues. The morphological changes (i.e. large nucleus, irregular in size/shape and scarce of cytoplasm) similar to human tumour cells in these cells after *in vitro* exposure can be directly linked to transcriptional alterations of key genes (Galimany and Sunila, 2008). In addition, Walker et al. (2009) reported method for mass culture and long-term storage of cancerous clam haemocytes (CCH) both *in vitro* and in liquid nitrogen. Following subculture, CCH can successfully transform from primary cultures to non-immortalised cancer cell lines that continue to proliferate and do not revert to the normal clam haemocyte phenotype (as defined for human cells in suspension culture by Schaeffer (1984) and Masters (2000)). The observation of normal clams to develop cancer after inoculation with different CCH/haemolymph combinations provides a transplantable tumour model for amplifying CCH *in vivo* (Ostrand-Rosenberg, 2004). By applying this technique, mussels with leukaemia or gonadal neoplasia which collected from contaminated sites can be used as the seed to produce cancerous mussel haemocyte (CMH) which can inoculate to normal healthy mussel to promote the development of tumour in inoculated individuals. In this case, the *in vivo* transcriptional
alteration analyses of selected genes can provide the direct link to the mechanism of
tumour formation in mussels. The establishment of the mass culture of CMH can also (1)
provide a more thorough understanding of the etiology of carcinogenesis in terms of
exposure pathways or molecular mechanisms; (2) establish marine mussel cell lines for
viral isolation and characterisation and for \textit{in vitro} studies of pathways of cell cycle
regulation, apoptosis, etc.; (3) identify the tissue source for cancerous mussel
haemocytes; and (4) clone the mussel genome and/or developing high-throughput
microarrays for the detection of mussel genes that are differentially expressed during the
transition between normal and cancerous cells and after environmental insults (Denslow

It is to be pointed out that in the present study expression of only selected genes were
analysed over a fixed time point and no genome wide expression arrays were performed
to determine a large number of genes instead of just a few, despite the fact that these
genes are involved in multiple pathways. Such microarray based studies has been
carried out in some aquatic species (Banni \textit{et al.}, 2011; Denslow \textit{et al.}, 2007; Dondero
\textit{et al.}, 2006; Hegedus \textit{et al.}, 2009) and would have perhaps indicated more precisely
whether observed effects in the present study reflect DNA damage, rather than general
stress or toxicity, or damage to proteins and lipids. It is also important to remember that
regulation of transcription in response to environmental stress is influenced by
epigenetic mechanisms, i.e. heritable changes in gene expression through somatic or
germ line transmission, other than changes in DNA sequences (Bonasio \textit{et al.}, 2010;
Egger \textit{et al.}, 2008). These modifications influencing gene expression include interlinked
mechanisms of DNA methylation, post-transcriptional modifications of histones,
binding of acidic proteins to DNA (Chan \textit{et al.}, 2008; Turner, 2009). In particular,
tissue- and sex-specific methylation have been reported in different fish species, with
particular reference to the genes involved in endocrine function hence target for those anthropogenic chemicals which can disrupt the endocrine system and could also simultaneously damage the DNA and influence the carcinogenesis process (Mirbahai et al., 2011a; Mirbahai et al., 2011b; Navarro-Martin et al., 2011; Strömqvist et al., 2010).

Whilst such phenomena have been reported in fish species, there is lack of information for aquatic invertebrates. It is however important to keep in mind the potential epigenetic factors which might influence the gene expression profile following exposure to environmental toxicants. Furthermore, ‘bystander effects’, a phenomenon which has been well established in the field of radiation genetics and referred to un-irradiated cells exhibit irradiated effects as a result of signals received from nearby irradiated cells (Mancuso et al., 2012), is being also demonstrated in aquatic organisms such as fish following exposure to anthropogenic chemicals (Liu et al., 2012). Whilst mechanism of this intriguing phenomenon is not very well understood, it is being proposed that epigenetic mechanisms (i.e. DNA methylation, histone modification, RNA silencing etc.) could play an important role in these processes (Mothersill and Seymour, 2012). In the context of present study, it is possible that biological responses observed in the individual mussels could be an indirect effect induced by the individuals directly impacted by the exposure of chemicals.

In conclusion, an integrated approach of applying biomarkers at different biological levels has been established and applied in the assessment of B(α)P exposure either alone or in combination with C₆₀ induced response in marine bivalve Mytilus sp.. Although clearance rate and histopathological analyses did not show statistically significant difference after exposures, it still indicated the impact in response to applied exposure. The observations indicate DNA strand break and transcriptional alteration analysis to be more sensitive and reliable tools to assess stress induced response in mussels.
Application of changes in expression of $p53$ and $ras$ genes in different tissues has been successfully employed for the analysis of molecular impact induced by variety of environmental pollutants. In combination with other biological responses, they can be employed in environmental monitoring programme and could help the environmental managers and regulators to implement effective management for environmental sustainability given that biological responses or biomarkers are being considered as robust tool to protect the human and environmental health (Moore et al., 2004). Potential signal transduction pathways in different tissues/cells of mussel were also proposed in this PhD project which could serve as a spring board to stimulate further research on environmental health and could also act as a surrogate or sentinel for human health.
Appendix I List of regents and solution preparation

1. Low melting point (LMP) agarose (0.75%)
   - Dissolve 75 µg LMP in 10 mL PBS;
   - Heat in microwave for 1 - 2 minutes at full power until fully dissolved;
   - Aliquot 2 mL samples into microcentrifuge tubes and store at 4 °C.

2. Normal melting point (NMP) agarose (1.5%)
   - Dissolve 0.75 mg NMP in 50 mL PBS (i.e. 0.75 g in 50 mL);
   - Heat in microwave for 1 - 2 minutes at full power until fully dissolved.

3. Lysing solution
   - To 1L distilled water (dH₂O) add: 2.5M NaCl;
     - 100mM EDTA;
     - 10mM Tris base;
     - 1% N-Lauroyl-sarcosine;
   - Stir mixture and adjust to pH 10 using NaOH;
   - Store at room temperature, but refrigerate for at least 2 h prior to use;
   - Immediately prior to use add: 1% Triton X 100;
     - 10% DMSO.

4. Electrophoresis buffer for comet assay
   - Must be freshly prepared for each set of gel runs;
   - To 2 L of dH₂O add: 0.3M NaOH;
     - 1 mM EDTA;
   - Check pH ≥13 and then refrigerate for at least 2 h (preferably overnight).

5. Neutralization buffer for comet assay
   - To 1 L dH₂O add 0.4 M Tris base;
   - Adjust to pH 7.5 using concentrated HCl;
• Make up to 1 L and store at room temperature;
• Refrigerate before use.

6. Ethidium bromide (EB) staining solution (store in dark)
   • Stock solution: 2 mg/mL
   • Working solution: 20 µg/mL

7. Propidium Iodide (PI) for ‘dual stain’
   • Stock concentration: 500 µg/mL
   • Working concentration: 5 µg/mL

8. 4,6-Diamidino-2-phenylindole (DAPI) for ‘dual stain’
   • Stock concentration: 50 µg/mL
   • Working concentration: 5 µg/mL

9. Antifade Mounting Medium for apoptosis analysis
   • 0.5 M Carbonate buffer with equal volume glycerol

10. Cell Fixation Medium for apoptosis analysis
    • 4% Paraform Aldehyde

11. Mayer’s Haemalum working solution (1 L)
    • Haematoxylin powder: 1.0 g;
    • KAl(SO₄)₂: 50 g;
    • NaI: 0.2 g;
    • Citric Acid: 1.0 g.

12. tGSH extraction buffer – Trish-chloride buffer (20 mM)
    • KCl 0.15 M;
    • Sucrose: 0.5 M;
    • EDTA: 1 mM;
    • DTT: 1 mM
• Protease inhibitor cocktail: 100 μL
• pH=7.6.

13. 5-5’’-Dithio-bis(2-nitrobenzoic acid (DNTB) buffer
• DNTB 10 mM in 100 mM potassium phosphate
• EDTA: 5 mM;
• pH=7.5.
Appendix II Apoptosis (diagnosis at cellular level)

Apoptosis is a highly regulated cellular process. Dysregulation of apoptosis is involved in tumour formation. It may provide the basis for developing biomarkers to monitor non-lethal levels of xenobiotic-induced cellular stress and toxicity (Busch et al., 2004). Apoptosis is a characteristically orderly sequence of events that ultimately leads to the complete disassembly of the cell. It is distinguishable from the more non-specific mode of cell death known as necrosis. Necrosis is characterized by the swelling of mitochondria and immediate loss of plasma cell integrity, which can be observed by the uptake of normally membrane impermeable stains. In contrast, apoptosis can be induced in the normal course of growth and development of tissues, and can be induced by a vast array of compounds and conditions (Steinert, 1996). It is the major pathway of cell death and is necessary for the maintenance of the steady-state kinetics of healthy tissues. Changes in the level of apoptosis upon exposure to specific pollutants have been observed in numerous aquatic organisms (Blas-Machado et al., 2000). It can be used as biomarkers following exposure to carcinogenic environmental compounds at the early stage of tumour formation (e.g. at initiation process).

There are various techniques available for screening and detection of apoptosis. Dual stain of cell with fluorescent dyes which bind with nuclear and visualized under microscope is a relative simple way to distinguish normal, apoptosis and dead cells based on their morphology change (Bouchier-Hayes et al., 2008). For example, 4’,6’-diamidino-2-phenylindole (DAPI) is one of the nuclear dyes which can pass through intact cell membrane and stain nuclear in both live and dead cells. As an apoptosis cell has increased condensation of nuclear material, it will show stronger fluorescence under microscope observation than normal cell. In contrast, another dye propidium iodide (PI), cannot pass the live cell membrane. It can bind with nuclear material only when the cell
process necrosis with the morphology of increased cell membrane permeability. Therefore, PI can stain necrosis cells. The combination of PI and DAPI can detect apoptotic cell levels which induced by genotoxic component. The detailed method for ‘dual stain’ was described in Chapter 2.5. Following dual staining the haemocytes treated of 1000 μM H₂O₂, normal and apoptosis cells were stained with DAPI in the color of blue. Apoptotic cells shows brighter blue in comparison with normal cells due to condensed nuclear in the process of apoptosis. Dead cells were stained with PI as of red because of the loss of cell membrane integrity (Figure A1).

The majority cells (80%) showed normal morphology under control condition. The percentage of normal cells in total examined cells was declined after exposure and reached to about 30% after in vitro exposure to the highest concentration of H₂O₂. Number of apoptosis cells was also decreased after exposure compared to control, except for exposure to 10 μM H₂O₂. The dead cell number was increased in line with increased H₂O₂ concentration (Figure A2). The results suggested that apoptosis detected using dual stain method is indeed reflecting the level of cell damage. Apoptosis will be initiated when cells under stress induced by H₂O₂. This defense can be dysfunctional when excessive stress induced (i.e. 10 mM H₂O₂) and is over the threshold of cellular tolerance, the majority cell status showed dead.
Figure A1 Dual staining image illustrating cell status after in vitro exposure to H$_2$O$_2$.
Blue-normal cells, red-dead cells and bright blue-apoptotic cells.

Figure A2 Percentage of normal, apoptosis and dead haemocytes after *in vitro* exposure to H$_2$O$_2$. 

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### Appendix III Table of primers designed for real-time qPCR and relevant PCR conditions tested for future work.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Tested T&lt;sub&gt;A&lt;/sub&gt; Range (°C)</th>
<th>Confirmed T&lt;sub&gt;A&lt;/sub&gt;(°C)</th>
<th>Amplicon size (bp)</th>
<th>Gene bank access number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAp63/73</td>
<td>F 5’ AATATGGAACTCCCGTCCAGATC 3’</td>
<td>55-60</td>
<td>58</td>
<td>66</td>
<td>DQ865150</td>
<td>Muttray et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>R 5’ TGTAATTATTATATCCTTGCAGC 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔNP63/73</td>
<td>F 5’ AAATTTGAGAGAATGGATTACCAACC 3’</td>
<td>55-60</td>
<td>59</td>
<td>141</td>
<td>DQ865151</td>
<td>Muttray et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>R 5’ TGTGATTGTATGATGAGCTTGAG 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyp</td>
<td>F 5’ CCTGTCATCACGACGAGA 3’</td>
<td>50-55</td>
<td>52</td>
<td>292</td>
<td>MGC00313</td>
<td>Designed</td>
</tr>
<tr>
<td></td>
<td>R 5’ GCCGGTGGCGAGCAACT 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyp3A1</td>
<td>F 5’ AGTATGCGGATGTAGTGTTG 3’</td>
<td>45-50</td>
<td>48</td>
<td>224</td>
<td>AB479539</td>
<td>Designed</td>
</tr>
<tr>
<td></td>
<td>R 5’ TTTTCAGATGAAATGTAGG 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chk1</td>
<td>F 5’ CTTGGGGAGGGAGGAGCTATGAG 3’</td>
<td>58-63</td>
<td>60</td>
<td>121</td>
<td>GU212861</td>
<td>Designed</td>
</tr>
<tr>
<td></td>
<td>R 5’ CTCTTTCCCTGACATTTCTG 3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TA: Annealing temperature; F: forward primer; R: reverse primer.
Appendix IV Summary of correlation analyses for paired genes.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene pair</th>
<th>Haemocytes</th>
<th>Digestive gland</th>
<th>Mantle</th>
<th>Adductor muscle</th>
<th>Gill</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>PC</td>
<td>P</td>
<td>PC</td>
<td>P</td>
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<tr>
<td>Biotransformation</td>
<td><em>p53 vs cyp</em></td>
<td>0.008</td>
<td>0.501</td>
<td>0.543</td>
<td>-0.122</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>ras vs cyp</em></td>
<td>0.012</td>
<td>0.475</td>
<td>0.602</td>
<td>-0.105</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>cyp vs jnk</em></td>
<td>0</td>
<td>0.936</td>
<td>0</td>
<td>0.942</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>cyp vs bax</em></td>
<td>0.033</td>
<td>0.412</td>
<td>0</td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>cyp vs rad</em></td>
<td>0.739</td>
<td>-0.067</td>
<td>0</td>
<td>0.915</td>
<td>0</td>
</tr>
<tr>
<td>Apoptosis</td>
<td><em>p53 vs bax</em></td>
<td>0.035</td>
<td>0.703</td>
<td>0.534</td>
<td>-0.125</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>jnk vs bax</em></td>
<td>0.009</td>
<td>0.493</td>
<td>0</td>
<td>0.897</td>
<td>0</td>
</tr>
<tr>
<td>DNA repair</td>
<td><em>p53 vs rad</em></td>
<td>0.242</td>
<td>0.233</td>
<td>0.455</td>
<td>-0.15</td>
<td>0</td>
</tr>
<tr>
<td>P53-regulation</td>
<td><em>p53 vs jnk</em></td>
<td>0</td>
<td>0.651</td>
<td>0.572</td>
<td>-0.114</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>p53 vs mdm</em></td>
<td>0.01</td>
<td>0.583</td>
<td>0.622</td>
<td>-0.099</td>
<td>0</td>
</tr>
<tr>
<td>Ras-regulation</td>
<td><em>ras vs jnk</em></td>
<td>0.2</td>
<td>0.254</td>
<td>0.635</td>
<td>-0.096</td>
<td>0</td>
</tr>
</tbody>
</table>

P: P-value;

PC: Pearson correlation
### Appendix V Table of associated academic activities.

<table>
<thead>
<tr>
<th>Date</th>
<th>Conference/seminar</th>
<th>Work present</th>
</tr>
</thead>
<tbody>
<tr>
<td>22 April 2010</td>
<td>Marine Biological Association (MBA) council visiting</td>
<td>Determination of physiological, histopathological and genetic responses of <em>Mytilus edulis</em> to chemicals (Poster)</td>
</tr>
<tr>
<td>31 May - 04 June 2010</td>
<td>6th International conference of marine pollution &amp; ecotoxicology</td>
<td>Tissue specific expression of <em>p53</em> and <em>ras</em> genes as molecular biomarkers to evaluate impact of Benzo(α)Pyrene in marine mussels, <em>Mytilus edulis</em> (Platform)</td>
</tr>
<tr>
<td>04-07 June 2010</td>
<td>SETAC Asia/Pacific 2010 conference</td>
<td>Integrated biological responses and tissue specific expression of <em>p53</em> and <em>ras</em> genes to evaluate impact of benzo(α)pyrene in marine mussels, <em>Mytilus edulis</em> (Platform)</td>
</tr>
<tr>
<td>12-18 September 2010</td>
<td>EEMS meeting 2010</td>
<td>Tissue specific expression of <em>p53</em> and <em>ras</em> genes as molecular biomarkers to evaluate impact of benzo(α)pyrene in marine mussels, <em>Mytilus edulis</em> (Poster)</td>
</tr>
<tr>
<td>10 November 2011</td>
<td>Peninsula Medical Centre annual conference</td>
<td>Tissue specific expression of <em>p53</em> and <em>ras</em> gene in marine mussels following B(α)P exposure: implications for human and environmental health (Poster)</td>
</tr>
<tr>
<td>16–18 July 2012</td>
<td>UKEMS 2012 annual meeting</td>
<td>Integrated biological responses and tissue-specific expression of <em>p53</em> and <em>ras</em> genes in marine mussels following exposure to C_{60} fullerenes and/or benzo(α)pyrene (poster)</td>
</tr>
</tbody>
</table>
Appendix VI Publications


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