EFFECTS OF OIL EXPOSURE AND OIL-RELATED COMPOUNDS ON 
THE IMMUNE SYSTEM OF THE ARCTIC AND TEMPERATE SCALLOPS, 
CHLAMYs ISLANDICA AND PECTEN MAXIMUS 

by 

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

DOCTOR OF PHILOSOPHY 

School of Biomedical and Biological Sciences 
Faculty of Science 

In collaboration with IRIS - Akvamiljø, Norway 

March 2010
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MARIE LYN HANNAM

Abstract

With the current expansion of offshore oil activities in Arctic regions, there is an urgent need to establish the potential effects of oil-related compounds on Arctic organisms. The scallop Chlamys islandica is distributed throughout the sub-Arctic and has been proposed as a sentinel species for this region. In addition, the temperate scallop Pecten maximus occurs in regions of oil activity and, with increasing sea temperatures, the limit of its distribution may extend further into sub-Arctic regions. P. maximus also accumulates contaminants to a greater extent than the current temperate sentinel Mytilus edulis. A hierarchical approach, similar to that adopted to assess vertebrate immune function, was used to determine immunocompetence in the Arctic scallop C. islandica and the temperate scallop P. maximus following exposure to oil and oil-related compounds. The Arctic scallop C. islandica demonstrated a reduced immunocompetence following both dispersed and acute oil exposure. Immunomodulation in the scallops exposed to low levels of dispersed oil appeared to be reversible following removal of the contaminant stress (Chapter 3). However, a simulated oil spill resulted in mortalities and it remains unclear if the organisms are able to recover from the substantial immune suppression observed (Chapter 4). A component of crude oil and the most abundant PAH in aquatic ecosystems, phenanthrene suppressed immune function in P. maximus. These results indicated a link between PAH-induced oxidative stress and the subsequent inhibition in haemocyte immune function (Chapter 5). However, the ability of scallop haemocytes to recognise and respond to a pathogen-associated molecular pattern was not affected by phenanthrene exposure (Chapter 6). The immune parameters used in this research were shown to be sensitive,
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Acknowledgements

I am grateful to Professor Mal Jones for his kind support and advice throughout the duration of this research and extra special thanks for providing me with continued encouragement even into his retirement. Thanks to Dr Shaw Bamber who welcomed me at Akvamiljø and made my time in Norway both productive and enjoyable. I would also like to thank Professor Tamara Galloway for her guidance during these studies. A huge thanks to Dr John Moody for agreeing to come onboard this project as Director of Studies so late into the research; his words of wisdom have helped greatly in the production of this thesis. I am grateful to the University of Plymouth for the HEIF3 Research Studentship and to IRIS-Akvamiljø for the financial support which made this PhD possible.

Many at the University of Plymouth have helped me along the way; in particular, thanks to Ben Eynon and Pete Russell for their assistance with livestock husbandry, William Vevers for general laboratory support and also to Dr Paul Sutton for help with the PAH analysis. Thanks to Lindsey Glew and Matthew Emery for their technical advice and assistance with the microbiology work. Also, thank you to all those at IRIS-Akvamiljø for their practical assistance with experimental exposures and water chemistry.

Huge thanks go to Awantha Dissanayake and Martin Canty who have provided unwavering support, kind words, welcome distractions, cups of tea and apples when I needed them most. Finally I must thank the tireless and unconditional support from my family. Mum, Dad and Lisa, you helped make this possible, thank you so much.
Author's Declaration

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

This study was financed with the aid of a University of Plymouth HEIF3 studentship in collaboration with IRIS-Akvamiljø, Stavanger, Norway.

Relevant scientific seminars and conferences were attended at which work was presented and four papers have been prepared for publication:

Publications (please refer to Appendix I for the full manuscripts)


**Platform presentations**


Immunotoxicity and oxidative stress in the Arctic Scallop *Chlamys islandica*: effects of acute oil exposure. SETAC Europe 19th Annual Meeting, Gothenburg, Sweden, 31st May - 4th June 2009.

**Poster presentations**


Effects of the model PAH phenanthrene on immune function and oxidative stress in the temperate scallop *Pecten maximus*. The 15th International Symposium on Pollutant Responses in Marine Organisms (PRIMO 15), Bordeaux, France, 17th – 20th May, 2009.


Word count of main body of thesis: 29 270

Signed

Date 20/07/10.
Abbreviations and Acronyms

Abs  absorbance
AMAP  Arctic Monitoring and Assessment Programme
ANOVA  analysis of variance
AP  alkaline phosphatase
ATP  adenosine triphosphate
BaP  benzo(a)pyrene
BCF  bioconcentration factor
BFC  Baker's formol calcium
BHT  2,6-di-tert-butyl-4-methylphenol
CFS  continuous flow system
CFU  colony forming unit
Cl  condition index
dH₂O  distilled water
DMSO  dimethyl sulphoxide
DTNB  5,5'-dithiobis(2-nitrobenzoic acid)
EDTA  ethylenediamine tetra acetic acid
Fisher's LSD  Fisher's least significant difference
FSW  filtered seawater
g  standard gravity
GC/MS  gas chromatography mass spectrometry
GSH  reduced glutathione
GSSG  oxidised glutathione
H & E  haematoxylin and eosin stain
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICP-MS  inductively coupled plasma mass spectrometry
Kₐw  octanol-water partition coefficient
LOOH  lipid hydroperoxide
LPO  lipid peroxidation
LPS  lipopolysaccharide
MDAe  malondialdehyde equivalents
NADPH  nicotinamide adenine dinucleotide phosphate
NR  neutral red (2-metyl-3-amino-7-dimethylamino-phenazine)
NRR  neutral red retention
OBM  oil-based mud
OD  optical density
OECD  Organisation for Economic Cooperation and Development
ΣPAH  total polycyclic aromatic hydrocarbon concentration
PAH  polycyclic aromatic hydrocarbon
PAMP  pathogen-associated molecular pattern
PBS  phosphate buffered saline
phys. sal.  physiological saline
PRRs  pattern recognition receptors
PSU  practical salinity units
PVC  polyvinyl chloride
PW  produced water
ROS  reactive oxygen species
SBM  synthetic-based mud

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>TBARS</td>
<td>thiobarbituric acid reacting substances</td>
</tr>
<tr>
<td>TBA</td>
<td>thiobarbituric acid</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TEF</td>
<td>toxic equivalency factor</td>
</tr>
<tr>
<td>THC</td>
<td>total haemocyte count</td>
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<tr>
<td>US EPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>WAF</td>
<td>water accommodated fraction</td>
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<tr>
<td>WBM</td>
<td>water-based mud</td>
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<td>w/v</td>
<td>weight/volume</td>
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1 Introduction

1.1 Marine oil pollution

The marine environment is a major sink for many potentially hazardous contaminants (Woodhead et al., 1999). An estimated 3.27 million t of oil enter the marine environment each year and oil is now a ubiquitous contaminant of all marine ecosystems. Anthropogenic sources are of major importance, accounting for more than 90% of inputs (Preston and Chester, 2001). As the world's demand for oil grows, the offshore oil industry continues to expand, with offshore production, tanker operations and accidental spills accounting for some 1.17 Mt y⁻¹ of oil entering the marine environment (Preston and Chester, 2001). Recent focus has been directed to the expansion of oil exploration in Arctic and sub-Arctic regions, with exploration projects in both the Euro-Asian Arctic, notably the Barents Sea, and on the Alaskan North Slope (AMAP, 2007). Operational discharges from offshore oil activities vary depending upon the phase of production; drill cuttings and fluids generally dominate waste throughout exploration and pre-production, whilst produced water is the primary discharge during operational oil extraction (Carroll et al., 2000). These drilling wastes and produced water are a major source of oil discharged into marine ecosystems, with petroleum hydrocarbons recorded at concentrations of 150 mg l⁻¹ in seawater in areas of oil exploration and production (ACOPS, 1995). High levels of petroleum hydrocarbons, up to 80 mg l⁻¹, have also been reported at a polluted coastal site in the Mediterranean Sea (Khedir-Ghenim et al., 2009).
Oil can have detrimental impacts on marine organisms with mortalities observed in various invertebrate species (Table 1.1). Aside from the acute effects of crude oil and its water-accommodated fraction (WAF), sublethal effects have also been reported across numerous phyla (Table 1.1). Organism behaviour is disrupted following exposure to WAF with reduced feeding frequency and duration in the American lobster *Homarus americanus* (Atema et al., 1982) and inhibited prey-detection in the seastar *Coscinasterias muricata* (Georgiades et al., 2003). Reproductive impacts have been reported following dispersed oil exposure with larval abnormalities and mortalities reported in the Northern Shrimp *Pandalus borealis* (Larsen, 2004) and *Mytilus edulis* (Baussant, 2004). The reproductive toxicity of crude oil WAF has also been demonstrated in polychaetes (Lewis et al., 2008) and copepods (Bejarano et al., 2006). In addition to crude oil and WAF exposures, oil components such as polycyclic aromatic hydrocarbons (PAHs) have also been shown to elicit acute and sublethal effects.
Table 1.1 Examples of the biological effects of crude oil and its water accommodated fraction (WAF) on selected marine invertebrate groups.

<table>
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<tr>
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<th>Organism affected</th>
<th>Reference</th>
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<tr>
<td>Acute Mortalities</td>
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</tr>
<tr>
<td><strong>Sublethal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reproductive effects</td>
<td>Crustacea</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Gammarus wiltzkii</em></td>
<td>amphipod</td>
</tr>
<tr>
<td></td>
<td><em>Amphiascus teniremis</em></td>
<td>copepod</td>
</tr>
<tr>
<td></td>
<td><em>Sphaeroma quadridentatum</em></td>
<td>isopod</td>
</tr>
<tr>
<td></td>
<td><em>Pandalus borealis</em></td>
<td>shrimp</td>
</tr>
<tr>
<td></td>
<td>Annelida</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Arenicola marina</em></td>
<td>polychaete</td>
</tr>
<tr>
<td></td>
<td><em>Nereis virens</em></td>
<td>polychaete</td>
</tr>
<tr>
<td></td>
<td>Cnidaria</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Actinia equina</em></td>
<td>sea anenome</td>
</tr>
<tr>
<td></td>
<td><em>Siderastrea siderea</em></td>
<td>coral</td>
</tr>
<tr>
<td></td>
<td><em>Stylophora pistillata</em></td>
<td>coral</td>
</tr>
<tr>
<td></td>
<td>Echinodermata</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Strongyllocentrotus droebachiensis</em></td>
<td>sea urchin</td>
</tr>
<tr>
<td></td>
<td>Mollusca</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Venus verrucosa</em></td>
<td>clam</td>
</tr>
<tr>
<td></td>
<td><em>Patella vulgaris</em></td>
<td>limpet</td>
</tr>
<tr>
<td></td>
<td>Crustacea</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Homarus americanus</em></td>
<td>lobster</td>
</tr>
<tr>
<td></td>
<td>Echinodermata</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Coscinasterias muricata</em></td>
<td>seastar</td>
</tr>
</tbody>
</table>
1.1.1 PAHs in the marine environment

PAHs are natural constituents of crude oil accounting for around 1.9% of its composition, and are widespread environmental contaminants (Neff, 2002). Concern over their potential toxicity to aquatic organisms has seen the inclusion of PAHs on the US EPA Priority Pollutant List (US EPA, 2009) and the EU Water Framework Directive Priority Substances List (Annex II of Directive 2008/105/EC) (EC, 2010). PAHs are organic compounds made up of 2 or more benzene rings with delocalized electrons, and are highly lipophilic (Walker, 2001). They can be broadly categorised into two groups, petrogenic or pyrogenic PAHs, dependent upon their source. Petrogenic PAHs are found in crude oil and refined petroleum products and are made up of 2-3 benzene rings. These relatively lower molecular weights PAHs include naphthalene, phenanthrene and fluorene (Table 1.2). Pyrogenic PAHs, such as pyrene and fluoranthene, have a 4-6 ringed structure and are largely derived from combustion of organic matter and industrial processes (Walker, 2001). Biogenic PAHs are limited, with perylene the most common in marine systems, formed through bacterial and algal synthesis (Dahle et al., 2006; Boitsov et al., 2009).

The higher molecular weight (pyrolytic) PAHs are reported to be potential carcinogens and mutagens, and have, therefore, received much attention regarding biological-effect studies. However, the petrogenic PAHs naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene and phenanthrene have also been indicated as PAHs of environmental concern.
Despite pyrolytic PAHs having higher octanol-water partition coefficients ($K_{ow}$), petrogenic PAHs demonstrated a greater bioavailability than pyrogenic PAHs to bivalves, including the marine clam *Mya arenaria* (Thorsen et al., 2004). However, there remains a paucity of studies focusing on the biological effects of these petrogenic PAHs compared to the pyrolytic compounds.

Table 1.2 Structures of petrogenic PAHs included on the US EPA Priority Pollutant List. Their log octanol-water partition coefficients ($\log K_{ow}$) are also given.

<table>
<thead>
<tr>
<th>PAH</th>
<th>Structure</th>
<th>$\log K_{ow}$ (Latimer and Zheng, 2003)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td><img src="image" alt="Naphthalene" /></td>
<td>3.37</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td><img src="image" alt="Acenaphthene" /></td>
<td>4.00</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td><img src="image" alt="Acenaphthene" /></td>
<td>3.92</td>
</tr>
<tr>
<td>Fluorene</td>
<td><img src="image" alt="Fluorene" /></td>
<td>4.18</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td><img src="image" alt="Phenanthrene" /></td>
<td>4.57</td>
</tr>
<tr>
<td>Anthracene</td>
<td><img src="image" alt="Anthracene" /></td>
<td>4.54</td>
</tr>
</tbody>
</table>
1.2 Evaluating biological impacts of pollution

Substantial increases in oil exploration and production have occurred over the last 25 years (Carroll et al., 2000) and, as a result, there has been a growing need for rapid and effective monitoring of the environmental impacts of offshore oil production. Direct chemical analysis of pollutants provides detailed information on concentrations, composition and spatial distribution (Woodhead et al., 1999); however, such analyses give little information on their biological consequences. Traditionally, benthic community analyses have been used as an indication of the impact of contaminants of biota (Serrano et al., 2006; Jorissen et al., 2009), but these are time consuming and costly. In addition, changes to community assemblage suggest that these higher levels of biological organisation have already been disrupted as a result of contaminant exposure (Walker et al., 2001).

1.2.1 Biomarkers in aquatic toxicology

Whilst the effects of chemical contaminants may be seen across many levels of biological organisation, pollutants first act by altering chemical, structural or functional properties essential to cellular functioning (Jagoe, 1996). As such, in order to better predict and understand the effects of pollutants on organisms, populations and communities, it is important to establish the effects at lower levels of biological organisation (Haux and Förlin, 1988). These sensitive biochemical, molecular, cellular or physiological endpoints, are known as biomarkers (Figure 1.1) and indicate exposure to and/or damage incurred by environmental pollutants (Depledge and Fossi, 1994).
Although biomarker responses occur at a lower level of ecological relevance than alterations at the population and community levels, they also occur over much shorter timescales (Figure 1.1), highlighting their potential as early-warning indicators of contaminant-induced stress.

Over the past two decades, a wide range of biomarkers have been developed for use in aquatic toxicology (Table 1.3) allowing subtle changes to be detected at the biochemical level right up to behavioural alterations. Significant relationships between biomarkers at the lower levels of biological organisation (molecular and cellular) and the higher levels (behavioural) have been demonstrated in the seastar *Asterias rubens* and the mussel *Mytilus edulis* following exposure to the pharmaceutical cyclophosphamide (Canty et al., 2009). The authors reported significant correlations between...
increased DNA damage and reduced feeding rate in *M. edulis*, and in
*A. rubens* an increased righting time was linked to elevated DNA damage
and micronuclei formation. Recent advances have highlighted the value of
using a suite of biomarkers to allow rapid assessment of contaminant
exposure and effect (Galloway et al., 2002; Galloway et al., 2006). In
addition, the use of biomarkers in sentinel species has also been recognised
as a cost-effective surrogate measure for assessing overall ecosystem
health (Depledge and Galloway, 2005).

Table 1.3 Examples of biomarkers at different levels of biological organisation used in aquatic
toxicology.

<table>
<thead>
<tr>
<th>Organisational level</th>
<th>Biomarker</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behavioural</td>
<td>feeding rate</td>
<td>Canty et al., 2009; May et al., 2010; Scarlett et al., 2008</td>
</tr>
<tr>
<td></td>
<td>burrowing behaviour</td>
<td>Hyslop and Davies, 1999; Matozzo et al., 2004; Shin et al., 2002</td>
</tr>
<tr>
<td></td>
<td>valve gape</td>
<td>May et al., 2010; Tran et al., 2010</td>
</tr>
<tr>
<td>Physiological</td>
<td>SFG</td>
<td>Dissanayake et al., 2008b; Pook et al., 2009</td>
</tr>
<tr>
<td></td>
<td>heart rate</td>
<td>Bamber and Depledge, 1997; Camus et al., 2002a; Fossi et al., 2000</td>
</tr>
<tr>
<td></td>
<td>respiration</td>
<td>Camus et al., 2002a; St-Amand et al., 1999</td>
</tr>
<tr>
<td></td>
<td>osmoregulation</td>
<td>Bamber and Depledge, 1997; Lignot et al., 1998</td>
</tr>
<tr>
<td>Cellular</td>
<td>cell membrane stability</td>
<td>Canty et al., 2007; Scarlett et al., 2008</td>
</tr>
<tr>
<td></td>
<td>phagocytosis</td>
<td>Dissanayake et al., 2008a; Dyrnda et al., 1998; Parry and Pipe, 2004</td>
</tr>
<tr>
<td></td>
<td>micronuclei</td>
<td>Jha et al., 2005; Hagger et al., 2005; Nitro et al., 2006</td>
</tr>
<tr>
<td>Molecular</td>
<td>DNA strand breaks</td>
<td>Canty et al., 2009; Jha et al., 2000; Petridis et al., 2009</td>
</tr>
<tr>
<td></td>
<td>chromosome aberrations</td>
<td>Cheung et al., 2006</td>
</tr>
<tr>
<td>Biochemical</td>
<td>glutathione</td>
<td>Al-Subiai et al., 2009; Regoli and Principato, 1995</td>
</tr>
<tr>
<td></td>
<td>lipid peroxidation</td>
<td>Pan et al., 2005; 2006; Quinn et al., 2005</td>
</tr>
<tr>
<td></td>
<td>AChE</td>
<td>Bonacci et al., 2008; Brown et al., 2004; Hannam et al., 2008;</td>
</tr>
<tr>
<td></td>
<td>TOSC</td>
<td>Bocchetti et al., 2008; Camus et al., 2002b; Regoli, 2000</td>
</tr>
<tr>
<td></td>
<td>metallothionein</td>
<td>Da Ros et al., 2007; Gorbi et al., 2008; Amiard-Triquet et al., 1998</td>
</tr>
</tbody>
</table>

Abbreviations: AChE, acetylcholinesterase; SFG, scope for growth; TOSC, total oxyradical scavenging capacity.
1.3 Immunotoxicity

Due to its complexity, an organism's immune system is extremely vulnerable to xenobiotic stress (Galloway and Depledge, 2001), and there is increasing evidence to suggest that numerous environmental contaminants can impact immune function in many organisms (Table 1.4). Growth, disease and survival of an organism are partly determined by the capability of the immune system (Blaise et al., 2002), therefore, immune function is important in assessing sublethal effects of contaminant exposure (Luengen et al., 2004). Such sublethal effects may be of great significance to the long-term success of a population. For example, following a major oil spill, no direct oil-induced mortalities in the mussel *Mytilus edulis* were recorded, but the same mussels exhibited significant immunosuppression (Wotton et al., 2003a). Whilst severely compromised immune systems can result in rapid mortality, subtle changes in its components can be used as early-warning indicators of environmental stress.
<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mercury</td>
<td>Mytilus edulis, Crassostrea gigas</td>
<td>Duchemin et al., 2008; Gagnaire et al., 2004</td>
</tr>
<tr>
<td>Silver</td>
<td>Mya arenaria</td>
<td>Gagnaire et al., 2004</td>
</tr>
<tr>
<td>Cadmium</td>
<td>Ostrea edulis, Asterias rubens, Mytilus edulis</td>
<td>Auffret et al., 2002; Coteur et al., 2005; Coles et al., 1995</td>
</tr>
<tr>
<td>Copper</td>
<td>Crassostrea virgincia, Mytilus edulis</td>
<td>Anderson et al. 1994; Pipe et al., 1999</td>
</tr>
<tr>
<td>Zinc</td>
<td>Mytilus edulis</td>
<td>Sauve et al., 2002</td>
</tr>
<tr>
<td>Lead</td>
<td>Mytilus edulis</td>
<td>Nieto-Fernandez et al., 2000</td>
</tr>
<tr>
<td><strong>Organometals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBT</td>
<td>Crassostrea virgincia, Tapes philippinarum</td>
<td>Anderson et al., 1996; Matozzo et al., 2002</td>
</tr>
<tr>
<td>DBT</td>
<td>Mytilus edulis</td>
<td>St-Jean et al., 2002a&amp;b</td>
</tr>
<tr>
<td><strong>Aromatic hydrocarbons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anthracene+fluoranthene+phenanthrene</td>
<td>Mytilus edulis</td>
<td>Grundy et al., 1996b</td>
</tr>
<tr>
<td>fluoranthene</td>
<td>Mytilus edulis</td>
<td>Coles et al., 1994</td>
</tr>
<tr>
<td>phenanthrene</td>
<td>Mytilus edulis, Cerastoderma edule</td>
<td>Wootton et al., 2003a</td>
</tr>
<tr>
<td>phenanthrene enriched PAHs</td>
<td>Mya arenaria</td>
<td>Frouin et al., 2007</td>
</tr>
<tr>
<td>fluorene</td>
<td>Crassostrea gigas</td>
<td>Bado-Nilles et al., 2008</td>
</tr>
<tr>
<td>pyrene</td>
<td>Crassostrea gigas, Carcinus maenas</td>
<td>Bado-Nilles et al., 2009; Dissanayake et al., 2008b</td>
</tr>
<tr>
<td>benzo(b)/fluoranthene</td>
<td>Mya arenaria</td>
<td>Frouin et al., 2007</td>
</tr>
<tr>
<td>benzo(a)pyrene</td>
<td>Haliotis diversicolor</td>
<td>Gopalakrishnan et al., 2009</td>
</tr>
<tr>
<td><strong>Halogenated hydrocarbons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCBs</td>
<td>Mytilus galloprovincialis, Chlamys farrei</td>
<td>Canesi et al., 2003; Liu et al., 2009</td>
</tr>
<tr>
<td><strong>Pesticides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malathion</td>
<td>Hommarus americanus</td>
<td>De Guise et al., 2004</td>
</tr>
<tr>
<td>Azamethiphos</td>
<td>Mytilus edulis</td>
<td>Canty et al., 2007</td>
</tr>
</tbody>
</table>

Abbreviations: TBT, tributyltin; DBT, Dibutyltin; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls.
Table 1.4 continued.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pesticides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorfenvinphos</td>
<td><em>Mytilus edulis</em></td>
<td>Rickwood &amp; Galloway, 2004</td>
</tr>
<tr>
<td>Pesticide mixture (atrazine, glyphosate,</td>
<td><em>Crassostrea gigas</em></td>
<td>Gagnaire et al., 2007</td>
</tr>
<tr>
<td>alachlor, metolachlor, fosetyl-aluminium,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>terbuthylazine, diuron and carbaryl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pharmaceuticals and PCPs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bezafibrate</td>
<td><em>Mytilus galloprovincialis</em></td>
<td>Canesi et al., 2007b</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td><em>Mytilus galloprovincialis</em></td>
<td>Canesi et al., 2007b</td>
</tr>
<tr>
<td>17β estradiol</td>
<td><em>Mytilus galloprovincialis</em></td>
<td>Canesi et al., 2006</td>
</tr>
<tr>
<td>Triclosan</td>
<td><em>Mytilus galloprovincialis</em></td>
<td>Canesi et al., 2007a</td>
</tr>
<tr>
<td><strong>Nanoparticles</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon black</td>
<td><em>Mytilus galloprovincialis</em></td>
<td>Canesi et al., 2008</td>
</tr>
<tr>
<td>C60 fullerene</td>
<td><em>Mytilus galloprovincialis</em></td>
<td>Canesi et al., 2010</td>
</tr>
<tr>
<td>TiO₂</td>
<td><em>Mytilus galloprovincialis</em></td>
<td>Canesi et al., 2010</td>
</tr>
<tr>
<td>SiO₂</td>
<td><em>Mytilus galloprovincialis</em></td>
<td>Canesi et al., 2010</td>
</tr>
<tr>
<td><strong>Complex mixtures</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude oil emulsion</td>
<td><em>Mytilus edulis</em></td>
<td>McCormick-Ray, 1987</td>
</tr>
<tr>
<td>Fuel oil</td>
<td><em>Crassostrea gigas, Mytilus galloprovincialis</em></td>
<td>Bado-Niles et al., 2008; Ordas et al., 2007</td>
</tr>
<tr>
<td>Produced water</td>
<td><em>Mytilus edulis</em></td>
<td>Hannam et al., 2009</td>
</tr>
<tr>
<td>Untreated sewage</td>
<td><em>Mytilus edulis</em></td>
<td>Akaishi et al., 2007</td>
</tr>
<tr>
<td>Contaminated sediment</td>
<td><em>Mytilus edulis, Asterias rubens, Mactromeris polyynma, Mya arenaria, Crassostrea virgina, Crangon crangon</em></td>
<td>Cajaraville et al., 1996; Coteur et al., 2005; Fournier et al., 2002; Oliver et al., 2001; Sami et al., 1993; Smith et al., 1995</td>
</tr>
</tbody>
</table>

Abbreviations: PCPs, personal care products; TiO₂, titanium dioxide; SiO₂, silicon dioxide
1.3.1 Invertebrate immunity

Invertebrates have evolved innate defence mechanisms that can identify and protect against non-self material, including various cell-mediated and humoral responses. These immune parameters provide an accessible means for monitoring immune function (Galloway and Depledge, 2001) and act to provide a multifaceted immune response (Pipe et al., 1995b). Due to the number of immune components and their overlapping functions that contribute towards the immune system (Galloway and Depledge, 2001), Livingstone et al. (2000) suggested that a tiered approach be used for assessing the impact of environmental stressors on invertebrate immune function (Figure 1.2). The first stage of a hierarchical approach is based on the apparatus responsible for immune defence (haemocytes, in the case of bivalves). Such studies involve assessing the immunopathology, including total and differential cell counts and cell viability. The second tier is centred on mechanisms of immunity, such as phagocytosis, cytotoxicity and the release of humoral factors. The third tier, and potentially the most important, determines the overall host immunocompetence through a bacterial challenge (Figure 1.2). This is of high ecological relevance, since the range of immune strategies available may mean that, even if one parameter is inhibited, other immune responses may compensate so the host resistance is not compromised.
Figure 1.2 Tiered approach to assessing invertebrate immunocompetence (adapted from Livingstone et al., 2000).

### 1.3.2 Biomarkers of immunotoxicity

#### 1.3.2.1 Immunopathology

**Haemocyte counts**

The immune system of invertebrates is largely dependent on the circulating cells that move throughout the tissues of an organism to protect against foreign material and carry out immuno-surveillance (Pipe et al., 1995b). In bivalves these cells are termed haemocytes, which were thought to originate in the connective tissues. However, there is now evidence of cell division also occurring in the haemolymph of bivalves including the mussel *Mytilus edulis* (Mayrand et al., 2005) and the clam *Tapes philippinarum* (Cima et al.,
2000; Matozzo et al., 2008). Haemocytes of invertebrates are less differentiated than the leucocytes of vertebrates (Hine, 1999). As such there has been no accepted haemocyte classification scheme applicable to all bivalves; however it is now thought that cells fall into two broad categories, the granulocytes and the hyalinocytes (Hine, 1999). Previous work on the oyster Crassostrea virginica divided these granulocytes into sub-populations based upon the staining of the cytoplasmic granules (Feng et al., 1971) but the use of electron microscopy later found these stained granules to be the same type (Cheng et al., 1974). Other studies on this same bivalve identified three subpopulations of granulocytes based on cell size (Renwrantz et al., 1979), although it is unclear if these represent different types of granular cells or cells at different stages of maturation (Cheng, 1981). Bivalves from the family Pectinidae differ from most other bivalves as they lack these granulocytes (Auffret, 1988; Mortensen and Glette, 1996). It is due to this controversy over bivalve cell classification that differential haemocyte counts were not conducted during the studies of this thesis.

The total number of circulating haemocytes can fluctuate widely (Livingstone et al., 2000) and there are contrasting reports of xenobiotics significantly increasing or decreasing cell counts. A reduction in total haemocyte count was observed in the crustacean Palaemon elegans after exposure to 20 mg l⁻¹ Cu and 10 mg l⁻¹ Cd (Lorenzon et al., 2001). In contrast, the mussel Mytilus edulis exposed to 0.05 mg l⁻¹ Cu and 0.4 mg l⁻¹ Cd had an elevated number of circulating haemocytes (Coles et al., 1995; Pipe et al., 1999).
These conflicting responses may reflect the large difference between exposure concentrations. The high Cu and Cd levels used in the *P. elegans* exposure may be acutely toxic to the haemocytes leading to cell death. In addition, high concentrations of xenobiotics including Cu and Cd can cause histopathological damage (Sunila, 1986; Da Ros et al., 1995), resulting in the infiltration of haemocytes into the tissues to carry out wound repair, subsequently decreasing the number of circulating haemocytes. Conversely, the lower concentrations used in the exposure of *M. edulis* may stimulate the migration of cells from the haematopoietic tissues (Pipe et al., 1999) elevating the numbers of circulating cells. These haemocytes may move into circulation to sequester metals from the haemolymph, detoxifying the metal in their lysosomes (Cajaraville and Pal, 1995) and through the metal-binding protein metallothionein (Roesijadi et al., 1997).

An increase in circulating cells is also reported in *M. edulis* after low level PAH exposure, with 0.2 and 0.4 mg l⁻¹ fluoranthene significantly elevating the THC (Coles et al., 1994). This is in agreement with results from *Crassostrea gigas* exposed to sublethal PAHs in vitro (anthracene, chrysene, fluorene, phenanthrene or pyrene) where possible cell migration from the tissues was indicated by a higher percentage of viable circulating haemocytes (Bado-Nilles et al., 2008). Due to the variable nature of haemocyte counts, it is recommended that they should be conducted in parallel with other immunopathology assays to allow better interpretations of the results.
**Cell membrane stability**

Cell membranes are essential in the functioning of cellular immune processes with several stages of phagocytosis (recognition, adherence and ingestion) dependent upon the membrane properties of the immune cells (Grundy et al., 1996b). Alterations in these cell membranes are often one of the earliest detectable changes induced by contaminant exposure (Moore, 1985). The measurement of cell membrane stability can, therefore, provide a sensitive indicator of the immune capability of an organism.

Originally developed for use with 3T3 mouse fibroblast cells, the neutral red uptake assay is an in vitro method for determining cell membrane stability (Borenfreund and Puerner, 1985). The weakly cationic, supravital dye, neutral red (NR) enters cells by passive diffusion across the cell membrane and accumulates in the lysosomes of living cells, however, cells with damaged membranes have a reduced ability to take up and retain the NR dye (Babich and Borenfreund, 1992). The amount of NR taken up by the cells can be determined spectrophotometrically after lysing the cells to extract the dye, with 'healthier' cells having a higher measured optical density corresponding to more dye being retained. This technique gives an indication of overall cell membrane stability rather than lysosomal stability as it does not distinguish between dye that has accumulated in the lysosomes and dye retained within the cytoplasm of the cell. Later work by Lowe and Pipe (1994), adapted this method for determining specific lysosomal membrane stability. This method involved observing the release of the NR
dye from the lysosomal compartment using a light microscope and assigning a NR retention time to express the stability of the lysosomal membrane. Whilst this technique has proved useful (Ringwood et al., 1999; Wedderburn et al., 2000; Hauton et al., 2001; Galloway et al., 2010), it is time demanding and restricts the number of samples than can be processed at any one time. Therefore, the spectrophotometric method of the NR uptake assay was applied in the studies of this thesis.

The spectrophotometric NR assay has now been included in the OECD guidelines for in vitro phototoxicity testing (Commission Directive 2000/33/EC), and has also been adapted for use in aquatic organisms using a microplate method (Pipe et al., 1995). Studies have shown cell membrane stability to be a sensitive indicator of contaminant-induced damage, with metals (Olabarrieta et al., 2001; Dailianis, 2009), pesticides (Rickwood and Galloway, 2004), PAHs (Grundy et al., 1996a; Gómez-Mendikute et al., 2002) and organotins (Hagger et al., 2005) reducing the cell membrane stability of marine invertebrates. However, at very low concentrations, contaminants may not compromise the cell membrane, with no effects observed in *M. edulis* exposed to 5 µg l⁻¹ branched alkylbenzenes (Scarlett et al., 2008). The mode of action of toxicants is also important; exposure of mussels (*M. edulis*) and seastars (*Asterias rubens*) to the anti-cancer drug cyclophosphamide had no impact on cell membrane stability, which the authors reasoned was due to the primary site of toxicity for this compound being the DNA rather than cell membranes (Canty et al., 2009). In contrast
lipophilic contaminants are likely to target cell membranes (Dyrynda et al., 1998) and can penetrate these membranes altering their fluidity (Nelson et al., 1990). This spectrophotometric NR assay has been used successfully across a range of marine invertebrates including crustaceans (Dissanayake et al., 2008a; Hagger et al., 2009), echinoderms (Békri and Pelletier, 2004; Canty et al., 2009) and molluscs (Coles et al., 1995; Pipe et al., 1995; Hannam et al., 2009b).

1.3.2.2 Mechanisms of immunity

Cell-mediated responses

Many organisms have developed an efficient internal defence system capable of eliminating potential pathogens. Invertebrates rely on innate immunity (Livingstone et al., 2000), where the most common, non-specific defence mechanism is phagocytosis (Pipe and Pulsford, 1995). This mechanism is the cellular ingestion of particulate matter by either nodulation (response to small particles) or encapsulation (response to larger foreign material) (Pipe and Coles, 1995), and involves 5 stages: recognition, chemotaxis, adherence, ingestion and destruction (Feng, 1988).

In bivalve haemocytes, the phagocytic capability can be measured directly through quantification of the uptake of dyed particles into haemocytes (Pipe and Coles, 1995). The uptake of neutral-red stained yeast particles (from *Saccharomyces cerevisiae*) has been used to measure phagocytic activity.
across a range of mollusc species including the bivalves *M. edulis* (Pipe et al., 1995; Dyrynda et al., 1998; Rickwood and Galloway, 2004; Hagger et al., 2005; Canty et al., 2007), *M. galloprovincialis* (Cajaraville et al., 1996; Olabarrieta et al., 2001; Canesi et al., 2007a), *Cerastoderma edule*, *Ensis siliqua* (Wootton et al., 2003b) and *Ruditapes decussates* (Ordas et al., 1999), and the gastropod *Littorina littorea* (Gorbushin and Lakovleve, 2007).

Numerous laboratory studies have demonstrated contaminant-induced phagocytic inhibition resulting from metal (Coles et al., 1995), PAH (Grundy et al., 1996b; Wootton et al., 2003a; Dissanayake et al., 2008a), pharmaceutical (Canesi et al., 2007b) and organophosphorus pesticide (Rickwood and Galloway, 2004) exposure. Whilst a reduction in phagocytosis appears to be a common result of pollutant exposure, some authors report an increase in phagocytic activity in *Mytilus* sp. associated with short-term chemical stress at low concentrations. For example, Canesi et al. (2007c) reported a stimulation of phagocytic activity in *M. galloprovincialis* following in vitro exposure to 0.1 µM synthetic estrogens, including bisphenol A, benzophenone and mestranol. A similar phagocytic increase has been observed in *M. edulis* following in vivo exposure to 0.2 mg l⁻¹ Cu (Pipe et al., 1999) and 0.125 % oil-well produced water (Hannam et al., 2009b) for 7 d. However, all the authors noted that elevated phagocytosis changed to phagocytic inhibition for all three pollutant types at higher exposure concentrations and longer exposure periods (Pipe et al., 1999; Canesi et al., 2007c; Hannam et al., 2009b). It was proposed that an...
initial low dose stimulation of phagocytosis may involve high energetic costs to an organism that cannot be maintained for extended periods. Pipe et al. (1999) also indicated that exposed organisms may have a threshold for contaminant exposure, with a concentration below which phagocytosis is stimulated and above which this immune mechanism is impaired.

A further mechanism of the invertebrate defence system, cytotoxic capability, is the ability of the haemocytes to lyse foreign cells (Parrinello et al., 1993). This is similar to the cytotoxic reactions of natural killer cells in vertebrates (Galloway and Depledge, 2001). Spontaneous cytotoxicity can involve soluble cytotoxic mediators found in plasma, or may require direct cell-to-cell contact (Parrinello et al., 1993). Cell-mediated cytotoxic reactions, requiring contact between the effector and target cells, will be the focus here and have been observed in tunicates (Parrinello et al., 1993; Raftos and Hutchinson, 1995), polychaetes (Porchet-Hennere et al., 1992), gastropods (Gorbushin and Lakovleva, 2007) and bivalves (Rickwood and Galloway, 2004; Hannam et al., 2009b).

Exposure to environmental contaminants can affect the ability of haemocytes to function properly through alterations in cell membrane stability, as discussed in Section 1.3.2.1. Hence, the ability of haemocytes to recognise and lyse allogenic target cells may also be altered and therefore a useful indicator of immune modulation following contaminant exposure. Studies have demonstrated a reduction in the haemocyte-mediated cytotoxicity of
M. edulis following in vivo exposure to 0.25% oil-well produced water (Hannam et al., 2009b), and a 24 h in vitro exposure to 7 and 15 µg l⁻¹ chlorfenvinphos (Rickwood and Galloway, 2004). A reduction in the cytotoxic capability of M. edulis haemocytes was also reported in mussels from the copper-contaminated Restronguet Creek (Smith, 2002), and in the mussel Geukensia demissa from New Bedford Harbour in areas contaminated with PCBs and PAHs (Galloway et al., 2002).

**Humoral factors**

Several types of humoral activities have been identified in marine invertebrates. These immune-related factors augment the cellular immune mechanisms, and are found in the cell-free fraction of the haemolymph (Smith, 1991). Humoral factors identified in marine bivalves include hydrolytic enzymes (McHenery et al., 1979; Xue et al., 2004), agglutinins (Olafsen et al., 1992), lectins (Olafsen, 1995; Leclerc, 1996) and antimicrobial peptides (Hancock et al., 2006; Li et al., 2007).

The hydrolytic enzyme alkaline phosphatase (EC 3.1.3.1) participates in the degradation and breakdown of invading non-self material (Liu et al., 2004) by catalysing the hydrolysis of phosphate esters (Georgiades et al., 2003). Whilst isoenzymes of AP have been reported in mammals (Moss, 1982) this has yet to confirmed in invertebrates, however the presence of AP has been reported across various invertebrate groups including echinoderms (Temara
et al., 1997a; Temara et al., 1997b; Georgiades et al., 2003) and bivalves (Xing et al., 2002; Liu et al., 2004; Xue et al., 2004; Jing et al., 2006). As well as being present within bivalve haemocytes, this enzyme is also secreted into the plasma as part of the organism’s immune response (Cheng and Rodrick, 1975; Gestal et al., 2008). The bivalves *Ruditapes philippinarum* and *Scrobicularia plana* exhibited a reduction in AP activity following Hg exposure (Blasco et al., 1993; Mazorra et al., 2002). Metal exposure also affected AP activity in the asteroid *Asterias rubens*, with 50 µg l⁻¹ Pb significantly inhibiting AP (Temara et al., 1997a). This inhibition of *A. rubens* AP activity was also demonstrated in organisms from areas of a fjord contaminated with metals, PAHs and PCBs (Temara et al., 1997b).

### 1.3.2.3 Host resistance

The ability of an organism to resist bacterial infection is a key factor in disease incidence. Most studies on invertebrate host resistance use *Vibrio* spp. as the bacterial inoculum which is injected into the test species. Clearance of these injected bacteria is then used as a measure of an organism’s susceptibility to infection. These bacterial challenge assays have been used across a range of invertebrates including the sea urchin *Strongylocentrotus purpuratus* (Yui and Bayne, 1983), the blue crab *Callinectes sapidus* (Macey et al., 2008), the mussel *Mytilus galloprovincialis* (Canesi et al., 2001; Parisi et al., 2008) and the clam *Ruditapes philippinarum* (Allam et al., 2002).
A contaminant-induced reduction in bacterial elimination has been reported in the bivalve Mercenaria mercenaria following long-term exposure to the organic pollutants pentachlorophenol and benzo(a)pyrene (Anderson et al., 1981). This increase in bacterial susceptibility was also observed in M. edulis as a result of TBT and DBT exposure, with exposed mussels demonstrating an impaired ability to clear V. anguillarum from the haemolymph (St-Jean et al., 2002b). When challenged with this same bacterium, mussels from contaminated field sites also had a reduced capacity to eliminate bacteria (Mayrand et al., 2005).

In addition to impaired bacterial clearance, pollutants have also been associated with increased mortalities following a bacterial challenge. TBT-exposed oysters, Crassostrea virginica, exhibited significant mortalities after being challenged with Perkinsus marinus (Anderson et al., 1996). Similar increases in mortality have also been reported for M. edulis challenged with Vibrio spp. after exposure to copper (Pipe and Coles, 1995) and untreated sewage (Akaishi et al., 2007). These studies clearly demonstrate that contaminant-induced changes in immune function can compromise host resistance to bacterial infection, and this could be related to disease outbreaks in polluted areas.

1.4 Scallops as a test organism

The Arctic Scallop, Chlamys islandica (Figure 1.3a), is a bivalve mollusc distributed throughout the sub-Arctic transitional zone of the North Atlantic
(Thorarinsdóttir, 1993) from Spitsbergen in the north (Wiborg, 1963) to near Bergen (Greve and Samuelsen, 1970). It is the northernmost species of family Pectinidae, and was previously exploited on a large scale in Iceland, Greenland and Norway (Shumway and Parsons, 2006). The Great Scallop *Pecten maximus* (Figure 1.3b), also commercially important for fisheries and aquaculture, is largely found around France and the British Isles, but also occurs along the European Atlantic from the Iberian Peninsula to northern Norway (Marshall and Wilson, 2008). This temperate scallop occurs in regions of oil activity and, with increasing sea temperatures, the limit of its northern distribution may be pushed further into sub-Arctic regions.
With low metabolic rates, bivalves have a tendency to accumulate pollutants in their tissues, with scallops reportedly concentrating toxic compounds to a greater extent than other bivalves, including the common sentinel species *M. edulis* (Young-Lai and Aiken, 1986), highlighting scallops as potentially valuable biomonitoring organisms. Not only economically valuable, scallops...
are also an important prey source for other organisms including sea stars and crabs, and in the past decade the use of scallops in biological-effect studies has increased. The scallop *Placopecten magellanicus* has been used to assess the effects of oil-related discharges and showed that exposure to 1 mg l⁻¹ oil based drilling mud (OBM) caused significant mortalities, whilst 0.5 mg l⁻¹ OBM and 10 mg l⁻¹ water-based mud (WBM) reduced somatic growth (Cranford et al., 1999).

Biomarkers have been applied to various scallop species (Table 1.5) to assess the effects of contaminants on biological parameters. Oxidative stress biomarkers, conducted using *Chlamys farreri*, demonstrated initial stimulation followed by inhibition of the antioxidant enzyme superoxide dismutase (SOD) following benzo(a)pyrene and benzo(k)fluoranthene exposure (Pan et al., 2005; Pan et al., 2006). In the Arctic Scallop *C. islandica*, reduced TOSC was measured following exposure to benzo(a)pyrene (Camus et al., 2002b) and crude oil (Baussant et al., 2009). Oxidative stress biomarkers have also been studied in the Antarctic Scallop *Adamussium colbecki* following metal exposure, with 20 μg l⁻¹ copper and 5 μg l⁻¹ mercury both reducing the activity of the antioxidant enzymes SOD and catalase (Regoli et al., 1998). *A. colbecki* has also been used as a candidate species for neurotoxicity biomarkers following organophosphorus exposure (Bonacci et al., 2004). Similarly, the neurotoxic effect of the organophosphorus pesticide chlorpyrifos (0.1 ng l⁻¹) has been demonstrated in the zigzag scallop *Euvola ziczac* through the inhibition of
acetylcholinesterase activity (Owen et al., 2002). Immune parameters have also been measured in the scallops Agropecten irradians and C. farreri, with temperature significantly impacting these biomarkers (Liu et al., 2004).

Limited field studies have been conducted using scallops as a biomarker test organism (bioindicator). The biological impact of a municipal dump discharge was investigated using Agropecten gibbus, with significant increases observed in DNA damage, metallothionein levels and vitellin-like proteins, suggesting that the dump was having a negative impact on organisms in the vicinity of the dump site (Quinn et al., 2005). Despite the few biomarker field studies conducted with scallops, laboratory studies indicate their potential as bioindicators (Regoli et al., 1998; Owen et al., 2002; Camus et al., 2002b).
Table 1.5 The application of scallops as a biomarker test organism.

<table>
<thead>
<tr>
<th>Species</th>
<th>Biomarker</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Adamussium colbecki</em></td>
<td>neurotoxicity</td>
<td>Bonacci et al., 2004</td>
</tr>
<tr>
<td></td>
<td>oxidative stress</td>
<td>Regoli et al., 1998; 2000; 2002; Viarengo et al., 1995</td>
</tr>
<tr>
<td><em>Agropecten gibbus</em></td>
<td>DNA damage</td>
<td>Quinn et al., 2005</td>
</tr>
<tr>
<td></td>
<td>endocrine disruption</td>
<td>Quinn et al., 2005</td>
</tr>
<tr>
<td></td>
<td>metallothionein</td>
<td>Quinn et al., 2005</td>
</tr>
<tr>
<td><em>Agropecten iradians</em></td>
<td>immune function</td>
<td>Liu et al., 2004</td>
</tr>
<tr>
<td><em>Chlamys farreri</em></td>
<td>DNA damage</td>
<td>Jing-jing et al., 2009; Liu et al., 2009</td>
</tr>
<tr>
<td></td>
<td>immune function</td>
<td>Chen et al., 2007; Liu et al., 2004; Liu et al., 2009</td>
</tr>
<tr>
<td></td>
<td>oxidative stress</td>
<td>Pan et al., 2005; 2006</td>
</tr>
<tr>
<td><em>Chlamys islandica</em></td>
<td>DNA damage</td>
<td>Baussant, 2004; Baussant et al., 2009</td>
</tr>
<tr>
<td></td>
<td>LMS</td>
<td>Baussant et al., 2009; Camus et al., 2002b</td>
</tr>
<tr>
<td></td>
<td>oxidative stress</td>
<td>Baussant et al., 2009; Camus et al., 2002b; Regoli et al., 1998</td>
</tr>
<tr>
<td><em>Euvola ziczac</em></td>
<td>neurotoxicity</td>
<td>Owen et al., 2002</td>
</tr>
<tr>
<td><em>Flexopecten flexosus</em></td>
<td>oxidative stress</td>
<td>Peña-Llopis et al., 2002</td>
</tr>
<tr>
<td><em>Nodipecten nodosus</em></td>
<td>immune function</td>
<td>Schleder et al., 2008</td>
</tr>
<tr>
<td><em>Pecten jacobaeus</em></td>
<td>neurotoxicity</td>
<td>Bonacci et al., 2008</td>
</tr>
<tr>
<td></td>
<td>oxidative stress</td>
<td>Regoli et al., 2000, Viarengo et al., 1995</td>
</tr>
<tr>
<td><em>Pecten maximus</em></td>
<td>LMS</td>
<td>Hauton et al., 2001</td>
</tr>
</tbody>
</table>

Abbreviations: AChE, acetylcholinesterase; AP, alkaline phosphatase; EROD, ethoxyresorufin-O-deethylase; GST, glutathione-s-transferase; GPx, glutathione peroxidase; LMS, lysosomal membrane stability; NRR, neutral red retention; PO, phenoloxidase; SOD, superoxide dismutase; TOSC, total oxyradical scavenging capacity.
1.5 Conclusions and project rationale

Oil is now a ubiquitous contaminant throughout the marine environment. With increasing oil activities in the Sub-Arctic and Arctic regions, discharges of wastes associated with oil extraction and the potential for accidental spills is also elevated. Therefore, research into the potential impacts of oil pollution on Arctic marine organisms is required.

As biomarkers offer several advantages over traditional environmental monitoring methods, there has been a rapid expansion in their application to environmental risk assessment in recent years. Often simple to apply and cost-effective, biomarkers provide biological detail that is lacking from chemical screening alone. Although community analyses provide an indication of the biological impacts of exposure, they are time consuming and costly, and generally indicate that higher levels of biological organisation have already been impacted and disrupted. Using suites of biomarkers offers the benefit of providing an early-warning indicator of such environmental stress, providing sufficient time to take action and mitigate impacts at higher levels of biological organisation.

The potential use of immune endpoints in biomonitoring is a positive line of research, and the immune competence of an organism has the potential to be both a sensitive and ecologically relevant measurement of the impact of oil derived chemicals. There is a large body of evidence of the immunotoxic properties of environmental contaminants and numerous immune biomarkers
have been developed. Despite this, the use of such immune biomarkers in Arctic bivalves is deficient, and the Arctic Scallop *C. islandica* may offer a potential sentinel species for this polar region. In addition, the commercially important temperate scallop, *P. maximus*, presently occurs in regions of offshore oil production, and with increasing sea temperatures, its northern limit may extend further into sub-Arctic regions. The use of scallops as biomarker candidates has grown in the last decade, demonstrating their potential as sensitive monitoring species.

### 1.6 Aims and objectives

This aim of this research programme was to establish if the immune system of the Arctic Scallop *Chlamys islandica* and the temperate scallop *Pecten maximus* was a sensitive endpoint that could be used to detect the effects of oil and oil-related compounds. To achieve this aim, a suite of immune parameters was applied to assess immunocompetence. A schematic outline of the thesis is given in Figure 1.4 and the research hypotheses tested within each chapter are also detailed.

The main objectives of this research were to:

(i) Develop and validate a range of biomarkers that can be applied to both temperate (*P. maximus*) and Arctic (*C. islandica*) scallop species to assess immune function (Chapter 2).
(ii) Provide an understanding of the immunotoxic effects of oil on the immune function of *C. islandica* as a result of:

a) A low level exposure (Chapter 3)

b) A simulated oil spill (Chapter 4)

(iii) Determine the impact of an oil-derived, model PAH on the temperate scallop *P. maximus* (Chapter 5).

(iv) Establish the effect of PAH exposure on host resistance in *P. maximus* through the use of a:

(a) pathogen-associated molecular pattern (Chapter 6)

(b) live bacterial challenge (Chapter 7)

(v) Determine the ecological relevance of alterations in immune response (Chapter 7).

(vi) Investigate the impact of other oil-related wastes on the immune function of *P. maximus* (Chapter 8).
Figure 1.4 Thesis outline and research questions tested in each experimental chapter.
Chapter 2

Materials and methods
2 Materials and methods

2.1 Collection and maintenance of test organisms

2.1.1 *Chlamys islandica*

Arctic Scallops, *C. islandica* (Figure 2.1a), of 70-100 mm shell length, were collected by divers (~30 m depth) from Porsangerfjord, Norway (70°1' N, 25°1' E) and packed into a polystyrene fish box between two layers of damp dense foam, before being transported by air to the exposure facility at Stavanger, Norway (58°57' N, 5°43' E) (Figure 2.2). The total transit time did not exceed 6 h and no mortalities were recorded during transportation.
Upon arrival, organisms were transferred to 600 l fibreglass tanks with a continuous flow of 35 PSU filtered seawater (FSW) at 5 ± 1 °C. Scallops were maintained in these holding tanks and fed daily (Instant Algae® Shellfish Diet) for a minimum of two weeks prior to their transfer into the exposure system.

Figure 2.2 Chlamys islandica collection site at Porsanger Fjord and location of exposure facility at Stavanger (Norway).
2.1.2 *Pecten maximus*

Unless stated otherwise, all *Pecten maximus* (Figure 2.1b), of 90-120 mm shell length, were diver-collected (~20 m depth) from Start Bay, Devon, UK (50°15'N, 3°37'E) (Figure 2.3) and obtained through Britannia Shellfish Ltd. Scallops were transported to the exposure facility at Plymouth in cool boxes of aerated seawater, with a maximum transit time of 1 h. No mortalities were recorded during transportation. Upon arrival, the animals were held in 50 l tanks containing 10 μm carbon filtered aerated seawater (FSW; 15 ± 1 °C, 34 PSU). Water changes were carried out every 2 days and animals were fed three times a week using the algal concentrate *Isochrysis* (Instant Algae®). Scallops were maintained in the holding tanks (~15 animals per tank) for at least two weeks prior to transfer into the exposure system.

![Start Bay](50°15' N, 3°37' E)

Figure 2.3 *Pecten maximus* collection site at Start Bay, Devon (UK).
2.2 Biological analyses

2.2.1 Haemolymph extraction and sample preparation

Haemolymph (~0.8 ml) was extracted from the striated region of the adductor muscle (Figure 2.4) using a 21 gauge needle (external diameter 0.8 mm). The samples were transferred to a siliconised microcentrifuge tube and stored on ice (or at -80 °C where stated) until analysis. For the total glutathione assay requiring haemocyte lysate, haemolymph samples were centrifuged at 200 × g for 5 min (4 °C). The supernatant was removed and the cells re-suspended in physiological saline (0.02 M HEPES, 0.4 M NaCl, 0.1 M MgSO4, 0.01 M KCl, 0.01 M CaCl2; pH 7.4) and the haemocytes then lysed through sonification (30% duty cycle, 3 × 15 s; Ultrasonic Processor W-385, Heat Systems Ultrasonics, USA) in an ice bath and stored at -80 °C until analysis.
2.2.2 Condition index
After haemolymph collection, scallop dimensions were recorded (length, height, width) and soft tissue removed. The tissue wet weight was recorded, before and after drying at 60 °C for 18 h, and the condition index (CI) calculated:

\[
Cl = \frac{\text{tissue dry weight (g)}}{\text{shell length (mm)}} \times 100
\]

2.2.3 Protein concentration
Plasma protein concentration was determined using a modified microplate method of Bradford (1976). Briefly, diluted haemolymph samples (1:3 physiological saline) were transferred in 5 μl aliquots to a microplate. Five μl aliquots of a blank (physiological saline) and 5 μl protein standards (0.2-1.0 mg ml^{-1} bovine serum albumin) were added in triplicate. Two hundred μl of diluted BioRad reagent (1:5 distilled water) was added to each well and the absorbance (595 nm) recorded after 20 min incubation at 20 °C.

2.2.4 Immunopathology
2.2.4.1 Total haemocyte count
Immediately after extraction, haemolymph samples were diluted 1 in 4 with Baker's formol calcium (BFC: 2% sodium chloride, 1% calcium acetate, 4% formaldehyde), to fix cells and prevent cell aggregation. Total haemocyte counts were carried out using an Improved Neubauer haemocytometer under × 40 magnification.
2.2.4.2 Cell membrane stability

The membrane stability of haemocytes was assessed by measuring the retention of Neutral Red (NR) dye (Babich and Borenfreund, 1992). Undiluted haemolymph samples (50 μl) were pipetted in triplicate onto a microplate. After 45 min incubation at 4 °C, non-adhered cells were removed by rinsing with physiological saline and 200 μl aliquots of NR solution (0.004% in physiological saline) were added to each well. After a 3 h incubation at 4 °C, excess NR solution was removed by rinsing with physiological saline, and 200 μl of acidified ethanol (1% acetic acid, 49% ethanol) was added to breakdown cellular membranes and resolubilise the dye. The optical density (OD) of the NR dye was measured spectrophotometrically at 550 nm and expressed as a function of protein content.

Validation of cell membrane stability

The PAH benzo(a)pyrene (BaP), a known mammalian immunosuppressant, is highly toxic and inhibits immune function in many invertebrate species (Gómez-Mendikute et al., 2002; Gopalakrishnan et al., 2009; Matozzo et al., 2009). Therefore, the NR assay was validated using BaP (0-10 μg ml⁻¹) as a model in vitro toxicant for both P. maximus and C. islandica. Exposure concentrations were based on previously published values reported to be sublethal to bivalve haemocytes for in vitro exposures (Gómez-Mendikute et al., 2002). Validation was conducted as shown in Figure 2.5 with an in vitro
exposure of haemolymph to BaP followed by the determination of cell membrane stability.

There was no significant difference in the cell membrane stability between the two scallop species tested \( (F_{1,31} = 1.82, \; P = 0.190) \), however, BaP exposure significantly reduced cell membrane stability in both species at all concentrations tested (Figure 2.6; \( F_{3,31} = 18.35, \; P < 0.001 \)).

Figure 2.5 Experimental procedure for in vitro validation of immune endpoints in *Pecten maximus* and *Chlamys islandica*. 

---

1. Haemolymph extracted from 4 individuals
2. 200 µl pipetted into siliconised Eppendorfs
3. 50 µl of BaP solution added (0.1, 1, 10 µg ml\(^{-1}\))
4. 30 min incubation on ice
5. immune endpoint determined
Figure 2.6 Cell membrane stability in *Pecten maximus* and *Chlamys islandica* exposed to benzo(a)pyrene (BaP) in vitro. Values displayed are means ± 1 SE (n = 4). Significant differences (P < 0.05) from the control for *P. maximus* (△) and *C. islandica* (•) are indicated.

### 2.2.5 Mechanisms of immunity

#### 2.2.5.1 Phagocytosis

Phagocytic activity of haemocytes was assessed by measuring the uptake of NR-stained heat-stabilised zymosan particles from *Saccharomyces cerevisiae* (Pipe et al., 1995). Haemolymph samples were pipetted in triplicate onto a microplate, with a further 50 µl of cell suspension added to three wells to act as negative controls. After 50 min incubation at 4 °C, 100 µl of BFC was added to the negative controls to kill the cells, and the microplate re-incubated at 4 °C for a further 10 min. Non-adhered cells were removed by rinsing with physiological saline (100 µl × 2), before adding 50 µl of dyed zymosan suspension (50 × 10⁷ particles ml⁻¹) to each well. The microplate
was incubated for 30 min at 20 °C, after which 100 µl BFC was added to fix the cells and stop the reaction. Excess zymosan suspension was removed by rinsing with physiological saline (until negative controls were clear) and 100 µl acidified ethanol was added to solubilise the dye before recording the absorbance at 550 nm. Phagocytosis of zymosan particles by haemocytes was determined against a standard curve constructed by using zymosan standards ranging from 1.56 to 50 (× 10⁷) particles ml⁻¹, and expressed as a function of protein content.

**Validation of phagocytosis assay**

For both *P. maximus* and *C. islandica*, the immunosuppressant BaP was used to validate the phagocytosis assay. An in vitro exposure was conducted at sublethal BaP concentrations in the range of 0-10 µg ml⁻¹ (Gómez-Mendikute et al., 2002). After haemolymph extraction, in vitro BaP exposure was carried out (Figure 2.5) and the phagocytic activity determined.

There was no significant difference in phagocytic activity between the two scallop species tested (*F*₁,₃₁ = 1.00, *P* = 0.328), however, 1 µg ml⁻¹ and 10 µg ml⁻¹ BaP significantly reduced phagocytosis in both *P. maximus* and *C. islandica* (Figure 2.7; *F*₃,₃₁ = 9.98, *P* < 0.001).
Figure 2.7 Phagocytic activity in *Pecten maximus* and *Chlamys islandica* haemocytes exposed to benzo(a)pyrene (BaP) in vitro. Values displayed are means ± 1 SE (n = 4). Significant differences (P < 0.05) from the control for *P. maximus* (*) and *C. islandica* (△) are indicated.

### 2.2.5.2 Cytotoxic ability

The ability of haemocytes to lyse target mammalian erythrocytes was used as a measurement of cytotoxicity (Raftos and Hutchinson, 1995; Rickwood and Galloway, 2004). Haemolymph samples were extracted and diluted to a concentration of $2 \times 10^6$ cells ml$^{-1}$ in physiological saline. One ml of sheep erythrocytes (TCS Biosciences Ltd, UK) was centrifuged at $200 \times g$ for 5 min before being resuspended in phosphate buffered saline (PBS). The suspension was centrifuged again and a cell pack volume of 125 µl was resuspended in 1 ml of PBS, this was further diluted with 15 ml of Tris-buffered saline. Triplicate 100 µl samples of diluted haemolymph were pipetted into round-bottomed microplates, along with duplicate controls of 100 µl PBS (for spontaneous release) and 100 µl PBS with 2 µl of 2%
Triton-X100 (for maximum release). One hundred µl of the erythrocyte suspension was added to each well and the plate was incubated at 25 °C for 1 h. After centrifuging the plate at 100 × g for 10 min the supernatant was transferred to a flat-bottomed microplate and the percentage lysis was determined by measuring the haemoglobin released into the supernatant by reading its absorbance at 405 nm. The cytotoxicity was expressed as a percent lysis relative to the maximum release observed using PBS with Triton-X100 and normalised for spontaneous release.

Validation of cytotoxic ability

Cytotoxic ability in *P. maximus* and *C. islandica* was validated using a sublethal in vitro BaP exposure (Figure 2.5). After 30 min incubation with BaP (0-10 µg ml⁻¹), the cytotoxic ability of the haemocytes was determined. There was no significant difference in the cell membrane stability between the two scallop species tested (*F*₁,₃₁ = 0.39, *P* = 0.536). In vitro exposure to 1 µg ml⁻¹ and 10 µg ml⁻¹ BaP significantly reduced the cytotoxic ability in *C. islandica*, whilst this was only inhibited in *P. maximus* from the highest BaP exposure of 10 µg ml⁻¹ (Figure 2.8; *F*₃,₃₁ = 5.76, *P* < 0.005).
Figure 2.8 Cytotoxic ability of haemocytes from *Pecten maximus* and *Chlamys islandica* exposed to benzo(a)pyrene (BaP) in vitro. Values displayed are means ± SE (n = 4). Significant differences (P < 0.05) from the control for *P. maximus* (△) and *C. islandica* (*) are indicated.

### 2.2.5.3 Alkaline phosphatase

Alkaline phosphatase (EC 3.1.3.1) activity was measured in the plasma obtained by centrifuging 150 μl of haemolymph at 200 × g for 5 min (4 °C). The supernatant was transferred onto a microplate in 40 μl aliquots and 180 μl of the liquid substrate p-nitrophenyl phosphate (pH 10) was added to each well. Following 20 min incubation at 20 °C, the absorbance was read at 405 nm and activity expressed as Δ abs × 100 mg⁻¹ protein.
Validation of the alkaline phosphatase assay

The alkaline phosphatase (AP) assay was validated using BaP (0-10 µg ml\(^{-1}\)) as a model toxicant for an in vitro exposure with *P. maximus* and *C. islandica* (Figure 2.5). Following the 30 min in vitro exposure, AP activity was determined in the cell-free haemolymph. There was no difference in the AP activity between the two scallop species tested (*F*\(_{1,31}\) = 0.54, *P* = 0.471); however, in vitro exposure to 10 µg ml\(^{-1}\) BaP significantly reduced AP activity in both *P. maximus* and *C. islandica* (Figure 2.9; *F*\(_{3,31}\) = 4.12, *P* < 0.05).

![Figure 2.9 Alkaline phosphatase (AP) activity in *Pecten maximus* and *Chlamys islandica* exposed to benzo(a)pyrene (BaP) in vitro. Values displayed are means ± 1 SE (n = 4). Significant differences (*P* < 0.05) from the control for *P. maximus* (▲) and *C. islandica* (*) are indicated.](image-url)
2.2.6 Host resistance

2.2.6.1 Bacterial clearance

**Baseline bacteria levels in test organism**

The baseline levels of bacteria present in the test organisms were determined prior to experimental use. Haemolymph was extracted as described in section 2.2.1, and 100 µl was plated out in triplicate onto marine agar (84% FSW, 14% dH₂O, 0.5% peptone, 0.1% yeast extract, 0.3% glycerol, 1.2% agar; pH 7.3). After 48 h incubation at 20 °C, the numbers of colony forming units (CFUs) were counted (Gerhardt et al., 1981). Low baseline levels of bacteria were counted with a mean level of 0.5 x 10³ CFU ml⁻¹ haemolymph (n = 5). This is similar to background levels reported in the oyster Crassostrea gigas, which were in the range of 0.14-0.56 x 10³ CFU ml⁻¹ haemolymph (Olafsen et al., 1993).

**Preparation and growth of Vibrio pectenicida**

A conical flask containing 50 ml of marine salts broth (85% FSW, 14% dH₂O, 0.5% peptone, 0.1% yeast extract, 0.3% glycerol; pH 7.3) was inoculated with freeze dried *Vibrio pectenicida* culture 13510 (NCIMB, Scotland) and incubated overnight at 20 °C. When a culture is grown on after being freeze dried this can result in an extended lag phase in the bacterial growth, therefore, 500 µl of this liquid culture was used to inoculate 50 ml of marine salts broth and grown overnight, and this process was repeated a further 2 times.
In order to determine when the bacteria reach the exponential growth phase, a standard curve for the growth of *V. pectenicida* was constructed. For this, 500 µl of the liquid culture was incubated in 50 ml of marine salts broth at the optimal temperature of 20 °C. This culture was sampled every hour and the absorbance at 600 nm was determined, identifying the initial lag phase in bacterial growth (Figure 2.10a). For sampling time points after the lag phase, 10 µl was pipetted in triplicate on to marine salts agar and the number of CFUs counted after 48 h incubation at 20 °C. It was determined that 1 Abs unit (at 600nm) represents $8 \times 10^8$ CFU ml$^{-1}$ during exponential growth (Figure 2.10b).
Figure 2.10 (a) Lag phase and exponential growth of *Vibrio pectenicida* at 20°C and (b) standard growth curve, where $A_{600} = 1$ is equivalent to $8 \times 10^8$ bacteria ml$^{-1}$.

**Bacterial challenge**

Prior to use, *V. pectenicida* was grown for 5-7 h at 20 °C to ensure it was in the exponential growth phase (Figure 2.10a). A 5 ml sample of the liquid culture was then centrifuged for 10 min (500 x g) and adjusted to $10^8$ bacteria ml$^{-1}$ using molluscan physiological saline (according to $A_{600} = 1$, which is equivalent to $8 \times 10^8$ bacteria ml$^{-1}$). Live scallops were inoculated with *V. pectenicida* through the injection of 100 μl ($10^7$ bacteria) into the adductor muscle using a 21 gauge needle.

**Clearance rate of V. pectenicida**

To determine the appropriate haemolymph sampling time after bacterial injection, a time course for the clearance of *V. pectenicida* from the haemolymph was established. Five animals were challenged with an injection of *V. pectenicida* and returned to their individual tanks. Haemolymph (100 μl) from each organism was sampled at a range of time...
points over the following 48 h, and 10 μl serial dilutions were transferred in triplicate onto marine agar plates. Following 48 h incubation at 20 °C, the number of CFU was then counted (Figure 2.11). There was an initial increase in the haemolymph bacteria content with the highest concentration recorded 1 h after injection \((5.58 \times 10^5 \text{ bacteria ml}^{-1})\). The bacterial content was significantly reduced 24 h post-challenge \((F_{7,39} = 3.51, P < 0.01)\) with the bacterial load reduced by more than 50% (Figure 2.12). As there was no further clearance of bacteria beyond the 24 h sampling point up to the end of the time course, 24 h post-injection was chosen as the haemolymph sampling point when conducting the bacterial clearance assay. This 24 h incubation period was sufficient to allow significant clearance of bacteria (Figure 2.12), but ensured good water quality was maintained in exposure tanks.

![Figure 2.11 Marine agar spread plates of Vibrio pectenicida colony forming units (CFUs) from serial dilutions of triplicate 10 μl Pecten maximus haemolymph samples after a bacterial challenge.](image)

\(x \times 10^{-2} \text{ dilution} \quad x \times 10^{-3} \text{ dilution} \quad x \times 10^{-4} \text{ dilution}\)
2.2.7 Oxidative stress

2.2.7.1 Total glutathione

Determination of total glutathione as an indication of a peroxidative challenge was conducted based on a cyclic reduction assay (Owens and Belcher, 1965). Haemolymph lysate samples (80 µl) were thawed on ice before adding 80 µl DTNB solution (10 mM DTNB, 100 mM KH₂PO₄, 5 mM EDTA). Aliquots of 40 µl DTNB-treated samples were transferred to a microplate and 210 µl of glutathione reductase solution (2.06 U ml⁻¹ glutathione reductase, 100 mM KH₂PO₄, 5 mM EDTA; pH 7.5) was added. After allowing samples to equilibrate for 1 min, 60 µl of 1 mM NADPH was added to start the reaction,
and the change in absorbance measured kinetically at 405 nm for 10 min. Concentrations of total glutathione were determined against a 40 μM GSH standard, and expressed per mg protein.

**Validation of the total glutathione assay**

The measurement of total glutathione was validated using an in vitro exposure with H₂O₂ (Figure 2.13) at concentrations sublethal to bivalve haemocytes in vitro (Cheung et al., 2006). Following a 30 min incubation with 0-100 μM H₂O₂, haemocyte lysate was prepared as described previously (section 2.2.1) and total glutathione was determined. There was a significant difference in total glutathione between the two scallop species tested ($F_{1,31} = 6.08, P < 0.05$), with *C. islandica* generally having a higher total glutathione content than *P. maximus* (Figure 2.14). In addition, in vitro exposure to 10 μM and 100 μM H₂O₂ significantly reduced the glutathione content in *P. maximus* compared to the control, whilst glutathione content in *C. islandica* was only reduced in the highest H₂O₂ exposure group of 100 μM ($F_{3,31} = 10.02, P < 0.001$).
Haemolymph extracted from 4 individuals

150µl pipetted into siliconised Eppendorfs

50 µl of H2O2 solution added (1, 10, 100 µM)

30 min incubation on ice in the dark

measurement of oxidative stress endpoint

Figure 2.13 Experimental procedure for the in vitro validation of oxidative stress in *Pecten maximus* and *Chlamys islandica*.

Figure 2.14 Total glutathione in *Pecten maximus* and *Chlamys islandica* haemocytes exposed to H2O2 in vitro. Values displayed are means ± 1 SE (n = 4). Significant differences (P < 0.05) from the control for *P. maximus* (▲) and *C. islandica* (*) are indicated.
2.2.7.2 Lipid peroxidation

Oxidative damage in the form of haemocyte lipid peroxidation (LPO) was assessed using a microplate method of the thiobarbituric acid reacting substances (TBARS) assay (Camejo et al., 1999). Haemolymph samples were thawed on ice and transferred in 40 μl aliquots onto a microplate containing 10 μl BHT (1 mM 2,6-di-tert-butyl-4-methylphenol in absolute ethanol) to prevent further LPO. One hundred μl of extraction buffer (20 mM Tris-chloride, 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA; pH 7.6) was added to each well, followed by 50 μl TCA solution (50% w/v trichloroacetic acid) and 75 μl TBA solution (1% w/v thiobarbituric acid in 50 mM NaOH). After 60 min incubation at 60 °C, the plate was cooled on ice and the absorbance of the malondialdehyde equivalents (MDA_e) at 530 nm recorded. Results were determined against a standard curve using 1,1,3,3-tetraethoxypropane (0-24 μM), and expressed per mg protein.

Validation of the lipid peroxidation assay

The measurement of LPO in *P. maximus* and *C. islandica* was validated using H_2O_2 (0-100 μM) as a model toxicant (Figure 2.13) and was measured following an in vitro exposure. There was no significant difference in LPO between the two scallop species tested (*F*\_1,31 = 0.01, *P* = 0.904); however, in vitro exposure to 10 and 100 μM H_2O_2 significantly increased the levels of LPO in both *P. maximus* and *C. islandica* (Figure 2.15; *F*\_3,31 = 12.75, *P* < 0.001).
Figure 2.15 Lipid peroxidation (LPO) in *Pecten maximus* and *Chlamys islandica* haemocytes exposed to H₂O₂ in vitro. Values displayed are means ± 1 SE (n = 4). Significant differences (P < 0.05) from the control for *P. maximus* (▲) and *C. islandica* (*) are indicated.

### 2.2.8 Histology

Following haemolymph extraction, the gonad was dissected out of the test organism and fixed in 10 % formalin. The samples were transferred to cassettes and dehydrated as described in Table 2.1 before embedding in paraffin. The tissues were cut into 7 µm sections and stained with hematoxylin and eosin (H & E) using a Leica AutoStainer XL.
Table 2.1 Histological preparation of tissue samples.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Treatment</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydration</td>
<td>70% alcohol</td>
<td>overnight</td>
</tr>
<tr>
<td></td>
<td>90% alcohol</td>
<td>2h</td>
</tr>
<tr>
<td></td>
<td>Industrial methylated spirit</td>
<td>2h</td>
</tr>
<tr>
<td></td>
<td>Absolute alcohol</td>
<td>2h</td>
</tr>
<tr>
<td></td>
<td>Absolute alcohol</td>
<td>overnight</td>
</tr>
<tr>
<td>Clearing</td>
<td>Histoclear</td>
<td>1h</td>
</tr>
<tr>
<td></td>
<td>Histoclear</td>
<td>0.5h</td>
</tr>
<tr>
<td></td>
<td>Histoclear</td>
<td>0.5h</td>
</tr>
<tr>
<td>Infiltration</td>
<td>Paraffin</td>
<td>1h</td>
</tr>
<tr>
<td></td>
<td>Paraffin</td>
<td>0.5h</td>
</tr>
</tbody>
</table>

In cases where the subsequent solution was identical, each transfer was made into a fresh solution.

2.3 Chemical analyses

2.3.1 PAH analysis

Water samples from oil exposure tanks were collected for PAH analysis. Samples were collected in 2 l amber bottles containing hydrochloric acid to maintain the pH < 2 and prepared for analysis within 48 h of collection. The 16 PAHs on the US EPA Priority Pollutant List (Table 2.2) were measured along with the alkyl homologues of naphthalene, chrysene, dibenzothiophene and phenanthrene/anthracene. Eight deuterated PAHs (naphthalene-d8, phenanthrene-d10, dibenzothiophene-d8, fluoranthene-d10, pyrene-d10, chrysene-d12, benzo(a)pyrene-d12 and dibenzo(a,h)anthracene-d14) were
added to the sample as quantitative internal standards (QIS) and mixed on a magnetic stirrer for 15 min. Liquid-liquid extraction was then carried out by stirring the sample and solvent on a magnetic stirrer for 30 min before pouring through a 2 l separating funnel. The water phase was drained back into the sampling flask and extracted a further two times. Combined extracts were dried with anhydrous NaSO₄, concentrated to 0.5 ml using a TurboVap 500 (Zymark Corporation, USA) and transferred to glass vials for analysis. Water chemistry PAH analysis was conducted using Gas Chromatography (HP5890, Hewlett Packard, USA) connected to a Mass Spectrometer (Finnigan SSQ7000, USA) and analysed in selected ion monitoring mode (GC/MS-SIM).

Where the PAH body burden of scallops was determined, whole tissue homogenate of 3 individuals was used. Each individual was opened and drained of seawater prior to the removal of all soft tissue. Whole body tissue was macerated using cyclohexane rinsed scissors and transferred to a glass vial pre-treated at 500 °C with Teflon Lock © and stored at -80 °C. Before analysis, scallop tissue was weighed and three quantitative internal standards were added before saponification with methanolic sodium hydroxide under reflux (2 h). Digest was then filtered and extracted three times with cyclohexane. Combined extracts were purified by normal-phase, solid-phase extraction, concentrated to 0.5 ml and analysed using GC/MS (HP5890 GC, Hewlett Packard USA; Finnigan SSQ7000 MSD, USA).
Table 2.2 Polycyclic aromatic hydrocarbons (PAHs) on the US EPA priority pollutant list. Details of the molecular weight, the method detection limit (MDL) and Toxic Equivalency Factor (TEF) are also given.

<table>
<thead>
<tr>
<th>PAH</th>
<th>Molecular weight (g)</th>
<th>MDL (µg l⁻¹)</th>
<th>TEF (Nisbet and LaGoy, 1992)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>128.2</td>
<td>0.005</td>
<td>0.001</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>152.2</td>
<td>0.005</td>
<td>0.001</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>154.2</td>
<td>0.005</td>
<td>0.001</td>
</tr>
<tr>
<td>Fluorene</td>
<td>166.2</td>
<td>0.005</td>
<td>0.001</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>178.2</td>
<td>0.005</td>
<td>0.001</td>
</tr>
<tr>
<td>Anthracene</td>
<td>178.2</td>
<td>0.005</td>
<td>0.01</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>202.3</td>
<td>0.005</td>
<td>0.001</td>
</tr>
<tr>
<td>Pyrene</td>
<td>202.1</td>
<td>0.005</td>
<td>0.001</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td>228.3</td>
<td>0.005</td>
<td>0.1</td>
</tr>
<tr>
<td>Chrysene</td>
<td>228.3</td>
<td>0.005</td>
<td>0.01</td>
</tr>
<tr>
<td>Benzo(b,j)fluoranthene</td>
<td>252.3</td>
<td>0.005</td>
<td>0.1</td>
</tr>
<tr>
<td>Benzo(k)fluoranthene</td>
<td>252.3</td>
<td>0.005</td>
<td>0.1</td>
</tr>
<tr>
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<td>252.3</td>
<td>0.005</td>
<td>1</td>
</tr>
<tr>
<td>Indeno(1,2,3-cd)pyrene</td>
<td>276.3</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>Benzo(g,h,i)perylene</td>
<td>276.4</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Dibenzo(a,h)anthracene</td>
<td>278.3</td>
<td>0.01</td>
<td>1</td>
</tr>
</tbody>
</table>

2.3.2 Phenanthrene analysis

Water samples were collected in 1 l amber Duran bottles and extracted through liquid-liquid extraction with 5 ml cyclohexane. Each extraction was carried out by mixing the sample and solvent through inversion and passed through a 1 l separating funnel. The water phase was drained back into the sampling flask and extracted a further two times. Combined extracts were dried with anhydrous NaSO₄, concentrated under nitrogen to 0.5 ml using an 1870 Pierce Reacti-VAPTMIII (Rockford, USA) and analysed using GC/MS (HP5890 series II GC, HP5970 MSD Hewlett Packard, USA).
2.3.3 Metal analysis

Preparation of ilmenite drilling mud for metal analysis involved acid digestion of the sample with 20 ml of 7 M HNO$_3$ at 120 °C for 30 min. Scallop tissue samples (gill and gonad) were also analysed for metal content. Following dissection, approximately 1 g of each tissue was placed in a Teflon vessel and digested with 5 ml of 15 M HNO$_3$ and 1 ml of 9 M H$_2$O$_2$ at 120 °C for 30 min. After digestion, indium was added (5 μg l$^{-1}$) to act as an internal standard, and metal content analysed using inductively coupled plasma mass spectrometry (ICP-MS: VG PlasmaQuad 2+, Fisons Elemental, UK).

2.4 Use of a solvent carrier

Exposure of aquatic organisms to hydrophobic contaminants requires the use of a solvent vehicle, and it has been recommended that the maximum solvent dose should not exceed 100 μl l$^{-1}$ (OECD, 2000). Phenanthrene used in the exposure of $P$. maximus was dissolved in DMSO (dimethyl sulphoxide). The final concentration of DMSO did not exceed 0.01 % of the total tank volume in accordance with the OECD guidelines.
2.4.1 Toxicity of solvent carrier

With recent reports that solvents may affect aquatic organisms (Hutchinson et al., 2006), a pilot study was conducted to ascertain the impact of DMSO on the measurement of immune and oxidative stress endpoints in the test organism *P. maximus*. Twenty scallops were subjected to a FSW or DMSO (0.01% total water volume) treatment for 7 d, before haemolymph was extracted and immune response and oxidative stress measures were conducted (Figure 2.16). No obvious effect was noted in either immune function or oxidative stress following exposure to 0.01% DMSO (Figure 2.17).

![Experimental procedure to assess the toxicity of the solvent carrier dimethyl sulphoxide (DMSO) to the test organism *Pecten maximus*.](image)

Figure 2.16 Experimental procedure to assess the toxicity of the solvent carrier dimethyl sulphoxide (DMSO) to the test organism *Pecten maximus*.
Figure 2.17 Biological responses of *Pecten maximus* to a 7 day 0.01 % dimethyl sulphoxide (DMSO) exposure: (a) total haemocyte count (THC), (b) cell membrane stability, (c) phagocytic activity, (d) total glutathione concentration and (e) lipid peroxidation (LPO). Values displayed are means ± 1 SE (n = 10) and one-way ANOVA results are also detailed.
2.5 Statistical analyses

All results in figures throughout the thesis are expressed as mean values ± 1 standard error. Statistical analyses were conducted using Statgraphics 5.1 (StatPoint Technologies Inc, USA). Data sets were checked for homogeneity of variance and univariate analysis was performed using ANOVA (unless otherwise stated). Post-hoc pairwise comparisons were carried out (Fisher’s LSD) to identify where significant differences occurred at or above the 95% confidence level (associated probability < 0.05).
Chapter 3

Immune function in the Arctic Scallop, *Chlamys islandica*, following dispersed oil exposure

Results from this chapter have been presented at the Arctic Frontiers 2008 Scientific Conference, Tromsø, Norway, Jan 2008 (poster presentation and 3 minute platform presentation) and the Marine Institute Conference 2007, Plymouth, UK, Dec 2007 (awarded best platform presentation). The results have also been published in Aquatic toxicology, 92, 187-194 (Hannam et al., 2009).
3 Immune function in the Arctic Scallop, *Chlamys islandica* following dispersed oil exposure

3.1 Introduction

With large quantities of oil entering marine systems from global offshore oil production, oil is a ubiquitous contaminant throughout the marine environment (GESAMP, 2007). Although methods of monitoring and assessing the impacts of oil on the coastal environment have been reported, most work has focused on the impact on temperate species (Laffon et al., 2006; Francioni et al., 2007; Culbertson et al., 2008; Hylland et al., 2008). With the expansion of oil exploration in Arctic and Sub-Arctic regions, notably the Barents Sea and the North Alaskan Slope (AMAP, 1998), there is an urgent need to develop methods to assess the potential impact of oil on Arctic species.

The ecological impact of oil in cold-water environments may vary from those observed in temperate and tropical regions as a result of environmental and biological factors characteristic of the Arctic environment. Cold-water marine invertebrates have reduced respiration rates (Ahn and Shim, 1998), altered cell membrane composition (Gillis and Ballantyne, 1999) and increased antioxidant defences (Regoli et al., 2000) compared to those from temperate regions. Although these adaptations enable them to survive at low temperatures, the same features may influence their susceptibility to oil-induced damage.
Whilst oil can be acutely toxic to marine organisms, disruptions in essential homeostatic mechanisms, such as the immune system, can also occur as a result of sublethal exposures (Pipe et al., 1999). Due to its complexity and integration with other physiological systems, the immune system is extremely vulnerable to xenobiotic stress (Galloway and Goven, 2006). A severely compromised immune system can result in rapid mortality, however, subtle changes in its function can be used as early-warning indicators of environmental stress and, therefore, immune function is important in assessing the sublethal effects of contaminant exposure (Luengen et al., 2004).

Bivalves are good candidates for immunotoxicology studies and have been studied widely (Pipe et al., 1999; Canesi et al., 2003; Wootton et al., 2003b; Cartier et al., 2004; Gagnaire et al., 2004; Auffret, 2005; Ordás et al., 2007). The Arctic Scallop, *Chlamys islandica*, is the northernmost member of family Pectinidae distributed widely throughout the Sub-Arctic transitional zone of the North Atlantic (Thorarinsdóttir, 1993) and has been successfully used in previous biomarker studies focusing on oxidative stress (Camus et al., 2002b). The aim of this study was to test the hypothesis that dispersed oil exposure may alter immune function in the Arctic Scallop *C. islandica* and, if immunomodulation occurs, to determine if it is reversible.
3.2 Materials and methods

3.2.1 Experimental design

In March 2007, Arctic Scallops, *Chlamys islandica*, were collected from Porsanger Norway, and transported by air to the exposure facility at IRIS-Akvamiljø in Stavanger, Norway (Section 2.1.1). In the laboratory, scallops were acclimated to laboratory conditions (Section 2.1.1) for 6 weeks prior to experimentation.

Scallops were exposed to dispersed Ekofisk crude oil in a continuous flow system (4 l min⁻¹, checked daily) to maintain the dispersion of oil in seawater, as described by Sanni et al. (1998). Crude oil stock was placed in the top section of a glass cylinder. Water was pumped into the lower section of the cylinder, pushing the piston upwards and thereby pumping oil into the flow of FSW (Figure 3.1). This dispersed oil was carried through capillary Teflon tubing (0.5 mm) to a header tank (5 mg l⁻¹) and delivered to exposure tanks using peristaltic pumps to achieve the required nominal concentrations.

Scallops were divided between three 600 l fibreglass tanks (~60 per tank) comprising 3 treatments: FSW control, low (0.06 mg l⁻¹) and high (0.25 mg l⁻¹) dispersed oil. Based on the OSPAR discharge limit of 30 mg l⁻¹ oil in produced water (PW) (OGP, 2006), the oil concentrations tested here (0.06 and 0.25 mg l⁻¹) represent 120-500 x dilutions of the maximum permissible level of oil in PW, and are comparable to the observed effect concentrations reported form previous studies using dispersed oil exposures (Baussant, 2004; Larsen, 2004). Scallops were exposed for up to 15 d; this
exposure time was selected based upon earlier studies reporting significant sub-lethal effects in temperate bivalve species (*Mytilus edulis*) following exposure to dispersed oil (Aas et al., 2002) and produced water (Hannam et al., 2009b). Exposures were maintained at 4 ± 0.5 °C and scallops were fed daily using the microalgae concentrate, Shellfish Diet (Instant Algae ®).

![Diagram of continuous flow system (CFS)](image)

**Figure 3.1** Principle of the continuous flow system (CFS) for dispersing oil in seawater.

### 3.2.2 Chemical measurements

Chemical (PAH) analyses were carried out on water samples taken from each exposure tank after 24 h and 7 d from the start of the exposure. In addition, PAH body burdens were determined in scallops from each treatment after 15 d exposure. The 16 PAHs on the US EPA priority pollutant list were measured together with the alkyl homologues of naphthalene, chrysene, dibenzothiophene and phenanthrene/anthracene using GC/MS (Section 2.3.1).
3.2.3 Biological measurements

From each treatment, ten scallops were sampled after 7 and 15 d exposure, and remaining scallops were transferred to clean FSW for 7 d. After this 7-day recovery period, a further 10 scallops were sampled from each treatment group. Haemolymph was extracted from the adductor muscle (Section 2.2.1), and total haemocyte counts (Section 2.2.4.1), protein concentration (Section 2.2.3) and cell membrane stability (Section 2.2.4.2) was determined, alongside phagocytic (Section 2.2.5.1) and alkaline phosphatase activity (Section 2.2.5.3) in order to assess immune function. All assays on haemolymph samples were conducted in triplicate and absorbances determined using a Labsystems Multiskan RC microplate reader (Labsystems, USA).

3.2.4 Statistical analyses

Immune parameters were measured in 10 individuals from each treatment group at each time point, with results expressed as mean values ± 1SE. Univariate analyses were carried out using two-way ANOVA; tests were performed on each immune parameter to determine significant differences due to interactions (treatment × time) or main factors (treatment and time). Where differences occurred at, or above, the 95% confidence level, post-hoc pairwise comparisons were also conducted.
3.3 Results

3.3.1 Chemical measurements

Whilst the nominal concentrations of dispersed oil were 0.06 and 0.25 mg l"^{-1}, the average ΣPAHs measured in the exposure tanks were 4.2 \times 10^{-4} and 2.4 \times 10^{-3} mg l^{-1} respectively, with the high treatment containing approximately 6 times higher ΣPAH levels than the low treatment group. An increase in ΣPAHs was also observed between the 24 h and 7 d sampling periods for both the low and high treatment groups (Table 3.1).

<table>
<thead>
<tr>
<th>PAH</th>
<th>24 h</th>
<th>7 d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>0.019</td>
<td>0.148</td>
</tr>
<tr>
<td>C1-Naphthalene</td>
<td>0.085</td>
<td>0.587</td>
</tr>
<tr>
<td>C2-Naphthalene</td>
<td>0.149</td>
<td>0.845</td>
</tr>
<tr>
<td>C3-Naphthalene</td>
<td>0.072</td>
<td>0.432</td>
</tr>
<tr>
<td>Fluorene</td>
<td>0.000</td>
<td>0.019</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>0.000</td>
<td>0.033</td>
</tr>
<tr>
<td>C1-Phen/Anthr</td>
<td>0.000</td>
<td>0.061</td>
</tr>
<tr>
<td>C2-Phen/Anthr</td>
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<td>0.066</td>
</tr>
<tr>
<td>Dibenzothiophene</td>
<td>0.000</td>
<td>0.005</td>
</tr>
<tr>
<td>C1-Dibenzothiophene</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>C2-Dibenzothiophene</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Σ PAHs</td>
<td>0.330</td>
<td>2.196</td>
</tr>
</tbody>
</table>

Naphthalene (and its alkyl homologues) accounted for a large proportion of the PAH content of the dispersed oil, with concentrations of the dominant PAH, C-2 naphthalene, ranging from 0.149 and 0.845 µg l^{-1} in the low and high treatments after 24 h exposure, up to 0.225 and 0.954 after 7 d (Table 3.1).
3.1). Only two other priority PAHs, fluorene and phenanthrene, were detected in the exposure tanks. However, these were considerably lower, with concentrations recorded at 13% (fluorene) and 22% (phenanthrene) of the naphthalene levels in the high-exposure treatment after 24 h.

PAHs were accumulated in the scallop tissues after 15 d exposure, with C-3 naphthalene recorded at the highest concentration of 1439 and 2422 µg kg⁻¹ in scallops from the low and high treatments respectively (Table 3.2), reflecting the high levels of naphthalene homologues present in the seawater. A total of 9 priority parent PAHs (as listed by the US EPA) were present in the tissues, of which the tricyclic PAH phenanthrene, was most strongly accumulated with a concentration of 56 and 105 µg kg⁻¹ observed in organisms from the low and high treatments (Table 3.2).

3.3.2 Biological measurements
The number of circulating haemocytes in Chlamys islandica was significantly increased following exposure to dispersed oil \( F_{2,81} = 7.11, P < 0.01 \). Total haemocyte counts were significantly higher in organisms from the high-treatment group following 15 d exposure, with THC of \( 16.9 \times 10^6 \text{ ml}^{-1} \) compared to \( 11.1 \times 10^6 \text{ ml}^{-1} \) in the control (Figure 3.2a). These increased cell counts did not return to control levels after the 7-day recovery period; THC were still significantly elevated, with \( 4.3 \times 10^6 \) more cells per ml of haemolymph relative to the control group.
Table 3.2 PAH body burdens (µg kg⁻¹ dry wt) in whole tissues of Chlamys islandica following exposure to low (0.06 mg L⁻¹) and high (0.25 mg L⁻¹) dispersed oil for 15 d. US EPA priority pollutants are indicated by an asterisk (*). Only PAHs present at detectable levels are shown.

<table>
<thead>
<tr>
<th>PAH</th>
<th>concentration (µg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>low treatment</td>
</tr>
<tr>
<td>*Naphthalene</td>
<td>5.16</td>
</tr>
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<td>C1-Naphthalene</td>
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</tr>
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<td>C2-Naphthalene</td>
<td>704.24</td>
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<td>C3-Naphthalene</td>
<td>1439.14</td>
</tr>
<tr>
<td>*Fluorene</td>
<td>8.57</td>
</tr>
<tr>
<td>*Phenanthrene</td>
<td>56.29</td>
</tr>
<tr>
<td>C1-Phenanthrene/Anthracene</td>
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</tr>
<tr>
<td>C2-Phenanthrene/Anthracene</td>
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</tr>
<tr>
<td>Dibenzothiophene</td>
<td>6.86</td>
</tr>
<tr>
<td>C1-Dibenzothiophene</td>
<td>60.07</td>
</tr>
<tr>
<td>C2-Dibenzothiophene</td>
<td>120.42</td>
</tr>
<tr>
<td>*Fluoranthene</td>
<td>5.43</td>
</tr>
<tr>
<td>*Pyrene</td>
<td>10.38</td>
</tr>
<tr>
<td>*Benz(a)anthracene</td>
<td>2.76</td>
</tr>
<tr>
<td>*Chrysene</td>
<td>27.95</td>
</tr>
<tr>
<td>C1-Chrysene</td>
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</tr>
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<td>C2-Chrysene</td>
<td>56.42</td>
</tr>
<tr>
<td>*Benz(o)fluoranthen</td>
<td>4.06</td>
</tr>
<tr>
<td>*Benz(b,j)fluoranthen</td>
<td>4.71</td>
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<tr>
<td>Σ PAHs</td>
<td>3365.28</td>
</tr>
</tbody>
</table>

Exposure of C. islandica to dispersed oil resulted in elevated plasma protein, with the highest mean concentration of 2.39 mg ml⁻¹ observed after 7 d in organisms exposed to 0.06 mg L⁻¹ (Figure 3.2b). Plasma protein levels were significantly elevated ($F_{4.81} = 3.93$, $P < 0.01$) in the haemolymph in exposed organisms relative to the control after both 7 and 15 d (Figure 3.2b). However, following the post-exposure recovery period, there was no significant difference in plasma protein between both the exposed and control groups, with mean concentrations ranging from 1.36-1.46 mg ml⁻¹.
Figure 3.2 Effect of dispersed oil on the biological response (mean ± 1 SE) of *Chlamys islandica* following 7 and 15 d exposure, and after a 7-day post-exposure recovery period: a) total haemocyte count (THC), b) plasma protein concentration, c) cell membrane stability, d) phagocytosis and e) alkaline phosphatase (AP) activity. Symbols indicate significant effects due to treatment (* denotes significant difference from the control, ▲ denotes significant difference from the low treatment), uppercase letters indicate significant effects due to time (A denotes a significant difference from 7 day, B denotes a significant difference from 15 day).
Whilst no significant change in cell membrane stability was observed in the control group throughout the exposure period (Figure 3.2c), cell membrane stability in exposed scallops was affected by both treatment and exposure time, with a significant interaction between these two factors ($F_{4,81} = 9.70$, $P < 0.001$). After 7 d exposure, there was no significant difference in cell membrane stability between oil-treated and control scallops. Membrane stability was compromised following 15 d exposure to 0.06 mg l$^{-1}$ and 0.25 mg l$^{-1}$ dispersed oil, with the OD of retained neutral red dye significantly decreased to 66% and 40% of the control group respectively (Figure 3.2c). This reduction in cell membrane stability was reversed after the post-exposure recovery period, with the OD significantly higher in organisms from the oil-exposed treatments compared to the control group, with a maximum OD of 10.90 mg$^{-1}$ protein.

Phagocytic activity in the haemocytes of C. islandica was significantly altered during exposure to dispersed oil, with an interaction between treatment and exposure time ($F_{4,81} = 4.52$, $P < 0.01$). After 7 d, phagocytic ingestion of zymosan in organisms from the oil-exposed treatments was not significantly different from the control group, with phagocytosis ranging from 24.83 - 28.33 x 10$^8$ particles mg$^{-1}$ protein (Figure 3.2d). However, 15 d exposure to 0.25 mg l$^{-1}$ oil (high treatment group) resulted in a significantly reduced phagocytic activity of 16.2 x 10$^8$ particles mg$^{-1}$ protein, only 48% of the level observed in the control group after 15 d. Following the recovery period, phagocytic ingestion in individuals from the high-treatment group (30.6 x 10$^8$
particles mg\textsuperscript{-1} protein) had returned to control levels (Figure 3.2d), a significant increase on that observed after 15 d exposure.

A maximum AP activity of 5.86 U mg\textsuperscript{-1} protein was recorded in the plasma of oil-exposed scallops after 7 d exposure at 0.06 mg l\textsuperscript{-1} (Figure 3.2e). However, no significant change in AP activity was observed as a result of an interaction between treatment and exposure time ($F_{4,81} = 0.59$, $P = 0.669$), and no significant effect of these main factors was detected; $F_{2,81} = 0.19$, $P = 0.831$ and $F_{2,81} = 1.29$, $P = 0.281$ for treatment and exposure time, respectively.

3.4 Discussion

An effective immune response is essential in maintaining the health of an organism and may, subsequently, affect growth, reproduction and, ultimately, survival (Blaise et al., 2002). The results of this study demonstrated how exposure to sublethal concentrations of dispersed oil alters the cellular immune function in the Arctic Scallop C. islandica. Various contaminants have been reported to exert immunotoxic effects on organisms, including oil (Ordás et al., 2007) and oil-related components such as PAHs (Coles et al., 1994; Wootton et al., 2003a; Frouin et al., 2007). The low levels of PAHs recorded in the seawater of the exposure tanks, relative to the nominal dispersed oil concentration, may reflect the partitioning of these hydrocarbons between the oil droplets and water (Skadsheim, 2004). The adsorption of oil onto both the surfaces of the tank and the organisms
themselves will also reduce the PAH concentrations measured in the seawater. This progressive loading of oil onto these surfaces may also account for the increase in ΣPAHs observed during the exposure period, with the adsorbed oil providing a secondary supply of PAHs to the seawater phase, increasing PAH concentrations over time (Skadsheim, 2004).

With PAHs having high octanol-water partition coefficients (log $K_{ow} >3$), they are readily taken up by organisms (Nielson et al., 1997). Bivalve molluscs have a limited ability to metabolise PAHs and they can therefore accumulate to high concentrations in the tissues (Moore et al., 1989). The uptake of PAHs by $C. islandica$ is likely to be the main cause of the reduction in PAH concentrations measured in the seawater, and is supported by the increased body burdens. Evaluating bioaccumulation is an important part in assessing the risk that contaminants pose to marine organisms. Based on the ratio of PAHs in biota and seawater (averaged from the low and high treatments), PAH bioconcentration factors (BCFs) were determined to provide an indication of the potential for bioaccumulation (Dimitrov et al., 2002; Arnot and Gobas, 2006). The BCFs of individual priority PAHs ranged from 169 for naphthalene up to 5434 for phenanthrene, a known immunotoxicant (Wootton et al., 2003a). Body burden analysis also indicated the accumulation of other priority PAHs in the tissues of $C. islandica$ including fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b,j)fluoranthene and benzo(b,j,k) fluoranthene, despite their concentrations in seawater being below the detection limit. As a result, BCFs were unable to be calculated for these higher molecular weight hydrocarbons. However, with PAH
hydrophobicity demonstrating a linear relationship with log $K_{ow}$ (Baussant, 2004), it can be assumed that these PAHs, some of which are reported immunotoxicants (Grundy et al., 1996a; Frouin et al., 2007; Bado-Nilles et al., 2008), may be strongly accumulated by organisms that lack an efficient detoxification mechanism.

Haemocytes play an important role in the defence mechanisms of the bivalve immune system (Pipe et al., 1995b). Whilst the haemocyte counts in *C. islandica* observed here are much higher than those reported for other bivalves such as mussels and clams, they are in line with numbers reported in other scallop species such as *Chlamys farreri* (Chen et al., 2007; Liu et al., 2009). The number of circulating cells often increases in response to contaminant stress (Coles et al., 1994; 1995) as observed in this study. Whilst increased total haemocyte count following 15 days exposure to 0.25 mg l$^{-1}$ dispersed oil may be a result of cell migration from the tissues, maintenance of elevated cell counts after the recovery period suggests stimulation of cell production (Pipe et al., 1999) which can occur in the haemolymph of bivalves (Mayrand et al., 2005; Matozzo et al., 2008). PAHs can cause cytolysis in lysosome-enriched cells such as haemocytes (McCormick-Ray, 1987) with an elevation in haemocyte number indicating compensation for cell lysis. Cell lysis may also contribute towards the significant increase in plasma protein concentration observed in organisms from the low and high treatments, a possible result of the release of cell contents into the plasma. Alternatively, induction of humoral factors such as lysozyme (Anderson and Beaven, 2001) and pro-phenoloxidase (Xing et al.,
may contribute to the increased protein concentration found in the oil-exposed animals.

Cell membrane stability is extremely important in the maintenance and functioning of cellular processes. PAHs can impair cell membrane function (French-McCay, 2004) with reduced cell membrane stability a common result of contaminant-induced cell membrane disruption (Moore, 1985). Within bivalve haemocytes, lysosomes sequester, accumulate and metabolise a range of xenobiotics (Hagger et al., 2005). However, this detoxification process is not effective if the storage capacity is exceeded and may result in damage to the cell (Moore et al., 1984). Overloading the detoxification process may account for the reduced membrane stability observed in the present study after 15 days exposure to both low and high dispersed oil treatments. A reduction in cell membrane stability is inversely correlated with lysosomal volume (Moore et al., 2006) and has been reported in marine molluscs following exposure to organic pollutants (Da Ros et al., 2007). If lysosomal enlargement occurred as a result of the reduced cell membrane stability and persisted after the 7-day recovery period, this would provide a larger volume for the neutral red dye to be taken up, accounting for the significant increase in neutral red retention of the oil-exposed recovery scallops compared to the control animals. Alternatively an increase in lysosomal turnover in response to the oil exposure may also contribute to the observed increase in neutral red retention after the recovery period.
Phagocytosis, an essential part of the invertebrate immune response, has been shown to be impaired by numerous organic contaminants including polychlorinated biphenyls (Fournier et al., 2002) and PAHs (Sami et al., 1993; Grundy et al., 1996a; Bado-Nilles et al., 2008), and was significantly reduced in *C. islandica* following 15 days exposure to 0.25 mg l⁻¹ dispersed oil. Phagocytic processes are dependent on membrane properties of the haemocytes (Grundy et al., 1996b), therefore, any alterations in the cell membrane may impact phagocytic activity. It has been suggested that disruption of cell membranes is more likely to occur from exposure to lipophilic organic contaminants rather than inorganic metals (Dyrynda et al., 1998), with PAHs (penetrating phospholipid monolayers in model membrane systems) altering membrane fluidity (Nelson et al., 1990). Haemocyte motility, or changes in membrane recognition, can also impair phagocytic ability (McCormick-Ray, 1987), with PAHs altering the expression of membrane receptors (Sami et al., 1993), which may interfere with the ability to recognise non-self material, essential to the phagocytosis process.

The enzyme alkaline phosphatase (AP) participates in the degradation and breakdown of invading non-self material (Liu et al., 2004), and has been recorded in various bivalve species (Xue and Renault, 2000; Xing et al., 2002; Liu et al., 2004; Jing et al., 2006). Previous work has reported increased AP activity in the oyster *Pinctada fucata* in response to copper exposure (Jing et al., 2006), however, dispersed oil exposure did not significantly alter AP activity in *C. islandica*. No effect on AP activity was also observed in the seastar *Coscinasterias muricata* exposed to naphthalene,
benzo(a)pyrene or the WAF of crude oil (Georgiades et al., 2003). AP is a polymorphic enzyme, with variable activity between organisms (Fahselt, 1987; Takeda et al., 1990). Such polymorphism has been reported in the coding loci for other enzymes in *C. islandica* (Fevolden, 1992) and may account for the large variability in AP activity observed in this species during the present study.

Whilst this study demonstrated an alteration in some aspects of immune function caused by exposure to sublethal, low-level concentrations of dispersed oil this modulation appears to be reversible upon removal of the contaminant stress. However, the impact on immune function of acute exposure, associated with accidental oil spills needs to be established and may have consequences for disease resistance and hence survival.
Chapter 4

Immunotoxicity and oxidative stress in the Arctic Scallop *Chlamys islandica*: effects of acute oil exposure

Results from this chapter have been presented at the 19th SETAC Europe Annual Meeting, Göteborg, Sweden, June 2009 (platform presentation). The results have also been accepted for publication in Ecotoxicology and Environmental Safety (Hannam et al., in press).
4  Immunotoxicity and oxidative stress in the Arctic Scallop *Chlamys islandica*: effects of acute oil exposure

4.1 Introduction

Currently, approximately one tenth of the world’s oil is supplied by production in Arctic regions, with cumulative production estimated at 13 400 million m³ (AMAP, 2007). Predicted increases in oil activity in Arctic regions will increase the risks of accidental oil spills, yet the ecological impacts of oil spills in Arctic environments are unclear. However, it is thought that the biological adaptations of cold-water species, may affect the susceptibility of Arctic organisms to oil-induced damage. In addition, biological recovery from such damage is predicted to be much slower than in temperate systems; a result of the low growth rates, higher generation turnover times and increased age at maturation that are characteristic of many Arctic organisms (AMAP, 1998).

Current expansion in oil production in the Arctic, therefore, raises growing concern over the effects of oil exposure on Arctic organisms. A zero discharge policy is in place in some areas of the Arctic such as the Barents Sea (OLF, 2006), however, there remains the possibility of tanker spills, blow outs and pipeline leaks; in addition, transport of the extracted oil extends the risk of accidental release beyond the production area (AMAP, 2007). For marine systems, oil spills pose the largest environmental threat, and when
difficult to contain, can cause impacts over large areas. Even small-scale oil spills can have substantial effects on local pollution levels and marine biota.

Bivalves have been used widely for pollution-effect studies, and the Arctic Scallop *C. islandica* has potential as a sentinel species in the Arctic and Sub-arctic region, where the commonly used *Mytilus edulis* is absent. Previous results indicate that immunotoxicity from low levels of dispersed oil is reversible in *C. islandica*; however, with the elevated risk of accidental spills due to increased oil exploration in Arctic regions, the consequences of an acute oil exposure are yet to be established. This study aims to assess if a simulated weathered oil spill will induce a peroxidative challenge and inhibit immune function in *C. islandica*.

4.2 Materials and methods

4.2.1 Experimental design

Diver-collected Arctic Scallops were collected in March 2008 from Porsanger (Norway) and transported by air to the exposure facility in Stavanger (Norway) as described in Section 2.1.1. Upon arrival, organisms (~180 total) were transferred to 600 l flow through fibreglass tanks and acclimated to laboratory conditions for 6 weeks prior to use in the exposure system (Section 2.1.1).
Scallops were exposed to the water accommodated fraction (WAF) of North Sea crude oil via a bead column (Figure 4.1) to simulate a weathered oil spill (Carls et al., 1999). This column exposure system delivers an initial high concentration of PAHs to the exposure tanks followed by a progressive decline in PAH exposure (Kennedy and Farrell, 2005; Camus and Olsen, 2008; Olsen et al., 2008). The polyvinyl chloride (PVC) column was initially
filled with a 15 cm layer of large glass beads (Ø 15 mm), before adding 20 kg of soda glass beads (Ø 3 mm) coated in crude oil (35 ml oil kg⁻¹ beads). FSW was passed through the column for 24 h to remove the most volatile and soluble fractions that would usually evaporate during the initial hours after a spill. The column was then connected into a continuous flow system (CFS), with a flow rate of 0.5 l min⁻¹ entering the exposure tanks. Scallops were subjected to this treatment over 21 days (8 °C ± 1) and fed daily on the microalgae concentrate Shellfish Diet 1800 (Instant Algae®).

4.2.2 Chemical measurements
PAH analyses were conducted on seawater samples collected from the exposure tank at time zero and after 1, 2, 4, 7, 14, and 21 days exposure. In addition to the 16 priority PAHs, the alkyl homologues of naphthalene, chrysene, dibenzophiothene and phenanthrene/anthracene were also measured using GC/MS as described in Section 2.3.1.

4.2.3 Biological measurements
Eight animals from the exposed and control (FSW) treatments were sampled at time zero and after 1, 2, 4, 7, 14 and 21 days exposure. Haemolymph was extracted from the adductor muscle using a 21 gauge needle and transferred to a siliconised microcentrifuge tube. Samples for determination of total haemocyte counts (Section 2.2.4.1), protein content (Section 2.2.3), cell membrane stability (Section 2.2.4.2) and phagocytosis (Section 2.2.5.1) were stored on ice to minimise cell aggregation. Haemolymph samples for
oxidative stress analysis (Section 0) were stored at -80 °C until required. All measurements conducted on haemolymph samples were carried out in triplicate. All absorbances were determined using a Labsystems Multiskan RC microplate reader (Labsystems, USA). Following haemolymph extraction, the condition index of the 8 individuals was also established (Section 2.2.2).

4.2.4 Statistical analysis

Biological endpoints were measured in 8 individuals from each treatment at each time point with results expressed as mean values ± 1 standard error. Data sets were checked for homogeneity of variance and univariate analysis was performed using two-way ANOVA; tests were performed on each biological endpoint to determine significant differences due to interactions (treatment x time) or main factors (treatment and time). Post-hoc pairwise comparisons were conducted (Fisher's LSD) to identify where significant differences occurred at or above the 95% confidence level.

4.3 Results

4.3.1 Chemical measurements

Chemical analysis indicated that after an initial increase in the total PAH (ΣPAH) exposure concentration in the first 48 h, levels then decreased over the remaining exposure period (Figure 4.2). The maximum ΣPAH concentration was observed 48 h after the start of the exposure at 163.4 µg l⁻¹, this level then decreased to just 8% of this maxima after 21 d, with a recorded ΣPAH concentration of 13.3 µg l⁻¹. Naphthalene, and its alkyl
homologues, dominated the PAHs recorded accounting for ~96% of the ΣPAH concentration (Table 4.1). Only 5 other priority PAHs were recorded in the exposure system over the 21 d, of which, only fluorene and phenanthrene were measured at levels in excess of 1 μg l⁻¹. However, the highest concentration of these two PAHs was observed after 7 d and did not coincide with the maximum ΣPAH which was recorded after 48 h (Table 4.1).

Figure 4.2 Decrease in total PAH concentration in the exposure tanks as a function of time.
**4.3.2 Biological measurements**

Mortalities were recorded during the exposure with the survival reduced by almost 40% after 21 d (Figure 4.3a). The condition index of *C. islandica* was also significantly affected by oil exposure ($F_{6,97} = 2.62$, $P < 0.05$) with a significant reduction in scallop CI observed after 2 days. The lowest CI was recorded after 4 d exposure; 28% less than the value recorded for the control scallops. The significant reduction in CI persisted up to day 14, after which the CI returned to similar values to those observed in the control organisms (Figure 4.3b).

### Table 4.1 PAH water concentrations in the exposure tank at different time points after the start of the exposure. US EPA priority pollutants are indicated by an asterisk (*).

<table>
<thead>
<tr>
<th>PAH</th>
<th>1 h</th>
<th>24 h</th>
<th>48 h</th>
<th>96 h</th>
<th>7 d</th>
<th>14 d</th>
<th>21 d</th>
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</thead>
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</tbody>
</table>
Figure 4.3 (a) Cumulative mortality recorded and (b) condition index of surviving Arctic Scallops (Chlamys islandica) after an acute exposure to the water accommodated fraction of crude oil for up to 21 d. CI data is expressed as mean values (n = 8) ± 1 standard error and significant differences from the control (P < 0.05) are indicated by an asterisk (*).
Acute oil exposure significantly altered the number of circulating haemocytes in *C. islandica* (*F*<sub>6,97</sub> = 3.14, *P* < 0.01). After 24 h the haemocyte count was increased, with the highest THC of 13.78 × 10<sup>6</sup> ml<sup>-1</sup> observed after 2 days exposure (Figure 4.4a). However, on day 4, the cell count declined to a level below that of the control scallops to 9.06 × 10<sup>6</sup> ml<sup>-1</sup>, with 35% fewer cells observed compared to that recorded 2 days previous. As the exposure time progressed to 7 days, the THC returned to levels similar to scallops from the control treatment, and no significant difference in THC between exposed and control organisms was observed for the remainder of the 21 d experimental period (Figure 4.4a). Cell membrane stability was significantly affected by acute oil exposure (Kruskal-Wallis, *H* = 38.84, *P* < 0.001). After 24 h the cell membrane stability in exposed animals was impaired relative to the control, with a significant reduction observed after 2, 4, 7 and 21 days (Figure 4.4b). The greatest decrease in membrane stability was observed after 4 days exposure, and was 52% lower than the control scallops sampled for that respective time point.

Phagocytic activity in *C. islandica* was significantly altered by acute oil exposure (Kruskal-Wallis, *H* = 66.05, *P* < 0.001), with the number of particles phagocytosed increasing from 26.0 × 10<sup>6</sup> at the start of the exposure to 47.5 × 10<sup>8</sup> in oil-treated scallops after 24 h (Figure 4.5a). This stimulation of phagocytosis changed to inhibition after 2 days exposure, with phagocytic ingestion reduced to 10.1 × 10<sup>8</sup> particles mg<sup>-1</sup> protein.
Figure 4.4 Immunopathological effects in *Chlamys islandica* following acute oil exposure: (a) total haemocyte count (THC) and (b) cell membrane stability. Data is expressed as mean values (n = 8) ± 1 standard error and significant differences from the control (P < 0.05) are indicated by an asterisk (*).
Figure 4.5 Immune function modulation in *Chlamys islandica* following acute oil exposure: (a) phagocytic activity and (b) cytotoxic ability. Data is expressed as mean values (n = 8) ±1 standard error and significant differences from the control (P < 0.05) are indicated by an asterisk (*)..

Lipid peroxidation (LPO) increased significantly in scallops exposed to the acute oil exposure and remained elevated up to day 21. Exposed organisms had elevated 14 days at 9.1 nmol mg⁻¹ protein in exposed animals compared to that of the control (P < 0.05).
Impairment of the haemocyte phagocytic capability persisted throughout the remaining exposure period, with oil-exposed scallops exhibiting phagocytosis at 41-57% of the levels observed in the control groups for each time point (Figure 4.5a). The ability of haemocytes to elicit a cytotoxic response to foreign cells was reduced in oil-exposed animals after just 24 h. Cytotoxic capability demonstrated the greatest inhibition after 2 days exposure, with 31.4% cytotoxicity compared to 57.0% in the control group (Figure 4.5b). However, oil-exposed scallops demonstrated increased variation in the cytotoxic response and the differences observed between exposed and control organisms were not found to be statistically significant at any time point ($F_{1,97} = 7.38, P = 0.078$).

A significant depletion in total glutathione was observed in C. islandica subjected to the acute oil exposure ($F_{1,97} = 7.46, P < 0.01$). Total glutathione levels were lowest after day 2 at 6.2 nmol mg$^{-1}$ protein; 40% less than the concentration recorded in scallops from the control group. This reduction in glutathione was also observed up to day 7, after which levels returned to that of the control for the remainder of the exposure period (Figure 4.6a). Lipid peroxidation (LPO) increased after 2 days in scallops subjected to the acute oil exposure and remained elevated up to day 21. Exposed organisms showed elevated LPO concentrations between 23 and 67% above the respective control groups (Figure 4.6b). However, only the highest level of LPO, recorded after 14 days at 8.1 nmol mg$^{-1}$ protein in exposed animals was significantly higher than the control value of 4.8 nmol mg$^{-1}$ protein ($F_{1,97} = 8.48, P < 0.005$).
Figure 4.6 Oxidative stress in *Chlamys islandica* following acute oil exposure: (a) total glutathione concentration and (b) lipid peroxidation (LPO). Data is expressed as mean values (n = 8) ± 1 standard error and significant differences from the control (P < 0.05) are indicated by an asterisk (*).
4.4 Discussion

PAHs are known to be a toxic component of the WAF of crude oil and were present at a concentration of 150 µg l⁻¹ at the beginning of the exposure (24 h) followed by a decline in concentration over the 21 days of the experiment. After an oil spill, PAH concentrations in the water column will vary according to environmental conditions and will also be dependent upon the quantity of oil released (Lee and Anderson, 2005). Total PAH concentrations in the water column have been reported to be in excess of 500 µg l⁻¹ after the Ekofisk blow out (Law, 1978), and Kingston (1999) reported high seawater levels of ~1600 µg l⁻¹ following the Braer oil spill. Other studies have reported much lower concentrations after accidental oil spills, with levels of 6 µg l⁻¹ detected after the Exxon Valdez spill; however, the latter may reflect the rapid reduction in seawater PAHs post-spill, since these measurements were made 14 d after the initial release (Short and Harris, 1996). After the North Cape spill, Reddy and Quinn (1999) reported PAH levels of 115 µg l⁻¹, but predicted the initial concentrations following the spill to be in > 200 µg l⁻¹, indicating the PAH concentrations in the current study to be environmentally realistic.

Whilst PAHs concentrations were measured in the current study, many other compounds are also present in crude oil (Neff et al., 2000); PAHs only account for 1.6% of the composition of North Sea oil (Baussant et al., 2009). Unbranched alkanes (n-alkanes) are a constituent of crude oil, although work by Skadsheim et al. (2000) only detected their presence in crude oil droplets,
not in the WAF, so *n*-alkanes are unlikely to be a source of toxicity in the current study. Alkylphenols are often present in the WAF of crude oil (Tollefsen et al., 2008). Whilst alkyated phenols may have contributed towards the immunotoxic effects reported here, the sublethal toxicity normally associated with alkylphenols concerns estrogenic (Aarab et al., 2004) and genotoxic effects (Baršiene and Andreikenaite, 2007). Often, conventional GC analysis of crude oil indicates the presence of unresolved complex mixtures (UCM), which have also been reported in the WAF (Melbye et al., 2009). Branched alkylaromatic hydrocarbons, such as branched alkylbenzene, are likely to be present in this UCM and can be accumulated by marine bivalves (Booth et al., 2007); however, these branched alkylbenzenes were not found to have any effect on the cell membrane stability of *M. edulis* (Scarlett et al., 2008), so it is concluded that they are unlikely to be a contributing factor towards the immunotoxic effects observed here. Polar compounds are also a major component of oil and are reported to dominate the WAF of Norwegian crude, accounting for 70% of the organic compounds present (Melbye et al., 2009). These polar fractions are largely made up of cyclic and aromatic sulphoxide compounds, and have been shown to be genotoxic to fish (Tollefsen et al., 2008). Since polar compounds dominate WAF of crude oil, it should be noted that in addition to the measured PAHs, polar compounds may also account for a proportion of the toxicity to *C. islandica* observed in the current study.

Acute oil exposure of *Chlamys islandica* resulted in ~40% mortality after 21 d, with similar oil-spill induced mortalities reported for the bivalves
Protothaca staminea (Fukuyama et al., 2000) and Mya arenaria (Gilfillan and Vandermuelen, 1978). Organisms from the present study that survived the initial high dose of PAHs had a reduced condition index, with many individuals exhibiting mantle retraction and narcosis. Such a reduction in tissue condition has been reported previously for bivalves transplanted to an oiled environment (Culbertson et al., 2008) and exposed to oil-based drilling mud (Cranford et al., 1999). In scallops, glycogen is an essential metabolic energy reserve important in maintaining tissue condition (Barber and Blake, 2006). The metabolism of PAHs in the oil-exposed scallops may deplete these glycogen stores, limiting the energy available for tissue growth. A contaminant-induced increase in glycogen utilisation may therefore be a contributing factor in the observed reduction in condition index. Previous studies have also demonstrated a significant correlation between tissue glycogen content and condition index in mussels along a pollution gradient (Smolders et al., 2004), with mussels subjected to heavily contaminated sites having lower glycogen levels and an impaired condition index (Pridmore et al., 1990; Smolders et al., 2004). In addition to the lethal effects, and visible signs of tissue deterioration from the acute oil exposure, exposed C. islandica also showed sublethal cellular effects.

The immune system of bivalve molluscs is largely dependent on the circulating haemocytes that carry out immune surveillance (Pipe et al., 1995). The number of circulating haemocytes can fluctuate under stressed conditions (Livingstone et al., 2000). For example, increased haemocyte numbers have been reported in bivalves following an oil spill (Dyrynda et al.,
1997a) and also as a result of fluoranthene exposure (Coles et al., 1994). Present results, showing elevated haemocyte counts during the initial stages of exposure, provide further evidence for contaminant-induced changes in cell count. In this study it is likely that increased haemocyte numbers is a compensatory mechanism for the compromised cell function demonstrated by the reduced cell membrane stability observed at the beginning of the exposure period. These results are concordant with the results from the previous chapter, where a significant increase in THC corresponded with a reduction in immune function in *C. islandica* after exposure to low concentrations of dispersed oil (Chapter 3). However, in addition to the increase in cell count, acute oil exposure in the current study resulted in a decrease in the number of circulating cells as the exposure period progressed. With PAHs reported to cause cytolysis in lysosome-enriched cells such as haemocytes (McCormick-Ray, 1987), the ability to maintain the elevated haemocyte numbers was reduced with the significant decrease in THC on day 4 the likely result of PAH-induced cell death. Other studies have reported decreased haemocyte counts persisting for up to day 20 in bivalves following PAH exposure (Jeong and Cho, 2005; Pichaud et al., 2008). Therefore, the recovery in cell numbers to those in the control, observed as early as day 7, is likely to reflect the reduction in PAH concentration throughout the exposure and the proliferation of new haemocytes (Pipe et al., 1999). The fluctuation in cell counts observed here suggests that the toxicity of oil to haemocytes, and the subsequent effect on the number of circulating immuno-surveillance cells, is dependent upon exposure period and concentration.
One of earliest detectable cellular changes caused by contaminants is associated with alterations in cell membranes (Moore, 1985). With PAHs able to penetrate model membrane systems (Nelson et al., 1990), the reduced cell membrane stability observed here, may represent the binding of lipophilic PAHs to membrane lipids, altering membrane fluidity and ionic pumps (Camus et al., 2002b). A decrease in cell membrane stability has also been reported in bivalves as a result of PAH (Camus et al., 2002b), produced water (Hannam et al., 2009b) and dispersed oil exposure (Baussant et al., 2009).

Phagocytosis is the most common non-specific immune defence mechanism in invertebrates and can be subject to modulation by a range of xenobiotics (Livingstone et al., 2000). In some instances, low exposure concentrations or short exposure times can result in an immuno-stimulatory effect within bivalves, with acute oil exposure initially inducing a stimulation of the phagocytic activity in *C. islandica* in the present study. A short-term low dose stimulation of phagocytosis has also been reported in bivalves in response to other contaminants including produced water (Hannam et al., 2009b), pesticides (Rickwood and Galloway, 2004), WAF of diesel (Hamoutene et al., 2004) and metals (Pipe et al., 1999; Sauvé et al., 2002). Whilst the increase in phagocytosis observed here in *C. islandica* was not associated with a low-dose exposure, it occurred after just a relatively short exposure on day 1, corresponding with an increase in the number of circulating haemocytes and
prior to the compromised cell membrane stability observed from day 2 onwards. Due to the high energetic costs associated with phagocytic activity (Cheng, 1981), an initial immune stimulation cannot be maintained and often changes towards suppression after longer exposure periods (Cheng and Sullivan, 1984; Pipe et al., 1999) as supported by the current results.

When molluscs are exposed to oil and PAHs their phagocytic activity is reduced (Sami et al., 1992; Dyrynda et al., 1997a; Wootton et al., 2003a; Frouin et al., 2007; Bado-Nilles et al., 2008; Pichaud et al., 2008; Gopalakrishnan et al., 2009; Matozzo et al., 2009). Present results also demonstrated inhibition of phagocytosis after 2 d exposure to the WAF of oil. Thus, it appears that phagocytic reduction is a common response in molluscs to contaminant exposure. The reduced phagocytosis observed here, corresponded with an impaired cell membrane stability, indicating that the phagocytic process is dependent upon the membrane properties of haemocytes; PAHs can interfere with the fluidity of cell membranes (Camus, 2002), restricting the deformation of the membrane essential to the phagocytic endocytosis process (Grundy et al., 1996a). The motility of the phagocytic cells places a large demand on intracellular ATP (Galloway and Depledge, 2001) as does endocytosis and intracellular digestion (Cheng, 1981) making phagocytosis an energy demanding process. If the current acute oil exposure reduced the feeding rate, as reported for marine bivalves exposed to WAF of crude oil (Gilfillan, 1975; Widdows et al., 1982), the energy available for such immune processes would be limited, possibly contributing towards the observed reduction in phagocytosis. Whilst the
inhibition in phagocytosis observed in *C. islandica* following an acute oil exposure is similar to that reported for exposure to low levels of dispersed oil (Chapter 3), in the current study phagocytic activity remained suppressed even at the end of the exposure period despite the reduction in ΣPAH exposure concentration to 13 μg l⁻¹. In contrast, the reduction in phagocytosis as a result of low levels of dispersed oil appeared reversible, with phagocytosis returning to control levels after just a 7 d recovery period (Chapter 3). These results indicate that acute oil exposure may result in prolonged immunotoxic effects that persist beyond the initial exposure period even after high PAH levels have subsided.

Normal cellular metabolism involves the production of potentially harmful reactive oxygen species (ROS), to neutralise these ROS organisms have developed antioxidant defences. Glutathione is a key component in an organism’s antioxidant defence system, with increased total glutathione concentrations reflecting an up regulation of antioxidant defences as a result of the increased ROS production from PAH metabolism (Cheung et al., 2001). However, hepatocytes exposed to naphthalene, the main component of the WAF used here, resulted in a rapid reduction in intracellular glutathione (Buonarati et al., 1989). A reduction in intracellular glutathione concentration may indicate the antioxidant capacity was overwhelmed; mass oxidation of GSH results in excretion of the oxidised molecule (GSSG) from the cell (Regoli et al., 1998). Results from the acute oil exposure in the present study suggest stimulated ROS production through PAH metabolism exceeded the neutralising capabilities of the antioxidant system after day 2.
and reduced the total glutathione concentration. A similar reduction in total glutathione has been reported in organisms exposed to a range of contaminants including organophosphorus compounds (Peña-Llopis et al., 2002), metals (Canesi et al., 1999) and PAHs (Vijayavel et al., 2004).

The targeting of cell membranes by ROS can cause an autocatalytic oxidation process known as lipid peroxidation (LPO) (de Almeida et al., 2007). LPO can perturb membrane structure and function by altering membrane fluidity, compromising membrane integrity, inactivating membrane-bound enzymes and disrupting surface receptor molecules. In addition, the early stages of LPO form lipid hydroperoxides (LOOH) which also participate in redox reactions, and can exacerbate peroxidative cell injury. During LPO, aldehydes such as malonaldehyde are formed as byproducts and these can react with DNA bases forming DNA adducts (Halliwell and Gutteridge, 2007). Whilst the total glutathione concentration recovered to control levels by day 14, a significant increase in LPO was observed at this time point. This delay in the occurrence of LPO following the overwhelmed antioxidant capacity observed on days 2-7, suggests that a lowered total glutathione concentration is a precursor to the oxidative damage of lipid membranes as proposed by Ringwood et al. (1999). The occurrence of LPO in bivalves following PAH exposure has been widely reported (Cheung et al., 2004; Pan et al., 2005; Kaloyianni et al., 2009) and can perturb membrane structure and function, which may contribute toward the reduced cell membrane stability observed during the later stages of the exposure period despite the reduction in PAH concentration.
In cold-water regions, the increased levels of dissolved oxygen may provide an increased source of oxyradicals (Viarengo et al., 1995). In addition, polar organisms often exhibit a higher degree of unsaturated fatty acids in cell membranes in order to maintain fluidity and functioning at cold temperatures (Viarengo et al., 1994), which may make them more susceptible to oxidative stress (Camus et al., 2002b). It has therefore been proposed that polar species have elevated antioxidant capacities to cope with oxidative stress (Regoli et al., 1997). Whilst previous studies have indicated a higher oxyradical scavenging capacity in *C. islandica* compared to temperate species (Regoli et al., 2000), the results here suggest that this species is still susceptible to oxidative damage following an acute oil exposure.

Increased oil production in Arctic regions inevitably means an elevated risk of an accidental oil spill into the environment. Whilst the previous chapter demonstrated the ability of *C. islandica* to recover from the effects of low-level dispersed oil exposure, acute oil exposure causes mortalities in *C. islandica*. In addition, the individuals that survived the initial high dose of oil were then subjected to immunotoxic effects and oxidative damage. Even 21 d after the initial exposure, no recovery of phagocytosis or cell membrane stability was observed despite the reduction in PAH exposure. Whilst it is likely that a recovery in immune function may be observed after the acute effects of an initial spill have subsided, chronic seepage from residual oil in sediments can also mean PAH levels remain elevated in benthic
invertebrates unable to metabolise and excrete these compounds which may have long-term sublethal effects. It should be noted that the mortalities associated with such an acute exposure may simply reflect the removal of weaker organisms from a population leaving only the more robust individuals, and therefore acute effects may be of less importance than chronic impacts.

The previous chapters highlight the immunotoxic effects of low-level and acute oil exposure in the Arctic Scallop *C. islandica* (Chapter 3 & 4). Whilst previous studies have reported similar effects in temperate bivalve species including mussels, clams and oysters (Grundy et al., 1996a; Grundy et al., 1996b; Oliver et al., 2001; Wootton et al., 2003a; Auffret et al., 2004; Frouin et al., 2007; Ordás et al., 2007; Pichaud et al., 2008), the impacts on temperate scallops are unknown. With inputs of PAHs into marine systems increasing, and bioaccumulation of contaminants even greater in Pectinid scallops than commonly used mussel species (*Mytilus* sp.) (Young-Lai and Aiken, 1986), the potential impacts on the commercial scallop species *Pecten maximus* may be significant and will, therefore, be addressed in the next chapter.
Chapter 5

Effects of the model PAH phenanthrene on oxidative stress and immune function in the temperate scallop *Pecten maximus*

The results from this chapter have been presented at the 15th International Symposium on Pollutant Responses in Marine Organisms (PRIMO15), Bordeaux, France, May 2009 (poster presentation). The results have also been published in Chemosphere 78, 779-784 (Hannam et al., 2010).
Effects of the model PAH phenanthrene on oxidative stress and immune function in the temperate scallop *Pecten maximus*

### 5.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous class of organic contaminants found throughout the marine environment, with the majority of inputs arising from anthropogenic sources (Law et al., 1997). Natural constituents of crude oil at around 19 000 mg kg\(^{-1}\), PAHs are both toxic and biologically persistent (Neff, 2002). PAH concentrations in excess of 600 µg l\(^{-1}\) have been reported in seawater following an accidental blow out of an offshore oil platform (Law, 1978), and PAH levels of 1700 µg l\(^{-1}\) can result from oil spills (Boehm and Page, 2007). Due to their hydrophobicity, PAHs are readily accumulated by organisms (Neff and Anderson, 1981), and their toxicity and widespread environmental presence has seen the inclusion of 16 PAHs on the US EPA priority pollutant list. The tricyclic PAH, phenanthrene, is considered the most abundant PAH in the aquatic environment (Yin et al., 2007). With input levels of phenanthrene increasing since the 1990s (Lima et al., 2003), elevated concentrations, up to 1460 µg l\(^{-1}\), have been recorded in seawater samples near areas of crude oil exploration (Anyakora et al., 2005). This low molecular weight PAH is readily bioavailable and highly toxic to marine organisms (Neff and Anderson, 1981). Whilst tissues such as the hepatopancreas, gonad and gill may be targets for PAH toxicity in bivalves, contaminants reach these organs through the haemolymph circulatory system. This acts as a transfer medium for pollutants and their metabolic
products, leaving the haemocytes susceptible to potential deleterious effects (Pan et al., 2006).

Once a PAH has been taken up by an organism it may be subjected to biotransformation reactions. In bivalve molluscs, PAH metabolism occurs largely through radical oxidation involving reactive oxygen species (ROS) (Stegeman and Lech, 1991) which can be generated at various metabolic stages (Livingstone, 1991). ROS are produced continually in living cells and are essential in maintaining cell function in biological systems. However, an imbalance between formation and neutralisation of these reactive species can induce oxidative damage (Valavanidis et al., 2006). Adverse effects of PAHs may also result in sublethal alterations of homeostatic mechanisms such as the immune system (Pipe et al., 1999). The complexity and integration with other physiological systems makes the immune system particularly sensitive to environmental contaminants with increasing evidence of immunotoxic properties in a range of xenobiotics including PAHs and crude oil (Galloway and Goven, 2006).

The Great Scallop *Pecten maximus* is commercially exploited by fisheries and aquaculture, with estimated UK landings of 20 000 t y⁻¹ worth £30 million (Briggs, 2000). Distributed along European Atlantic coasts, the northern limit of *P. maximus* may be pushed further into Arctic regions as a result of increasing sea temperatures, where a rapid increase in oil exploration activity is predicted (AMAP, 2007). Despite the economic importance of this species,
knowledge of the biological effects of contaminant exposure on *P. maximus* is severely lacking. This chapter investigated the sublethal effects of the model PAH phenanthrene on the oxidative status and immune function of *P. maximus* using a suite of biological endpoints.

### 5.2 Materials and methods

#### 5.2.1 Experimental design

*Pecten maximus* were obtained from Start Bay, Devon, UK through Britannia Shellfish Ltd, and individuals were acclimated in 50 l tanks (Section 2.1.2) for two weeks prior to transfer into the exposure system. Scallops were exposed to nominal phenanthrene concentrations of 50, 100 and 200 µg l⁻¹, and a vehicle control in 6 l aerated tanks (Figure 5.1). Phenanthrene was dissolved in dimethyl sulfoxide (DMSO); the final concentration of DMSO did not exceed 0.01% of the total tank volume. Scallops were subjected to each treatment for 7 d, with the seawater renewed and spiked with the PAH stock solution every 24 h to maintain water quality and exposure concentrations.
5.2.2 Chemical measurements

To confirm the PAH exposure concentration, and any decrease in PAH concentration over 24 h, 1 l water samples were collected from the exposure tanks immediately after the initial PAH spike and 24 h post spike prior to seawater renewal. Samples were processed and analysed using GC/MS (HP5890 series II GC, HP5970 MSD Hewlett Packard, USA) as described in Section 2.3.2.

5.2.3 Biological measurements

After 7 days phenanthrene exposure, haemolymph was collected from six scallops at each exposure concentration as described previously (Section 2.2.1) and total haemocyte counts were conducted (Section 2.2.4.1).
Oxidative stress measures of total glutathione (Section 2.2.7.1) and lipid peroxidation (Section 2.2.7.2) were carried out alongside measurements of protein concentration (Section 2.2.3), cell membrane stability (Section 2.2.4.2) and phagocytosis (Section 2.2.5.1) with all absorbance measurements conducted using an Optimax Tuneable microplate reader (Molecular Devices, USA).

5.2.4 Statistical analysis
All biological measurements were conducted in 6 individuals from each treatment with results expressed as mean values ± 1 standard error. Data sets were checked for homogeneity of variance and univariate analysis was performed using one-way ANOVA. Post-hoc pairwise comparisons were conducted (Fisher’s LSD) to identify where significant differences occurred at or above the 95 % confidence level.

5.3 Results

5.3.1 Chemical measurements
Phenanthrene concentrations recorded in the exposure tanks after the initial PAH spike were 45.8, 83.2 and 163.2 µg l⁻¹ for the 50, 100 and 200 µg l⁻¹ treatments, respectively (Table 5.1). These measured phenanthrene concentrations represented between 82% and 92% of the nominal exposure concentrations, with a reduction in this percentage with increasing nominal concentrations. Phenanthrene concentrations in the exposure tanks
decreased after 24 h, with a 73-75% reduction in the initial phenanthrene concentration across all treatment groups (Table 5.1).

Table 5.1 Mean (n = 6) water concentrations of phenanthrene (± 1 SE) in the three exposure treatments following the initial PAH spike (0 h) and after 24 h.

<table>
<thead>
<tr>
<th>Phenanthrene concentration (µg l⁻¹)</th>
<th>nominal 50.0</th>
<th>100.0</th>
<th>200.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>45.8 (1.52)</td>
<td>83.2 (7.35)</td>
<td>163.9 (9.33)</td>
</tr>
<tr>
<td>24 h</td>
<td>12.0 (1.65)</td>
<td>20.9 (2.45)</td>
<td>41.6 (4.06)</td>
</tr>
</tbody>
</table>

5.3.2 Biological measurements

Total glutathione concentration ranged from 13.9 nmol mg⁻¹ protein in scallops from the control group to 8.6 nmol mg⁻¹ protein in 200 µg l⁻¹ PAH-exposed scallops (Figure 5.2a). This reduced glutathione level in exposed scallops was significantly lower than the levels in the control, 50 µg l⁻¹ and 100 µg l⁻¹ treatments (F₃,₂₃ = 4.40, P < 0.05) by 39%, 28% and 31% respectively (Figure 5.2a). Lipid peroxidation (LPO) significantly increased with exposure to phenanthrene (F₃,₂₃ = 3.71, P < 0.05) with maximum LPO, 6.6 nmol mg⁻¹ protein, observed in the 200 µg l⁻¹ phenanthrene treatment (Figure 5.2b). Despite these endpoints indicating a peroxidative challenge in P. maximus following 200 µg l⁻¹ phenanthrene exposure, neither total glutathione nor LPO was significantly altered following exposure to the lower PAH concentrations of 50 and 100 µg l⁻¹ (Figure 5.2a and b).
The total number of circulating haemocytes (THC) in *P. maximus* increased following in vivo exposure to phenanthrene (Figure 5.2c). Whilst the small increase of $0.4 \times 10^6$ cells ml$^{-1}$ observed in the 50 µg l$^{-1}$ treatment was not significantly different from the control, exposure to 100 and 200 µg l$^{-1}$ phenanthrene significantly elevated THCs ($F_{3,23} = 3.73, P < 0.05$). The lowest THC, $10.8 \times 10^6$ cells ml$^{-1}$, was recorded in the control group, whilst exposure to 200 µg l$^{-1}$ increased the number of haemocytes by 32% with a THC of $14.2 \times 10^6$ cells ml$^{-1}$ (Figure 5.2c). Increasing phenanthrene concentrations also produced a trend of increasing plasma protein, with levels ranging from 1.04 mg ml$^{-1}$ in the control group to 1.71 mg ml$^{-1}$ in organisms exposed to 200 µg l$^{-1}$ (Figure 5.2d). However, there was a large degree of variability in the data, and the measured increase in protein concentrations was not statistically significant ($F_{3,23} = 1.74, P = 0.191$).

A trend of declining cell membrane stability was observed with increasing phenanthrene concentration (Figure 5.2e), with exposure to 50, 100 and 200 µg l$^{-1}$ reducing membrane stability by 11, 29 and 46%, respectively, relative to the control group. However, only exposure to the highest phenanthrene concentration tested, 200 µg l$^{-1}$, resulted in a significant reduction in cell membrane stability after 7 days ($F_{3,23} = 4.24, P < 0.05$) with an OD of 3.36 mg$^{-1}$ protein, compared to the control (6.27 mg$^{-1}$ protein) and 50 µg l$^{-1}$ (5.59 mg$^{-1}$ protein) treatments.
Figure 6.2 Biological response of *Pecten maximus* following 7 d phenanthrene exposure: (a) total glutathione concentration, (b) lipid peroxidation (LPO), (c) total haemocyte counts (THC), (d) plasma protein concentration, (e) cell membrane stability and (f) phagocytic activity. Values are displayed as means ± 1 SE (n = 6). Significant differences identified by Fisher's LSD post hoc comparisons are indicated by different upper case letters.
The phagocytic activity of *P. maximus* haemocytes was significantly reduced following exposure to 200 µg l⁻¹ phenanthrene (*F*₃,₂₃ = 3.20, *P* < 0.05), with the uptake of zymosan at 13.7 × 10⁸ particles mg⁻¹ protein, just 50% of the levels observed in the control group (Figure 5.2f). Despite this reduction observed with the high PAH concentration, phagocytosis was not significantly affected at the lower exposure concentrations of 50 and 100 µg l⁻¹ after 7 days (Figure 5.2f).

### 5.4 Discussion

The reduction in phenanthrene concentrations over 24 h observed in the exposure tanks in the present study is likely to reflect the bioavailability and uptake of this compound. With a log *K*ₐ = 4.57 (Skadsheim et al., 2009), phenanthrene is readily taken up by organisms (Neff and Anderson, 1981). To maintain phenanthrene exposure levels, despite the large uptake of this PAH by *P. maximus*, the water was renewed and spiked at regular intervals (every 24 h). The limited ability of bivalve molluscs to metabolise PAHs often results in accumulation in the tissues. Whilst body burdens were not determined in this study, evidence from previous work confirmed the accumulation of phenanthrene in the bivalves *Mytilus edulis* (Law et al., 1999; Moore et al., 2007), *Mytilus galloprovincialis* (Valavanidis et al., 2008) and *Crassostrea virginica* (Elder and Dresler, 1988).

The xenobiotic-induced proliferation of ROS through PAH metabolism, and subsequent peroxidative challenge and LPO, has been suggested as a
mechanism of contaminant toxicity in exposed organisms. Whilst there is no universal marker for oxidative stress, alterations in the antioxidant defence systems may be the best indicator of oxidative stress in vivo (Halliwell and Gutteridge, 2007). Glutathione is regarded as an essential antioxidant defence mechanism of cells, acting as an oxynradical scavenger, removing hydrogen peroxide (\(\text{H}_2\text{O}_2\)) by coupling the reduction to \(\text{H}_2\text{O}\) with the oxidation of glutathione (Halliwell and Gutteridge, 2007). An increase in glutathione may, therefore, indicate up regulation of antioxidant defences, and this has been correlated with increased PAH tissue burdens in the bivalve *Perna viridis* (Cheung et al., 2001). Conversely, the results from this study indicate a reduction in glutathione following phenanthrene exposure indicating enhanced formation of ROS overwhelming the antioxidant defence system. Phenanthrene exposure induces a similar decrease in glutathione in the fish *Carassius auratus* (Yin et al., 2007), and a reduction in glutathione has also been reported in the Antarctic scallop, *Adamussium colbecki*, following metal exposure (Regoli et al., 1998).

A decrease in glutathione may also represent indirect effects of PAH-induced oxidative stress at the cellular level; energetic costs of antioxidant biosynthesis to neutralise increased ROS may result in a decrease in the pool of reducing NADPH molecules available (Winston and Di Giulio, 1991). Glutathione also plays an important role in detoxification reactions as a key conjugate of electrophilic intermediates, catalysed by the enzyme glutathione-S-transferase (Van der Oost et al., 2003). Metabolised PAH intermediates, in the form of quinones, have a strong affinity for cellular thiol...
such as glutathione (Xue and Warshawsky, 2005) and previous studies have suggested that phenanthrene metabolites can form glutathione conjugates (Yin et al., 2007). Therefore, the reactivity of phenanthrene intermediates to conjugate with the glutathione molecule may also contribute to reduced levels of total glutathione observed in this present study.

Depletion of glutathione is seen as a sign of oxidative stress and the results from Chapter 4 suggested this was a predisposing factor in adverse effects of oxidative damage. An increase in oxidative damage (LPO) was observed in this current study following exposure to the highest phenanthrene concentration tested (200 µg l⁻¹). The elevated LPO corresponded with a reduction in intracellular glutathione, providing further evidence that depletion of glutathione is a predisposing factor in adverse effects of oxidative damage, as proposed by Ringwood et al. (1999). Previous work has also demonstrated an increase in LPO as a result of PAH exposure in sea bream Sparus aurata (Kopecka-Pilarczyk and Correia, 2009) and sea bass Dicentrarchus labrax (Ahmad et al., 2008) as well as in the bivalves M. galloprovincialis and Mya arenaria (Frouin et al., 2007; Kaloyianni et al., 2009).

Whilst no significant increase in LPO was observed following exposure to 50-100 µg l⁻¹ phenanthrene, this is not evidence against the occurrence of oxidative stress. The TBARS assay applied here measures the terminal products in the peroxidative breakdown of lipids, and organisms exposed to
lower PAH concentrations may be able to mount efficient antioxidant defences, so that the level of peroxidative damage does not reach these terminal products. However, it is the early stages of oxidative damage, including the formation of conjugated dienes and LOOH (Pannunzio and Storey, 1998), that can be responsible for the disruption of cellular metabolism and cause cell death (Girotti, 1998).

The innate immune defences of invertebrates, such as phagocytosis, rely on the membrane function of haemocyes which illicit the cellular immune response (Pipe et al., 1995). As such, any changes in the properties of the cell membrane due to LPO, may also alter these immune parameters. In bivalve molluscs, phagocytosis consists of 5 stages including recognition, chemotaxis, attachment, ingestion and destruction (Pipe and Coles, 1995). The phagocytic activity relies on effective receptor binding, and is susceptible to interference by xenobiotics (Thiagarajan et al., 2006). A change in membrane recognition may result in an impaired phagocytic response, with previous work demonstrating that PAHs alter the expression of membrane receptors in the oyster Crassostrea virginica (Sami et al., 1993).

An increase in the number of circulating haemocytes was reported in P. maximus following phenanthrene exposure in excess of 100 µg l⁻¹, a trend which appears to be a common response to xenobiotic stress (Chapters 3 & 4). Despite the increase in cell count, low phagocytic activity was observed.
after phenanthrene exposure, suggesting cell proliferation, with immature haemocytes having a reduced phagocytic capability.

The results from this study demonstrate the disruption of the oxidative status resulting in cellular lipid peroxidation, and reduced immune function in the Great Scallop *P. maximus* exposed to the model PAH, phenanthrene. Compared to other PAHs, phenanthrene is considered relatively less toxic, with a toxic equivalency factor of 0.001 relative to benzo(a)pyrene (Nisbet and LaGoy, 1992). However, the ubiquitous distribution of phenanthrene in the aquatic environment, and its tendency for accumulation within organisms, suggests the potential for deleterious effects.

Since a bivalve’s ability to mount an efficient immune response is reliant upon the integrity and efficient functioning of haemocytes, PAH-induced stimulation of ROS production may be a contributing factor in the reduction in an organism’s immunocompetence. Whilst the results from this chapter demonstrated a clear reduction in the basal immune function of *P. maximus* following phenanthrene exposure, it is also important to determine if PAH exposure will affect the organism’s ability to recognise and respond to invading bacteria and this will be the topic of the next chapter.
Chapter 6

Functional immune response in *Pecten maximus*: combined effects of a pathogen-associated molecular pattern and PAH exposure

The results from this chapter have been presented at the Plymouth Marine Sciences Partnership Symposium 2009, Plymouth, UK, April 2009 (poster presentation). These results have also been published as a short communication in Fish and Shellfish Immunology, 28, 249-252 (Hannam et al., 2010).
Functional immune response in *Pecten maximus*: combined effects of a pathogen-associated molecular pattern and PAH exposure

6.1 Introduction

The causative agents of disease in bivalve molluscs can include protozoans, fungi or bacteria (Paillard et al., 2004). These microorganisms have molecular structures that are not shared by host organisms, allowing the host's immune system to recognise invading microorganisms. These molecular structures are known as pathogen-associated molecular patterns (PAMPs) and play an important role in initialising an immune response (Kline, 2009). One such group of PAMPs are the lipopolysaccharides (LPS) that constitute an essential part of the outer membrane of Gram-negative bacteria and stimulate the innate immune system of invertebrates (Hernroth, 2003). Whilst previous studies have investigated the immune response in organisms challenged with various PAMPs (Hernroth, 2003; Aladaileh et al., 2007; Costa et al., 2008; Costa et al., 2009; Pei-Feng et al., 2009), the effect of contaminant exposure on an organism's ability to recognise and respond to a PAMP is unknown.

Previous studies have indicated the incidence of bivalve disease and associated abnormalities may be linked to environmental pollution (Sindermann, 1979). Enhanced disease expression of *Perkinsus marinus* has been observed in the oyster *Crassostrea virginica* exposed to PAH-contaminated sediments (Chu et al., 2002) and Hillman (1993) reported
a link between PAH field contamination and the occurrence of neoplasms in *Mytilus edulis*. Therefore, the ability of an organism to recognise and respond to the bacterial PAMP may be inhibited by exposure to environmental pollutants, resulting in an increased susceptibility to disease.

The Great Scallop *Pecten maximus* is a commercially important bivalve for both fisheries and aquaculture. Results from the previous chapter (Chapter 5) have indicated the susceptibility of its baseline immune parameters, including total haemocyte count, cell membrane stability and phagocytosis, to a contaminant stressor. However, it is unclear if such effects will impair the ability to respond to a PAMP challenge. Here, the functional immune response of *P. maximus* after stimulation with PAMP is determined. In addition the effect of phenanthrene exposure on the ability of *P. maximus* to recognise and respond to a PAMP is also investigated.

### 6.2 Materials and methods

#### 6.2.1 Experimental design

Diver-collected scallops, *Pecten maximus*, from Start Bay, Devon, UK, were exposed to 200 μg l\(^{-1}\) phenanthrene and a filtered seawater (FSW) control (15 ± 1 °C, 34 PSU) in 6 l static tanks (24 tanks in total, 1 animal per tank) for a total of 9 days. The seawater was renewed and spiked with the phenanthrene stock solution every 24 h to maintain water quality and exposure concentration. After 7 days, six scallops from each treatment were
subjected to an immune challenge through the injection of 100 μl LPS from *Escherichia coli* 055:B5 (100 μg ml⁻¹ in physiological saline [0.02 M HEPES, 0.4 M NaCl, 0.1 M MgSO₄, 0.01 M KCl, 0.01 M CaCl₂, pH 7.4]) into the adductor muscle. A further six animals from each treatment were injected with 100 μl physiological saline (phys. sal.) to act as controls (Figure 6.1a). Scallops were returned to their individual exposure tanks (Figure 6.1b) for a further 48 h before biological assays were conducted.

### 6.2.2 Chemical measurements

Water was sampled from the exposure tanks after the PAH spike on day 1 and day 7 of the exposure to determine any changes in phenanthrene concentration over time. In addition, water was sampled 24 h after the initial PAH spike to determine any decrease in PAH concentration between the water renewal and PAH spike. All samples were collected using 1 L amber Duran bottles and analysed for phenanthrene concentrations using GC/MS as described in Section 2.3.2.

### 6.2.3 Biological measurements

Haemolymph (~0.8 ml) was extracted from the striated region of the adductor muscle using a 21-gauge needle and stored in siliconised microcentrifuge tubes until analysis. A tiered approach to assess immune function was adopted. The first stage was based on the immune apparatus responsible for the immune defence, in this case the haemocytes. Therefore, total haemocyte counts (Section 2.2.4.1) and cell membrane stability (Section
2.2.4.2) were measured in addition to protein concentration (Section 2.2.3). The second tier was centred on mechanisms of immunity, with the determination of phagocytic activity (Section 2.2.5.1) and the ability of the haemocytes to lyse foreign cells (cytotoxic capability), as described in Section 2.2.5.2. All absorbance measurements were conducted using an Optimax Tuneable microplate reader (Molecular Devices, USA).

6.2.4 Statistical analysis

Immune parameters were measured in six individuals from each of the four final treatment groups (FSW + phys. sal., FSW + LPS, PAH + phys. sal., PAH + LPS) with results expressed as mean values ± 1 standard error. Data sets were checked for homogeneity of variance and univariate analyses were performed using two-way ANOVA; tests were performed on each immune parameter to determine significant differences due to interactions (exposure treatment x injection challenge) or main factors (exposure treatment and injection challenge). Where differences occurred at, or above, the 95 % confidence level, Fisher's LSD post hoc pairwise comparisons were conducted.
Figure 6.1 (a) Experimental design and (b) exposure of *Pecten maximus* used in the investigation of the stress on stress effect of LPS challenge and PAH exposure.
6.3 Results

6.3.1 Chemical measurements

Phenanthrene concentrations recorded in the exposure tanks after the initial PAH spike on day 1 were measured at 171 ± 15.6 µg l\(^{-1}\) (Table 6.1); concentrations decreased over 24 h by 75 ± 3.7%. No significant difference in the exposure concentration was observed over the exposure period (t-test: \(t = -0.92, \ P = 0.365\)), with 180 ± 18.5 µg l\(^{-1}\) recorded on day 7.

Table 6.1 Mean (n = 12) phenanthrene concentrations (± 1 SE) in the exposure tanks on day 1 following the initial PAH spike (0 h), after 24 h (prior to water renewal) and after the PAH spike on day 7.

<table>
<thead>
<tr>
<th>phenanthrene concentration (µg l(^{-1}))</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>nominal</td>
<td>200</td>
</tr>
<tr>
<td>0 h</td>
<td>171 (5.64)</td>
</tr>
<tr>
<td>24 h</td>
<td>42  (2.68)</td>
</tr>
<tr>
<td>day 1</td>
<td>171 (5.64)</td>
</tr>
<tr>
<td>day 7</td>
<td>180 (5.33)</td>
</tr>
</tbody>
</table>

6.3.2 Biological measurements

The LPS challenge significantly increased the response of some biological parameters in *P. maximus*, with a 25% increase in total haemocyte count (THC) and a 41% increase in phagocytic activity (Figure 6.2 a and d). However, no significant change in plasma protein concentration was observed (Figure 6.2b). The change in haemocyte counts following LPS stimulation was also significantly impacted by phenanthrene exposure (interaction effect: \(F_{1,20} = 6.00, \ P < 0.05\)), with PAH-exposed scallops unable to further increase the number of circulating cells after the PAMP challenge (Figure 6.2a).
Figure 6.2 (a) Total haemocyte counts (THC), (b) plasma protein, (c) cell membrane stability, (d) phagocytic activity and (e) cytotoxic ability in Pecten maximus following 7 d phenanthrene exposure and subsequent LPS challenge. Values are displayed as means ± 1 SE (n = 6). Significant differences are denoted by different lowercase letters.
Despite the immunosuppressive effect of phenanthrene, this PAH did not significantly affect cell membrane stability or the ability of scallops to illicit a phagocytic or cytotoxic response to the LPS challenge (interaction effect: $F_{1,20} = 0.01$, $P = 0.955$, $F_{1,20} = 0.19$, $P = 0.666$ and $F_{1,20} = 0.01$, $P = 0.926$ respectively). Whilst the LPS PAH-exposed scallops had reduced cell membrane stability, phagocytic and cytotoxic activity compared to LPS unexposed animals (Figure 6.2c-e), this is a result of the PAH exposure, not a decreased ability to respond to the PAMP, since a similar decrease was also observed in scallops not subjected to the LPS challenge.

6.4 Discussion

It is well documented that the limited ability of bivalve molluscs to metabolise PAHs often results in accumulation in the tissues (Meador, 2003). Although body burdens were not determined in this study, phenanthrene is known to be readily taken up by organisms (Neff and Anderson, 1981). Based on similar reductions in phenanthrene concentration in all the exposure tanks over 24 h, it is assumed that uptake of phenanthrene occurred. This is in agreement with evidence from previous work confirming the accumulation of phenanthrene in bivalves (Elder and Dresler, 1988; Law et al., 1999; Moore et al., 2007; Valavanidis et al., 2008).

*P. maximus* subjected to the LPS challenge had elevated THC. Whilst crustaceans show a decrease in cell counts in response to LPS (Lorenzon et
al., 1999), LPS was not found to have any toxic effect on the haemocytes of the mussel *Mytilus edulis* (Hernroth, 2003). In addition, the initial decrease in cell counts of crustaceans, changed to elevated THC 48 h post challenge (Lorenzon et al., 1999) in accordance with what was observed after 48 h in the present study. This increase in cell counts is likely to reflect the initiation of an immune response to the invading non-self material through migration of haemocytes from the tissues and cell proliferation. This is further evidenced in the current study by the parallel increase in phagocytosis following the LPS challenge, and has also been reported in the rock oyster *Saccostrea glomerata* in response to PAMPs (Aladaileh et al., 2007).

Many previous studies have demonstrated the immunotoxic effects of PAH to various bivalves (Coles et al., 1994; Wootton et al., 2003a; Jeong and Cho, 2005; Frouin et al., 2007; Bado-Nilles et al., 2008; Pichaud et al., 2008) and the findings are in agreement with results from the present study with phenanthrene exposure significantly reducing cell membrane stability and phagocytic and cytotoxic activity. This inhibitory effect could be a result of direct effects on the immune capacity of the haemocytes. Pollutants can disrupt cell metabolism and ATP production (Buttgereit et al., 2000), reducing haemocyte motility and, therefore, phagocytic activity. In addition, phagocytic processes are dependent on the membrane properties of the haemocytes (Grundy et al., 1996a) and the cytotoxic capability relies on direct cell-to-cell contact between haemocytes and the target cells (Raftos and Hutchinson, 1995). With PAHs reportedly inducing peroxidative injury to cellular
membranes of *P. maximus* (Chapter 5), changes observed in the cell membrane stability are also likely to alter these immune parameters.

The recognition of PAMPs relies on pattern recognition receptors (PRRs) such as the toll-like receptors reported in the scallops *Chlamys farreri* (Qiu et al., 2007) and *Agropecten irradians* (Song et al., 2006). PRRs occur on the surface of haemocytes and therefore alterations in cell membrane stability resulting from phenanthrene exposure may be expected to interfere with PRRs. However, the results from the present study indicate the ability of *P. maximus* to recognise PAMPs (such as LPS) is unaffected by phenanthrene exposure.

The results from this chapter demonstrated the ability of *P. maximus* to recognise the PAMP, LPS, associated with Gram-negative bacteria. The scallops were able to effectively respond to this LPS challenge through the activation of the innate immune system. Whilst the ability to recognise and respond to a PAMP was not compromised by PAH exposure, the overall level of phagocytic and cytotoxic activity was lowered in phenanthrene-exposed scallops due to the immunosuppressive effects of this PAH. Such an effect of contaminant exposure may consequently increase the susceptibility of this commercial species to bacterial infection and disease incidence.
Chapter 7

Immune modulation and ecological relevance: linking immunotoxic responses at different levels of ecophysiological significance

An abstract based on the results from this chapter has been accepted for a platform presentation at the 6th International Conference on Marine Pollution and Ecotoxicology, Hong Kong, June 2010.
7 Immune modulation and ecological relevance: linking immunotoxic responses at different levels of ecophysiological significance

7.1 Introduction

The invertebrate immune system consists of various parameters that together make up an integrated host defence system (Livingstone et al., 2000). In bivalves, haemocytes are largely responsible for immune function; therefore, alterations in cell numbers, viability and membrane stability may modulate immune function. In addition, cell-mediated mechanisms such as phagocytosis and cytotoxicity, and humoral factors are important aspects of bivalve immunity. With so many parameters contributing to an organism’s immune system, alterations in some parameters may have more of a detrimental effect on the animal than others and therefore be of more ecological relevance.

Due to the multifaceted immune system, the use of a tiered system to assess the integrated functioning of the invertebrate immunity has been adopted in the previous chapters of this thesis, similar to those developed and used for assessing vertebrate immunotoxicology (Luster et al., 1988). Using a tiered assay approach can give a good overview of the functioning of an organism’s immune system. The results from previous chapters have demonstrated the use of immunopathological and immune function assays in C. islandica and P. maximus, and their sensitivity to xenobiotics, including crude oil and the
PAH phenanthrene. Whilst measuring these immune parameters provides useful evidence of cellular alterations in toxicity studies, it gives little information on whether host resistance may or may not be affected (Mayrand et al., 2005). With so many parameters contributing to an organism's immune response, it is likely that there is some overlapping and redundancy in the various responses (Galloway and Depledge, 2001), so if one parameter is inhibited other immune parameters may compensate for this and it is unclear if such alterations will affect the overall immunocompetence.

Currently, there is a lack of information on how changes in the immune parameters relate to alterations in host resistance. Whilst some studies have suggested a link between reduced immune function and decreased host resistance, they have measured the immune responses and bacterial susceptibility in separate individuals (St-Jean et al., 2002b; Mayrand et al., 2005). This approach can be problematic, with immune responses in bivalves known to vary widely between individuals (Pipe et al., 1995; Pipe et al., 1999; Carissan-Lloyd et al., 2004). To date, no correlative studies have been conducted in an attempt to elucidate a direct link between impaired immune function and compromised host resistance. The work in this chapter was aimed at determining the effect of phenanthrene on the ability of Pecten maximus to eliminate Vibrio pectenicida following a bacterial challenge. In addition, any relationships between immunopathological and immune function responses and bacterial clearance were identified to assess if changes in these widely used immune parameters are indicative of alterations in host resistance.
7.2 Materials and methods

7.2.1 Experimental design
Scallops (*Pecten maximus*) were hand collected by divers from Start Bay, Devon, UK (50°15'N, 3°37'E) and purchased through Britannia Shellfish Ltd. Scallops were maintained as described in Section 2.1.2 for 2 weeks prior to use in the experimental system. After this acclimation period, scallops were transferred to individual aerated glass tanks (18 in total) containing 6 l of filtered seawater (FSW; 15 ± 1 °C, 34 PSU). The tanks were then spiked with the solvent carrier, dimethyl sulphoxide, or phenanthrene stock solution to achieve nominal phenanthrene exposure concentrations of 0, 100 and 200 µg l⁻¹, with the solvent carrier not exceeding 0.01 % of the total seawater volume. The scallops were held in these static exposure tanks (6 tanks per treatment), for a total of 16 d with the water renewed and spiked every 24 h to maintain exposure concentration and water quality.

7.2.2 Chemical measurements
One litre water samples were collected from the exposure tanks on day 1, 5 and 10 immediately after the PAH spike to confirm phenanthrene exposure concentrations. Samples were processed using liquid-liquid extraction with cyclohexane and analysed using GC/MS (HP5890 Series II GC, HP5970 MSD, Hewlett Packard, USA) as described in Section 2.3.2.
7.2.3 Biological measurements

To determine the gonad maturation of experimental animals, 10 stock animals were chosen at random and histological sections of the male and female gonad were made as described in Section 2.2.8; these were stained with haematoxylin and eosin, and assessed using light microscopy under oil immersion at ×100 magnification. The stage of gametogenesis in individual scallops was determined based on the cytological criteria described by Mason (1958).

After 14 d exposure, haemolymph (~0.7 ml) was collected from the striated region of the scallop adductor muscle (Section 2.2.1) for determination of total haemocyte count, cell membrane stability (Section 2.2.4), phagocytosis and cytotoxic ability (Section 2.2.5). Scallops were then returned to their respective exposure tanks for 24 h before being subjected to a live bacterial challenge (Figure 7.1). Scallops were injected with 100 μl of *Vibrio pectenicida* suspension ($10^7$ bacteria; Section 2.2.6.1) into the adductor muscle. Twenty four hours post-challenge, haemolymph (~0.7 ml) was collected and the bacterial clearance was determined (Section 2.2.6.1).
Scallops divided between three treatments: 0 (FSW control), 100, 200 µg l⁻¹ phenanthrene

14 d

Haemolymph extracted from 6 individuals from each treatment (n = 18) and returned to exposure tank

Immune apparatus assays: THC, cell membrane stability
Immune mechanism assays: phagocytosis, cytotoxicity

24 h

Scallops injected with live bacterial challenge (Vibrio pectenicida, 10⁷) and returned to exposure tank

24 h

Haemolymph extracted from 6 individuals from each treatment (n = 18)

Host resistance assay: bacterial clearance

Figure 7.1 Experimental design to investigate PAH-induced alterations in the bacterial susceptibility of Pecten maximus.

7.2.4 Statistical analysis

Biological measurements were taken from 6 scallops from each treatment and results expressed as mean values ± 1 standard error. Data sets were checked for equal variance and analysed for significant differences between treatment groups using one-way ANOVA for biological parameters. Chemical data were analysed using two-way ANOVA. Fisher's LSD pairwise comparisons were conducted post-hoc to identify where significant differences occurred at or above the 95 % confidence level.
7.3 Results

7.3.1 Chemical measurements

Seawater phenanthrene concentrations measured in the exposure tanks were in excess of 94 and 88% of the nominal concentrations for the 100 and 200 µg l⁻¹ treatments, respectively (Table 7.1). No significant difference in exposure concentration was observed over the experimental period (two-way ANOVA: $F_{2,35} = 0.75$, $P = 0.75$) with levels in the range of 94-98 µg l⁻¹ and 176-179 µg l⁻¹ within the respective treatment groups (Table 7.1).

Table 7.1 Mean ($n = 6$) phenanthrene concentrations (± 1 SE) in the exposure tanks after the PAH spike at various time points over the experimental period.

<table>
<thead>
<tr>
<th>time (d)</th>
<th>0</th>
<th>100 (± SE)</th>
<th>200 (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;0.01</td>
<td>94.0 (3.39)</td>
<td>175.7 (5.83)</td>
</tr>
<tr>
<td>5</td>
<td>&lt;0.01</td>
<td>98.0 (3.49)</td>
<td>177.7 (7.15)</td>
</tr>
<tr>
<td>10</td>
<td>&lt;0.01</td>
<td>97.6 (4.03)</td>
<td>179.2 (5.44)</td>
</tr>
</tbody>
</table>

7.3.2 Biological measurements

Histological analysis of *P. maximus* gonads (Figure 7.2) showed characteristics indicative of stage VI of gametogenesis, with all experimental animals at a pre-spawning stage of gonad maturation; the gonads were dominated by large, well developed spermatic follicles filled with spermatozoa (Figure 7.2a), and turgescent female ovarian follicles packed with mature polygonal oocytes (Figure 7.2b). Few haemocytes were present in the connective tissue of both the male and female sections of the gonad (Figure 7.2a and b).
Phenanthrene exposure resulted in significant effects on the immune cells of *P. maximus* with elevated haemocyte counts (*F*₂,₁₇ = 4.92, *P* < 0.05) and reduced cell membrane stability (*F*₂,₁₁ = 5.35, *P* < 0.05). Following aquatic exposures of 100 and 200 µg l⁻¹, cell counts were elevated by 39 and 35%, respectively, compared with numbers recorded in control scallops (Figure 7.3a). This increased haemocyte count corresponded to impaired cell membrane stability, with stability in scallops from the 200 µg l⁻¹ treatment reduced to 50% of the control (Figure 7.3a). Cell-mediated responses demonstrated that phagocytic activity of *P. maximus* haemocytes was significantly reduced following 14 d in vivo exposure to phenanthrene (Figure 7.3b; *F*₂,₁₇ = 4.30, *P* < 0.05). However, no significant effect on the cytotoxic ability of the haemocytes was detected (Figure 7.3b; *F*₂,₁₁ = 2.36, *P* = 0.129). Whilst only one of the immune mechanisms measured was modulated by phenanthrene exposure, the bacterial susceptibility of *P. maximus* was altered by this PAH (*F*₂,₁₁ = 4.62, *P* < 0.05). Significantly higher numbers of CFUs (6.3 x 10⁵ ml⁻¹) were present in the haemolymph of 200 µg l⁻¹ exposed scallops 24 h after a bacterial challenge with the clearance of bacteria reduced by up to 65% in the exposed scallops (Figure 7.3c).
Figure 7.2 Histological section of (a) male gonad and (b) female gonad (haematoxylin and eosin stained) from *Pecten maximus* demonstrating stage VI gametogenesis.
Figure 7.3 PAH-induced alterations in (a) the immune apparatus (total haemocyte count and cell membrane stability), (b) mechanisms of immunity (phagocytosis and cytotoxic ability) and (c) bacterial susceptibility (presence of colony forming bacterial units (CFU)) in Pecten maximus after 14 d phenanthrene exposure.
There was a significant relationship between PAH-induced responses in the immune apparatus and the phagocytic immune mechanism (Figure 7.4), with a significant negative correlation between phagocytic activity and total haemocyte count \( (R = -0.471, \ P < 0.05) \). However, since the correlation coefficient was < 0.5 this indicated only a weak relationship between these two immune parameters, with the THC only accounting for 23% of the variation in phagocytic activity. A significant positive correlation was found between phagocytosis and cell membrane stability \( (R = 0.856, \ P < 0.001) \), with the fitted regression model accounting for 73% of the variation. In contrast, the cytotoxic capability of the haemocytes was not significantly related to either cell count or membrane stability (Figure 7.4), with total haemocyte number only accounting for 5%, and cell membrane stability accounting for 21%, of the variation in cytotoxicity.

The phagocytic mechanism of immune function was significantly related to bacterial susceptibility in *P. maximus* (Figure 7.5; \( R = -0.852, \ P < 0.001 \); a low level of phagocytosis was correlated with an increase in the presence of bacterial CFU with the linear regression model accounting for 73% of the variability in CFU counts. Whilst the relationship between cytotoxic capability and bacterial susceptibility was also significant (Figure 7.5: \( R = -0.648, \ P < 0.005 \)), cytotoxicity only accounted for 42% of the variation in CFU counts based on the fitted regression model.
Figure 7.4 Linear regression between the PAH-induced responses of immune apparatus and immune mechanisms in *Pecten maximus*. The solid line represents a linear regression, with dashed lines indicating 95% confidence limits.
7.4 Discussion

The reproductive status of an organism can greatly impact immune function, with the energetic costs of gamete production likely to result in immune suppression (Galloway and Depledge, 2001). Such a reduction in phagocytosis was observed in the bivalve *Crassostrea gigas* during gametogenesis (Delaporte et al., 2006). Inhibition of phagocytosis has also been reported in *M. edulis* post-spawning (Cartier et al., 2004), when the haemocytes are heavily involved in gamete resorption and restructuring of gonad tissue (Delaporte et al., 2006). The experimental animals used here were identified to be at a similar reproductive stage with mature gametes in a pre-spawning state. Results from this study suggest that even when the
The immunopathological and immune function alterations of *P. maximus* following PAH exposure were shown to ultimately impair the organism's capacity for bacterial clearance. Previous studies have also reported reduced bacterial clearance in bivalves as a result of contaminant exposure. The mussel *M. edulis* exposed to tributyltin, dibutyltin and untreated sewage waste exhibited a reduced capacity for bacterial elimination when injected with *Vibrio anguillarum* (St-Jean et al., 2002b; Akaishi et al., 2007). The PAH benzo(a)pyrene also causes impaired clearance of *Flavobacterium* sp. in the clam *Mercenaria mercenaria* (Anderson et al., 1981).

The phenanthrene-induced immunomodulation observed here may indicate a potential for increased disease susceptibility. The reduced immunocompetence may aid the proliferation of bacteria already present in the organism, as suggested for oil-exposed oysters (Barszcz et al., 1978). In addition, contaminant exposure can increase the pathogenicity of bacterial strains as reported for copper-exposed mussels challenged with *Vibrio tubiashi* (Pipe and Coles, 1995). A pollutant-induced increase in bacterial virulence was also reported for *Pekinsus marinus* infections in the oyster *Crassostrea virginica* following exposure to the WAF of PAH-contaminated sediments (Chu and Hale, 1994).
Gram-negative bacteria, notably members of Vibrionaceae, are the most common cause of reported bacterial infections in scallops (McGladdery et al., 2006), and are common in marine and coastal waters (Urakawa and Rivera, 2006). Despite most vibrios being largely non-pathogenic to adult bivalves, they are considered a useful model for pathogen clearance assays (St-Jean et al., 2002b). The results from this study suggest an increased susceptibility of phenanthrene-exposed scallops to bacterial infection, demonstrated by a reduced capacity for elimination of *V. pectenicida*. In adult *P. maximus*, *V. splendidus* induces shell deposits of conchiolin, deforming the scallop shell and showing signs of debilitating disease (Lambert et al., 1999). Other Vibrios also inhibit filtration rate in infected bivalves (Birkbeck et al., 1987) which in turn will have consequences for energy acquisition and metabolic function.

Whilst most bacteria associated with adult scallops do not cause mortalities in the organisms themselves, a number of species including *V. chloerae*, *V. vulnificus* and *V. parahaemolyticus* are well known human pathogens (Nishibuchi, 2006). Therefore, if reduced immunocompetence of *P. maximus* resulted in the infection by such strains, contaminated animals would not be permissible for consumption, having economical effects for the fisheries and aquaculture of this commercial species.
Measurements of immune parameters have potential as ecotoxicological monitoring tools to provide early warning indicators of environmental stress; however it is of added value if their ecological relevance can also be demonstrated. To assess the potential for disease incidence from pollution-induced stress, through the use of biochemical and cellular perturbations in immunity, it is essential to determine the relative importance of such immune modulations on the ability to eliminate pathogenic bacteria (Livingstone et al., 2000). Suggestions of a link between suppressed immune function and impaired bacterial elimination have been proposed (Akaishi et al., 2007); however, no attempt has yet been made to directly elucidate the relationship between immune biomarker responses and bacterial susceptibility. Using a tiered system to assess immune function within individual organisms, the results from this chapter demonstrated a clear relationship between immunopathological changes and mechanistic immune function. Alterations in haemocyte membrane stability subsequently reduced the phagocytic activity of these cells. These cellular perturbations appear to contribute toward a limited capacity for bacterial elimination, with a strong correlation between reduced phagocytosis and impaired bacterial clearance.

Phagocytic activity, haemocyte counts and cell membrane stability are widely used immune parameters within ecotoxicology. The results obtained in this chapter show that these quick, simple, cost-effective and non-destructive techniques are reliable indicators of bacterial susceptibility. In addition, these parameters have been shown to be sensitive measures in detecting immunotoxicity associated with PAH and oil exposure. With the continued
increase in offshore oil exploration continued efforts are being made to assess and reduce the environmental impact of such activities. The immune parameters studied here may therefore be useful in assessing sublethal effects of other wastes associated with offshore oil production and will be used in the next chapter to assess the impact of water-based drilling mud.
Chapter 8
Ilmenite-weighted water based drilling mud: effect on cellular immune function in the scallop Pecten maximus

The results from this chapter were presented at the 15th International Symposium on Pollutant Responses in Marine Organisms (PRIMO15), Bordeaux, France, May 2009 (poster presentation).
8 Ilmenite-weighted water based drilling mud: effect on cellular immune function in the scallop *Pecten maximus*

8.1 Introduction

Offshore oil exploration requires the use of drilling mud to lubricate the drill string, control internal pressure, stabilise the well and carry drill cuttings to the surface (Carroll et al., 2000; Scholten et al., 2000; Breuer et al., 2004). In the North Sea, oil exploration and production has seen the use of oil-based mud (OBM), synthetic-based mud (SBM) and water-based mud (WBM) (Breuer et al., 2004). Previous discharges of OBM resulted in extensive long-term damage to benthic communities as a result of oil toxicity; a reduction in macrofaunal abundance and species number was observed in the vicinity of a North Sea OBM discharge (Daan et al., 1990). The impact of the OBM on the benthic community was still apparent 3 years after the discharges had ceased, with reduced species diversity and a decreased total biomass compared to sites outside the discharge area (Kröncke et al., 1992). Also, there have been concerns over the environmental acceptability of some SBM components, which resulted in a zero discharge policy of both OBM and SBM being introduced in the North Sea in December 2000 (Breuer et al., 2004). Currently, only cuttings from sections drilled with WBM can be discharged into the marine environment, yet they still account for around half the total cuttings produced (Scholten et al., 2000); around 80 000 t y\(^{-1}\) are discharged into the sea from offshore drilling on the Norwegian continental shelf (OLF, 2006). Upon discharge, WBM separates into two plumes, with 90 % (by weight) of the material descending through the water column and
accumulating on the sea floor (Barlow and Kingston, 2001). It is estimated that drilling mud is diluted to concentrations in the range of 10-50 mg l⁻¹ in the lower 10 m of the water column at a distance of 500 m from the release site (Bechmann et al., 2006), with concentrations reduced to < 10 mg l⁻¹ at distances greater than 1 km from the discharge (Neff, 1987).

Barite (BaSO₄) has been used extensively in the oil industry as a weighting material in WBM (Ruus et al., 2005) and, with a high specific gravity (4.2), contributes directly to discharged materials accumulating on the seafloor. Although barite is listed as posing little or no risk to the environment (OSPAR, 2004), elevated levels of barium have been recorded in invertebrates in the vicinity of oil installations (Sadiq et al., 1990). Barium accumulation was also observed along with elevated levels of lead and aluminium in the polychaete Nereis diversicolor and the gastropod Hinia reticulata following exposure to barite-spiked sediments (Schaanning et al., 2002; Ruus et al., 2005). Biological impacts of barite have been reported in various molluscs; gill damage was reported in Cerastoderma edule and Macoma balthica, and a reduced scope for growth was reported in Placopecten magellanicus (Cranford et al., 1999). Exposure of Mytilus edulis and Pecten maximus to pure barite (23 mg ml⁻¹) and WBM weighted with barite (0.5-20 mg ml⁻¹) resulted in reduced lysosomal membrane stability and scope for growth, and increased oxidative stress in both bivalves, whilst P. maximus also showed an increased level of DNA damage (Bechmann et al., 2006).
Since concerns have been raised over the environmental impact of barite, mainly linked to its metal content (Neff, 2005), alternative weighting materials have been proposed. The crystallised form of iron titanium oxide (FeTiO₃), known as ilmenite, has been recommended for use as a weighting material in oil-drilling operations. The high specific gravity (4.7) of ilmenite would mean that in WBM discharges, ilmenite would descend through the water column similarly to barite and deposit on the seafloor, where drilling muds are considered to pose the greatest impact on benthic biota (Neff, 1987). However, ilmenite is used as a much finer particulate than barite (Barlow and Kingston, 2001) and has lower metal concentrations (Neff, 2005). Considered to pose little or no risk to the environment, ilmenite has been proposed as a replacement for barite in drilling mud discharged into the North Sea (OSPAR, 2004), but as yet little information is available on the effects of ilmenite on benthic biota. Benthic filter feeders are considered the most at risk from effects of drilling mud discharges (Neff, 2005), and previous studies have shown scallops to be particularly sensitive to such wastes (Cranford et al., 1999). The aim of the work in this chapter was to investigate the effect of ilmenite-weighted WBM on the cellular immune response of *P. maximus* following a long-term chronic exposure.
8.2 Materials and methods

8.2.1 Experimental design

Cultured *P. maximus* (100-120 mm in shell length) from Toskasundet, Radøy, Norway (60°38'N, 4°59'E) were purchased through Helland Skjell AS, Norway. Animals were maintained in a continuous flow system (CFS) with filtered seawater (9 ± 1 °C) for 5 weeks prior to the exposure. Organisms were exposed to used WBM with ilmenite as the weighting material in a CFS system, at 3 l min⁻¹, to simulate conditions in the water column following offshore drilling operations (Bechmann et al., 2006). A stock solution of ilmenite weighted WBM, diluted in FSW (50 g l⁻¹), was stored in a header tank and maintained in suspension using a propeller. The stock solution was then carried through Teflon tubing (1.9 mm φ) to the exposure tanks (500 l) using a peristaltic pump (Figure 8.1a). Scallops were exposed to a nominal concentration of 4 mg l⁻¹ (dry weight) ilmenite WBM and a FSW control for 100 days. In addition, exposed organisms were transferred to FSW for a 30 day post-exposure recovery period. All treatment tanks were maintained at 9 ± 1 °C and scallops fed daily using the micro-algae concentrate, Instant Algae® Shellfish Diet.

8.2.2 Chemical measurements

Samples of the WBM and scallop tissue were prepared for metal analysis through acid digestion (7 M HNO₃ at 120 °C for 30 min) and metal concentrations were determined using ICP-MS as described in Section 2.3.3.
The particle concentration in the exposure tank was determined using a Whatman GF/F 0.7 μm filter weighed prior to filtration, and again after drying at 80 °C for 24 h. Particle size distribution of the ilmenite WBM was carried out using laser analysis (Mastersizer 2000, Malvern Instruments, UK) to measure particle diameters in the range of 0.02-2000 μm.

Figure 8.1 (a) Experimental set up for the exposure of Pecten maximus to suspended ilmenite-weighted WBM (4 mg l⁻¹) in a continuous flow system.

8.2.3 Biological measurements

After 100 d exposure to ilmenite WBM, haemolymph was extracted from 10 scallops from both the control and ilmenite WBM treatments. Ten further scallops were sampled after a 30 d recovery period in FSW. Total haemocyte counts (Section 2.2.4.1), plasma protein concentration (Section 2.2.3), cell
membrane stability (Section 2.2.4.2) and phagocytic activity (Section 2.2.5.1) was then determined.

8.2.4 Statistical analysis
After checking for homogeneity of variance, univariate analyses were carried out using one-way ANOVA or Kruskal-Wallis where appropriate. Fisher’s LSD post-hoc pairwise comparisons were conducted to identify where significant differences occurred at the 95% confidence level.

8.3 Results
8.3.1 Chemical measurements
The concentration of WBM in the exposure tanks was \(3.7 \pm 0.34\) mg l\(^{-1}\), representing > 90% of the desired nominal concentration. The majority (85%) of the ilmenite WBM consisted of particles less than 15 \(\mu\)m in diameter with a volume-weighted mean particle size of 9 \(\mu\)m (Figure 8.2).

![Figure 8.2 Size distribution of particles in ilmenite WBM (mean particle size = 9 \(\mu\)m).](image)
Despite a low metal loading of the ilmenite WBM, Al, Fe, Ni, Ba, and Pb concentrations were elevated compared to sediment metal content reported for marine sites (Table 8.1). Despite Al being the dominant metal in the 4 mg l⁻¹ ilmenite WBM exposure (Table 8.2), Fe and Ba were measured at much higher concentrations than Al in the tissues of P. maximus after 100 d exposure to ilmenite WBM (Table 8.2); this is reflected by the higher bioconcentration factors shown in Table 8.2 for Fe and Ba compared to that for Al.

Table 8.1 Metal concentration (mg kg⁻¹) in ilmenite WBM and metal loading of sediments from marine sites. NR = not reported.

<table>
<thead>
<tr>
<th>metal</th>
<th>ilmenite WBM</th>
<th>marine sediments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(current study)</td>
<td>Stevenson, 2001</td>
</tr>
<tr>
<td>Al</td>
<td>~25000</td>
<td>NR</td>
</tr>
<tr>
<td>Fe</td>
<td>24711</td>
<td>NR</td>
</tr>
<tr>
<td>Ni</td>
<td>39.1</td>
<td>12</td>
</tr>
<tr>
<td>Ba</td>
<td>3730</td>
<td>336</td>
</tr>
<tr>
<td>Pb</td>
<td>10.3</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 8.2 Metal exposure concentration (ppb) and Pecten maximus tissue concentration (ppb) following exposure to 4 mg l⁻¹ ilmenite WBM for 100 d. The bioconcentration factors (BCF) for these metals have also been calculated according to Mountouris et al. (2002).

<table>
<thead>
<tr>
<th>metal</th>
<th>exposure concentration µg kg⁻¹ (C_EXPOSURE)</th>
<th>tissue concentration µg kg⁻¹ (C_BIOTA)</th>
<th>BCF (C_BIOTA/C_EXPOSURE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>100</td>
<td>54680</td>
<td>547</td>
</tr>
<tr>
<td>Fe</td>
<td>99.0</td>
<td>283980</td>
<td>2868</td>
</tr>
<tr>
<td>Ni</td>
<td>0.16</td>
<td>770</td>
<td>4813</td>
</tr>
<tr>
<td>Ba</td>
<td>14.9</td>
<td>183290</td>
<td>12301</td>
</tr>
<tr>
<td>Pb</td>
<td>0.04</td>
<td>500</td>
<td>12500</td>
</tr>
</tbody>
</table>

¹Calculated values based on 4 mg l⁻¹ ilmenite drilling mud nominal exposure.

²Total content measured in Pecten maximus gonad and gill.
Following the long-term WBM exposure, metals with accumulated levels in the gonad (Figure 8.3) showed a much greater accumulation in the gills, with the exception of Fe, which was measured in similar concentration in the gonad, mg kg⁻¹, and gill, 138 mg kg⁻¹. The metal concentrations in the gonads were significantly elevated for 3 metals (Figure 8.3). In the control group, the concentration of Fe, Mg, and Mn was similar to the control group, with percentage increase of Fe, Mg, and Mn. However, the levels of Cu, Zn, and Cd were significantly lower than the control group. The breakdown analysis of the metal concentrations was conducted using a Student's t-test and analysis of variance (ANOVA). The results indicated that the metal concentrations in the gonad and gill were significantly different between the control and exposed groups.

**Figure 8.3** Metal content in the gonad (□) and gill (■) of *Pecten maximus* after a 100 d ilmenite WBM exposure and a 30 d recovery period.
Following the long-term ilmenite WBM exposure, metals were accumulated in the gonad to a much greater extent than in the gills (Figure 8.3), with the exception of Fe, which was measured at similar concentration in the gonad, (148 mg kg\(^{-1}\)) and gill (136 mg kg\(^{-1}\)) (Figure 8.3). The metal concentrations in the gonads were significantly elevated for all 5 metals (Table 8.3) compared to the control scallops, with increased levels of Fe, Ni and Pb persisting even after a 30 d recovery period in FSW (Table 8.3).

Table 8.3 Significantly elevated (↑) metal concentrations in the gonad of *Pecten maximus* following a 100 d ilmenite WBM exposure and a 30 d recovery period. A dashed line (—) indicates metal concentrations were not significantly different from the control. The Kruskal-Wallis (H) test statistic and associated probability is also detailed.

<table>
<thead>
<tr>
<th>metal</th>
<th>test statistic and associated probability</th>
<th>treatment</th>
<th>ilmenite exposed</th>
<th>30 d recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>(H_{2,24} = 15.41) (P &lt; 0.001)</td>
<td>↑</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>(H_{2,24} = 15.10) (P &lt; 0.001)</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Ni</td>
<td>(H_{2,24} = 17.38) (P &lt; 0.001)</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Ba</td>
<td>(H_{2,24} = 15.97) (P &lt; 0.001)</td>
<td>↑</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pb</td>
<td>(H_{2,24} = 15.99) (P &lt; 0.001)</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

8.3.2 Biological measurements

The highest haemocyte count (12.6 × 10\(^6\) ml\(^{-1}\)) was observed in scallops following 100 days exposure to 4 mg l\(^{-1}\) drilling mud (Figure 8.4a), although this was not significantly different from cell counts in control organisms (\(F_{2,27} = 0.17\), \(P = 0.848\)). Plasma protein was also unaffected by exposure to the drilling mud (\(F_{2,27} = 1.29\), \(P = 0.292\)) with concentrations ranging from 1.31 mg ml\(^{-1}\) in the control group to 1.58 mg ml\(^{-1}\) in the exposed organisms (Figure 8.4b). Long-term drilling mud exposure did, however, significantly reduce the cell membrane stability of *P. maximus* haemocytes (\(F_{2,27} = 12.16\), \(P < 0.001\)) to 39% of the control value (Figure 8.4c; 0.164 OD mg\(^{-1}\) protein).
A significant inhibition of phagocytosis was also recorded in exposed animals ($F_{2,27} = 9.59$, $P < 0.001$), with a mean phagocytic activity of $11.8 \times 10^7$ particles mg$^{-1}$ protein compared to $18.8 \times 10^7$ in control scallops (Figure 8.4d). Despite the significant reduction in these parameters, measurements obtained following a 30-day post-exposure recovery period, indicated both cell membrane stability and phagocytosis had returned to control levels (Figure 8.4c and d).

Figure 8.4 Biological responses of Pecten maximus following a 100 d ilmenite WBM exposure and 30 d recovery period. Results are expressed as mean values ± 1 SE (n = 10) for (a) total haemocyte count (THC), (b) plasma protein concentration, (c) cell membrane stability and (d) phagocytic activity. Significant differences are denoted by different uppercase letters.
8.4 Discussion

Weighting materials, such as barite or ilmenite, normally make up at least 90% of the drilling mud composition (Bechmann et al., 2006). However, these materials can also contain trace metal impurities (Neff, 2005) such as the metals measured in the ilmenite WBM used here. Whilst ilmenite reportedly contains lower levels of trace metals than other weighting materials (Neff, 2005), these levels are still elevated compared to those recorded in marine sediments (Stevenson, 2001; Rezende et al., 2002), suggesting that ilmenite may be a source of metal contamination in discharge areas. These metals contained in ilmenite may have a biological impact if they are available to the organism, resulting in toxic effects (Neff, 2005). The elevated metal content measured in the gonads of P. maximus after long-term exposure to ilmenite WBM, indicated that the metals were taken up by the scallops. It should be noted that the metals are also likely to have accumulated in the digestive gland of the organism (not measured here), and therefore the total metal body burden may be much higher than what is reported for the sum of the gonad and gill content. The availability of metals results in high concentrations in biota through bioconcentration of metals from the surrounding phases (Mountouris et al., 2002). In the current study, metals may have been taken up by the scallops in the dissolved phase (due to leaching from the ilmenite) or adsorbed onto the drilling mud particles. Through these routes of uptake Fe and Ba, with higher bioconcentration factors (BCFs), accumulated to the highest concentrations.
Numerous studies have demonstrated the immunotoxic effects exerted by metals in the bivalves *Mytilus edulis* (Coles et al., 1995; Pipe et al., 1999; Sauvé et al., 2002), *Crassostrea gigas* (Gagnaire et al., 2004) and *Mya arenaria* (Sauvé et al., 2002). The results from the present study demonstrated a reduced immunocompetence with an increased metal body burden. Indeed, metals detected in the ilmenite WBM used here have also been shown to have immunotoxic potential, with reduced immune activity observed in Pb-exposed *M. edulis* (Nieto-Fernandez et al., 2000). Pb exposure also causes lipid peroxidation in the mussel *Perna viridis* (Prakash and Rao, 1995); such oxidative damage has been linked to compromised cell membranes and reduced phagocytic capacity in *P. maximus* (Chapter 5). Ni, present in the ilmenite WBM, also impairs immune function with decreased phagocytic activity in the crab *Scylla serrata* (Vijayavel et al., 2009).

Whilst the metal content may be contributing towards the observed reduction in cell membrane stability and phagocytosis, immunotoxic effects previously reported for Ni and Pb were observed at much higher exposure concentrations than those in the current study. In addition, the trace metals present in the ilmenite, whilst taken up by the scallops, may not be bioavailable; the accumulation of metals within an organism does not provide information on their biological reactivity. The particle bound fraction of metals may be non-toxic to the animal or it may become bioavailable through desorption which has been reported to occur in the gastrointestinal tract of other filter feeders (Weltens et al., 2000). The measured body burdens also do not take into account the ability to metabolise, sequester, detoxify, and
store these metals (McGeer et al., 2003), and most accumulated metals remain in the tissues as insoluble, inert forms reducing the potential for adverse effects (Neff, 2005). Most metals associated with the weighting material of drilling mud have low bioavailability (Schaanning et al., 2002) and are, therefore, unlikely to be the main contributor to the impaired immune function observed here. This is also supported by the immune parameters returning to control levels after the recovery period, despite body burdens of Fe, Ni and Pb remaining elevated.

The physical characteristics of the fine particles in the drilling mud may contribute towards the observed reduction in cell membrane stability and phagocytosis in P. maximus. Due to the large upper size limit on particle selection in the gills of P. maximus (>200 μm), this scallop can ingest most suspended particles (Beninger and Le Pennec, 2006), and has a low rejection efficiency for non-organic particles less than 10 μm (Berland et al., 2006). With a mean particle size of 9 μm in the ilmenite WBM, these drilling mud particles are likely to be easily taken up by the scallops. Once within the organism, scallops may enclose the particles within haemocytes (Berland et al., 2006), using this phagocytic process as a mechanism to remove particles from the epithelial surface (Beussink, 2007). This phagocytic removal of the drilling mud particles may then limit the capacity to phagocytise the zymosan particles used in the phagocytosis assay of the current study.

Optimum particle size for feeding in P. maximus is 5-6 μm (Berland et al., 2006), and therefore the scallops exposed to ilmenite WBM may be taking up
drilling mud particles rather than the algae provided as a food source in the experimental setup. Such small inorganic particles can impact the ciliary activity of the gills (Stevens, 1987) reducing the feeding rate (Cranford and Gordon, 1992). If the uptake of ilmenite WBM particles resulted in a similar reduction in the feeding rate, the energy available for such immune processes would be limited, possibly contributing towards the observed reduction in immune function.

Results obtained here demonstrated that although ilmenite WBM contains relatively low concentrations of metals compared to other weighting materials, its metal content is still higher than levels recorded in marine sediments, suggesting it may be a source of metal contamination in discharge areas. Whilst ilmenite is considered to pose little or no risk to the environment, altered immune function was observed in P. maximus exposed to relatively low concentrations (4 mg l⁻¹) of ilmenite WBM; a concentration expected to be found at distances greater than 1 km from the discharge site. Whilst this may, in part, be a result of metal toxicity, the low bioavailability of metals in weighting materials, means the observed immunotoxic effect is likely to be largely attributed to the physical characteristics of the drilling mud particles.
Chapter 9

General Discussion
9 General Discussion

This thesis focused on evaluating the immunotoxic effects of oil-related compounds on the Arctic and temperate scallops, *Chlamys islandica* and *Pecten maximus*. The multifaceted nature of the immune system offers potential for the development of a large number of assays to determine immunocompetence (Livingstone et al., 2000). The parameters used in the experimental chapters (Table 9.1) focused on the cell-mediated immune functions which are reported to be more sensitive to stress than humoral factors (Auffret, 2005). The wide range of components that make up the invertebrate immune system would render it impractical, if not impossible, to attempt to measure all immune parameters (Livingstone et al., 2000). Therefore, the assays used in this research were chosen to include immune apparatus, immune function and immune efficiency (Table 9.1). These immune parameters allowed the use of a hierarchical approach to determine immunocompetence, similar to that adopted to assess the integrated functioning of the vertebrate immune system (Luster et al., 1988).

Table 9.1 Comparison of the hierarchical approach used to assess invertebrate immunocompetence, adopted in this thesis, with the tiered vertebrate testing system (adapted from Galloway and Goven, 2006).

<table>
<thead>
<tr>
<th>Tier</th>
<th>Level of organisation</th>
<th>Invertebrate measurement</th>
<th>Vertebrate measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Immune apparatus</td>
<td>Total haemocyte count, cell membrane stability</td>
<td>Haematology, leucocyte proliferation</td>
</tr>
<tr>
<td>2</td>
<td>Mechanisms of immune function:</td>
<td>Phagocytic activity, Cytotoxic capacity, Alkaline phosphatase</td>
<td>Phagocytic activity, T-cell cytotoxicity, natural killer cell activity, Plaque forming assay, enzyme-linked immunosorbent assay (ELISA)</td>
</tr>
<tr>
<td></td>
<td>• cell-mediated response</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• humoral factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Immune efficiency</td>
<td>Bacterial clearance</td>
<td>Bacterial, viral and parasitic infection</td>
</tr>
</tbody>
</table>
9.1 Mechanisms of immunotoxicity

Biological-effect studies that focus on alterations at the cellular and biochemical level often help to provide a mechanistic understanding of toxicity (Hinton et al., 2005). The current research suggests that a contaminant-induced peroxidative challenge may be a contributing factor in the mechanism of immunotoxicity. This mechanistic link has already been proposed in vertebrates (Koner et al., 1997; Koner et al., 1998), but had not been previously demonstrated in bivalves, which are commonly used in invertebrate immunotoxicology (Auffret, 2005). Due to the observed effects of PAH exposure on cell membranes, it was hypothesised that PAH exposure may impair immune function by altering cell membrane receptors (as reported by Sami et al. (1993)) and therefore interfere with the ability to recognise the pathogen-associated molecular pattern (PAMP) of invading microorganisms. However, results obtained here demonstrated that the ability of P. maximus to recognise and respond to a PAMP was not impaired by sublethal phenanthrene exposure, indicating damaged cell membrane receptors are probably not a mechanism of PAH immunotoxicity.

9.2 Immune modulation in Arctic and temperate bivalves

The immunotoxic effects of oil exposure observed in the Arctic species C. islandica, during the studies of this thesis, are similar to those reported for temperate bivalves. In addition to laboratory oil exposures causing a reduced immune function in the temperate oyster Crassostrea gigas (Bado-Nilles et al., 2008; Bado-Nilles et al., 2009), immune suppression was also reported in
Mytilus spp. following the Prestige (Novas et al., 2007) and Sea Empress oil spills (Dyrynda et al., 1997b). However, it is difficult to establish whether the immunotoxic effects of oil are comparable among the organisms detailed above, as different exposure scenarios and concentrations were used. In addition, the above studies used different bivalves as their test organism, which may have different baseline immune responses (Wootton et al., 2003b) and can vary in their susceptibility to immunotoxic contaminants (Wootton et al., 2003a). Therefore, conducting an exposure using both Arctic and temperate species of the same type of bivalve, is essential to be able to directly compare any differences in sensitivity between organisms from these two regions. Only when such work has been carried out can it be determined whether extrapolating data based on temperate species is a valid approach for assessing the risks of oil production in Arctic regions.

When determining the possible differences between Arctic and temperate species in their susceptibility to oil pollution, it is important to consider that the effects of an oil spill may also vary due to the environmental factors. The low temperatures in Arctic regions may alter the behavior of oil, as oil dissolution is temperature dependent (Payne et al., 1991). During winter, photochemical degradation of petroleum hydrocarbons may be more restricted in the Arctic compared with temperate regions due to the former experiencing <50% of the annual solar radiation received by temperate areas (AMAP, 1998). However, during summer months there may be an increase in the degradation of spilled oil due to the increased UV radiation associated with ozone depletion in polar regions (Rozema et al., 2005). In addition, the
interaction with sea ice may not only alter oil dispersion, but oil trapped under an ice layer will have inhibited evaporation of volatile components, prolonging its toxicity (Engelhardt, 1994). These abiotic factors influence the distribution, composition and physical state of the oil, which in turn determines the bioavailability of its components. Therefore, future work should focus on the environmental fate of oil and the bioavailability of its components in Arctic regions, as this will inevitably play a key part in the biological effects of oil spills.

9.3 Immunotoxicology: environmental risk assessment and biomonitoring

At present, environmental risk assessment (ERA) is the widely accepted process on which environmental management decisions are based. ERA consists of four main steps: hazard identification, dose-response assessment, exposure assessment and risk characterisation (Figure 9.1). The results obtained from the studies of this thesis contribute towards the first stage in the ERA procedure through identification of the immunotoxic effects of dispersed oil and a simulated oil spill on C. islandica; these biological-effect studies are an essential part of establishing the risks associated with increased oil production in Arctic and Sub-arctic regions.

Whilst immunocompetence assays have been recognised as promising tools for biomonitoring (ICES, 2007), in field situations, it can be difficult to assess the extent to which immunotoxicity has occurred due to the inherent
Scientific data

Laboratory studies on adverse effects

Laboratory studies to determine dose-response relationship

Field studies, estimated exposure

Environmental Risk Assessment

1. Hazard identification:
   Does the substance cause adverse effects?

2. Dose-response assessment:
   What is the relationship between dose and adverse effect?

3. Exposure assessment:
   What exposures are predicted?

4. Risk characterisation:
   What is the estimated incidence of adverse effects occurring?

Figure 9.1 The Environmental Risk Assessment process for chemical substances: linking ecotoxicological research to environmental decision making (adapted from US EPA, 2010).

The multifactorial nature of the immune system (Galloway and Depledge, 2001). Stimulation or inhibition of an organism's immune parameters may be sensitive to the presence of pathogens (Parry and Pipe, 2004), environmental temperature (Liu et al., 2004; Monari et al., 2007), and contaminant exposure period and concentration (Hannam et al., 2009b). These factors will inevitably lead to variation in an organism's immune response in the field. Therefore, the use of an immune index to describe the combined results of different immune parameters was proposed by Auffret et al. (2004). Each immune parameter was assigned an ecophysiological significance factor (EPSF); a weighting based on the perceived ecological importance of changes in the given parameter. The immune index was then calculated as the cumulative variation of each immune response from a reference value, which was then multiplied by the assigned EPSF. This immune index has been used in field studies to assess the
immunocompetence of bivalves at different sites (Auffret et al., 2004; Auffret et al., 2006). Such an approach requires the collection of species-specific baseline data over long-term surveys. Whilst this was beyond the scope of this PhD, obtaining such base-line data on *C. islandica* is an essential next step if this species, proposed as a Sub-Arctic sentinel species (Baussant et al., 2009), is to be used in biomonitoring studies.

As with most biomarkers, measurements of individual immune parameters are only of environmental relevance if changes in these parameters ultimately impair organismal function, which in turn will impact at higher levels of biological organisation (Depledge and Fossi, 1994). Due to the range of parameters that contribute towards the invertebrate immune response there is likely to be some degree of redundancy in the various mechanisms (Galloway and Depledge, 2001). Therefore, it is difficult to determine the extent to which individual immune parameters must be altered to result in increased disease susceptibility. Despite this, results from this thesis provide evidence that compromised immune cells, with reduced cell membrane stability, result in a reduced phagocytic activity. With phagocytosis the major immune mechanism in bivalves (Auffret, 2005), low phagocytic activity ultimately impaired host resistance, with organisms demonstrating a reduced capability for bacterial elimination. The correlation between individual immune parameters and bacterial clearance gives a good indication of which parameters are closely associated with bacterial susceptibility (Galloway and Depledge, 2001) providing essential information on which parameters are useful in assessing immunocompetence.
In order for immune assays to be used successfully as biomonitoring tools, the influence of other abiotic and biotic factors on immune function must be considered. Physiological factors that can affect immune function include reproductive stage (Pouvreau et al., 2003; Cartier et al., 2004; Duchemin et al., 2007) and nutritional state (Hégaret et al., 2004). The developmental stage of an organism can also affect its immune function, with juvenile Carcinus maenas exhibiting lower phagocytic activity than adults of the same species (Dissanayake et al., 2008b). This phagocytosis was then further reduced after PAH exposure (Dissanayake et al., 2008b). A reduced immunocompetence during early developmental stages is also apparent in bivalves, with larval stages more susceptible to pathogenic bacteria than adults (Paillard et al., 2004). It would, therefore, be interesting to establish the effects of oil exposure on the bacterial susceptibility of C. islandica larvae. A contaminant-induced increase in the bacterial susceptibility of larvae may be of great importance to C. islandica which, due to the colder temperatures, have an extended larval period of up to 10 weeks before metamorphosis occurs (Gruffydd, 1976), compared to the temperate P. maximus which spends ~5 weeks as planktotrophic larvae (Gruffydd and Beaumont, 1972). In turn, this may have a detrimental effect on the proliferation of C. islandica.

The immune parameters used here have been shown to be sensitive, reliable markers of immunocompetence which can be directly linked to host
resistance. Alterations in these immune parameters also appear to be largely reversible, highlighting their use as early warning indicators of contaminant-induced stress. These immune parameters, therefore, show potential as tools to be used in environmental monitoring.
Appendix I: Published Papers

Paper I

Immune function in the Arctic Scallop, *Chlamys islandica*, following dispersed oil exposure

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2009 Aquatic Toxicology 92: 187-194
Immune function in the Arctic Scallop, *Chlamys islandica*, following dispersed oil exposure

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**ARTICLE INFO**

Article history

Received 1 October 2008
Received in revised form 19 January 2009
Accepted 21 January 2009

Keywords:

Arctic Scallop
Chlamys islandica
Biomarker
Phagocytosis
Oil
Immunotoxicity

**ABSTRACT**

With the current expansion of offshore oil activities in Arctic regions, there is an urgent need to establish the potential effects of oil-related compounds on Arctic organisms. As susceptibility to growth, disease and survival is determined partly by the condition of an organism’s immune system, measurement of endpoints linked to the latter system provide important early warning signals of the sub-lethal effects of exposure to contaminants. This study assessed the impact of dispersed oil exposure on immune endpoints in the Arctic Scallop *Chlamys islandica*, using a combination of cellular and humoral biological responses. Laboratory exposures of *C. islandica* to sub-lethal dispersed oil concentrations (0.06 and 0.25 mg l⁻¹) were conducted over 15 days, followed by a 7-day recovery period in clean, filtered seawater. Cellular endpoints were significantly altered following dispersed oil exposure: haemocyte counts (*P* < 0.01) and protein levels (*P* < 0.001) were significantly elevated, whilst cell membrane stability (*P* < 0.01) and phagocytosis (*P* < 0.01) demonstrated a significant reduction. Whilst these results indicate alteration in the immune endpoints measured, this appears to be reversible upon removal of the contaminant stress. However, the impact of long-term continuous exposure and high-level acute exposure to oil is still unknown, and may have consequences for disease resistance and hence survival.

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1. Introduction

Oil is a ubiquitous contaminant throughout the marine environment with at least 20,000 tonnes entering marine systems from offshore oil production alone (GESAMP, 2007). Although there has been rapid progress towards monitoring and assessing the impacts of oil on the coastal environment, most work has focused on the use of temperate species (Laffon et al., 2006; Francioni et al., 2007; Culbertson et al., 2008; Hylland et al., 2008). With the expansion of oil exploration in Arctic and Sub-Arctic regions, notably the Barents Sea and the North Alaskan Slope (AMAP, 1998), there is an urgent need to develop methods to assess the potential impact of oil on Arctic species. The ecological impact of oil in cold-water environments may vary from those observed in temperate and tropical regions as a result of environmental and biological factors characteristic of the Arctic environment. For example, the low temperatures of Arctic regions may alter the behaviour of oil, as oil dissolution is temperature dependent (Payne et al., 1991). Also, photochemical degradation of petroleum hydrocarbons is more restricted in the Arctic compared with temperate regions due to the former region experiencing <50% of the annual solar radiation received by temperate areas (AMAP, 1998). Finally, interaction with sea ice may not only alter oil dispersion, but oil trapped under an ice layer will have inhibited evaporation of volatile components, prolonging its toxicity (Engelhardt, 1994). These abiotic factors influence the distribution, composition and physical state of oil, which in turn determines the bioavailability of its components. Cold-water marine invertebrates also possess adaptations enabling survival at low temperatures; these include low respiration rates (Ahn and Shim, 1998), altered cell membrane composition (Gillis and Ballantyne, 1999) and increased antioxidant defences (Regoli et al., 2000). The combination of altered chemical behaviour of oil at low temperatures, together with the biological adaptations of cold-water species, may affect the susceptibility of Arctic organisms to oil-induced damage. In addition, biological recovery from such damage is predicted to be much slower than in temperate systems: a result of the low growth rates, higher generation turnover times and increased age at maturation that are characteristic of many Arctic organisms (AMAP, 1998).

It is difficult to extrapolate how oil will impact on Arctic organisms from what has been reported for temperate species. Whilst oil can be acutely toxic to marine organisms, disruptions in essential homeostatic mechanisms, such as the immune system, can also occur as a result of sub-lethal exposures (Pipe et al., 1999). Due
to its complexity and integration with other physiological systems, the immune system is extremely vulnerable to xenobiotic stress (Galloway and Goren, 2006), and there is increasing evidence that numerous environmental contaminants impact immune function in a range of organisms (Galloway and Depledge, 2001; Auffret et al., 2002). A severely compromised immune system can result in rapid mortality, however, sudden changes in its function can be used as early warning indicators of environmental stress and, therefore, immune function is important in assessing the sub-lethal effects of contaminant exposure (Lueggen et al., 2004).

Bivalves are good candidates for immunotoxicology studies and have been studied widely (Pipe et al., 1999; Canesi et al., 2003; Wootton et al., 2003b; Cartier et al., 2004; Gagnaire et al., 2004; Auffret, 2005; Ordúñez et al., 2007). The Arctic Scallop, Chlamys islandica, is the northernmost member of family Pectinidae distributed widely throughout the Sub-Arctic transitional zone of the North Atlantic (Thorarinsdóttir, 1993) and has been successfully used in previous biomarker studies focusing on oxidative stress (Camus et al., 2002). With a low metabolic rate, scallops accumulate pollutants in their tissues to an even greater extent than other bivalves, including the common sentinel species Mytilus edulis (Young-Lai and Aiken, 1986). The aim of this study was to determine the effect of dispersed oil exposure on the immune function of the Arctic Scallop C. islandica.

2. Materials and methods

2.1. Experimental design

2.1.1. Animal collection and handling

In March 2007, Arctic Scallops, C. islandica (80–100 mm shell length), were hand collected by divers from Porsanger Norway (70°15’ N, 25°15’ E) and transported to the exposure facility at IRIS-Biolimjøen Stavanger, Norway (58°57’ N, 5°43’ E). The scallops were packed prior to flying in a polystyrene fish box, lying between two layers of damp dense foam. They were met from the flight and transported directly to the lab (total transit time ~6 h). No mortality was recorded during transportation. Upon arrival, organisms were transferred to 6001 fibreglass tanks with a continuous flow of seawater (Hannam et al., 2009). The dispersed oil was carried through the system in a glass vial pre-treated at 500°C with Teflon Lock® and stored at ~80°C. Before analysis, scallop tissue was weighed and three quantitative internal standards were added before saponification with methanolic sodium hydroxide under reflux (2 h). Digest was filtered and extracted three times with cyclohexane. Combined extracts were purified by normal-phase, solid-phase extraction, concentrated to 0.5 ml and stored in capped glass vials for analysis.

Water chemistry and biotic PAH analyses were conducted using Gas Chromatography (HP5890, Hewlett Packard, USA) connected to a Mass Spectrometer (Finnigan SSQ7000, USA) and analysed in selected ion monitoring mode (GC/MS-SIM).

2.2. Biological measurements

2.2.1. Haemolymph analysis

From each treatment, 10 scallops were sampled after 7 and 15 days exposure, and remaining scallops were transferred to clean FSW for 7 days. After this 7 days recovery period, a further 10 scallops were sampled from each treatment group. Haemolymph (approximately 0.8 ml) was extracted from the strialed region of the posterior adductor muscle using a 21-gauge needle. This was then transferred to a siliconised Eppendorf® and stored on ice until analysis to minimise cell aggregation. All assays on haemolymph samples were conducted in triplicate.

2.2.2. Tissue PAH analysis

Analysis of PAH body burden was conducted on whole tissue homogenate of three individuals from each treatment after 15 days exposure as described by Jonsson et al. (2004). An individual was opened and drained of seawater prior to dissecting out all soft tissue. Briefly, whole body tissue was macerated using cyclohexane-rinsed scissors and transferred to a glass vial pre-treated at 500°C with Teflon Lock® and stored at ~80°C. Before analysis, scallop tissue was weighed and three quantitative internal standards were added before saponification with methanolic sodium hydroxide under reflux (2 h). Digest was filtered and extracted three times with cyclohexane. Combined extracts were purified by normal-phase, solid-phase extraction, concentrated to 0.5 ml and stored in capped glass vials for analysis.

Water chemistry and biotic PAH analyses were conducted using Gas Chromatography (HP5890, Hewlett Packard, USA) connected to a Mass Spectrometer (Finnigan SSQ7000, USA) and analysed in selected ion monitoring mode (GC/MS-SIM).

2.3. Protein concentration

Protein concentration was determined using a modified microplate method (Bradford, 1976). Briefly, 5 µl diluted haemolymph samples (1:3 in physiological saline (0.02M NaCl)) were added to a 96-well microplate (Costar®) treated with a 1:3 dilution of BioRad Protein Assay Reagent A. The plate was incubated for 10 min at room temperature and 5 µl of BioRad Protein Assay Reagent B was added to each well and the sample plate was read at 595 nm. Concentration was calculated using a standard curve of bovine serum albumin (BSA) and converted to arbitrary units (AU) using calibration data.

2.4. Total haemocyte count (THC)

Immediately after extraction, sub-aliquots of haemolymph (20 µl) were diluted 1:3 with Baker’s Formol Calcium (2% sodium chloride, 1% calcium acetate, 4% formaldehyde) to fix cells and prevent aggregation. Total haemocyte counts were then carried out using an improved Neubauer haemocytometer under 40x magnification.

2.5. Protein concentration

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2.7. Total haemocyte count (THC)

Immediately after extraction, sub-aliquots of haemolymph (20 µl) were diluted 1:3 with Baker’s Formol Calcium (2% sodium chloride, 1% calcium acetate, 4% formaldehyde) to fix cells and prevent aggregation. Total haemocyte counts were then carried out using an improved Neubauer haemocytometer under 40x magnification.

2.8. Total haemocyte count (THC)

Immediately after extraction, sub-aliquots of haemolymph (20 µl) were diluted 1:3 with Baker’s Formol Calcium (2% sodium chloride, 1% calcium acetate, 4% formaldehyde) to fix cells and prevent aggregation. Total haemocyte counts were then carried out using an improved Neubauer haemocytometer under 40x magnification.

2.9. Total haemocyte count (THC)

Immediately after extraction, sub-aliquots of haemolymph (20 µl) were diluted 1:3 with Baker’s Formol Calcium (2% sodium chloride, 1% calcium acetate, 4% formaldehyde) to fix cells and prevent aggregation. Total haemocyte counts were then carried out using an improved Neubauer haemocytometer under 40x magnification.
0.4M NaCl, 0.1M MgSO4, 0.01M KCl, 0.01M CaCl2 (pH 7.4)) were transferred to a microplate. 5 µl aliquots of a blank physiological saline and 5 µl protein standards (0.2–1.0 mg ml⁻¹ bovine serum albumin) were also added in triplicate. 200 µl of diluted BioRad reagent (1:5 distilled water) were added to each well, and the absorbance at 595 nm recorded after 20min of incubation at 20°C.

2.3.4. Cell membrane stability

The membrane stability of haemocytes was assessed by measuring retention of neutral red (NR) dye (Babich and Borenfreund, 1992). Undiluted haemolymph samples (50 µl) were pipetted onto a microplate. After 45min incubation at 4°C, non-adhered cells were removed by rinsing with physiological saline, before adding 200 µl aliquots of NR solution (0.004% in physiological saline) to each well. After 3h of incubation at 4°C, excess NR solution was removed by rinsing with physiological saline and 200 µl of acidified ethanol (1% acetic acid, 99% ethanol) was added to breakdown cellular membranes and resolubilise the dye. The optical density (OD) of the NR was measured spectrophotometrically at 540 nm.

2.3.5. Phagocytosis

Phagocytic activity of haemocytes was assessed by measuring the uptake of neutral red stained zymosan particles (from Saccharomyces cerevisiae) (Pipe et al., 1995). Briefly, 50 µl of undiluted haemolymph was pipetted onto a microplate and incubated with the cells for 30 min at 20°C, after which 100 µl of Baker’s Formol Calcium was added to fix the cells and stop the reaction. Excess zymosan suspension was removed by rinsing twice with physiological saline, before adding 100 µl of acidified ethanol to resolubilise the dye. Phagocytic uptake of these dyed particles was determined spectrophotometrically at 540 nm. Protein concentration (mg ml⁻¹) was determined as described previously and the amount present in a 50-µl sample calculated, the OD of NR retention was then expressed as a function of this protein content.

2.3.6. Alkaline phosphates (ALP)

Alkaline phosphatase (EC 3.1.3.1) activity was measured in the cell-free plasma obtained by centrifuging 150 µl of haemolymph at 200 xg for 5min (4°C). The supernatant was transferred onto a microplate in 40 µl aliquots, and 180 µl of the liquid substrate p-nitrophenyl phosphate (SIGMA, UK) was added to each well. Following 20min incubation at 20°C, the absorbance was read at 405 nm and activity expressed as Aabs x 100 mg⁻¹ protein.

2.4. Statistical analyses

Immune parameters were measured in 10 individuals (with the mean value of each individual calculated from the triplicate samples) from each treatment group at each time point, with results expressed as mean values ±1 standard error. Univariate analyses of variance (ANOVA) were performed on each immune parameter to determine significant differences due to interactions (treatment × time) or main factors (treatment and time). Where differences occurred at, or above, the 95% confidence level, post hoc pairwise comparisons were also conducted.

3. Results

3.1. Chemical analyses

Whilst the nominal concentrations of dispersed oil were 0.06 and 0.25 mg l⁻¹, the average ∑PAHs measured in the exposure
Fig. 1. Effect of dispersed oil on biological endpoints (mean ± S.E.) in Chlamys islandica, following 7 and 15 days exposure, and after a 7-day post-exposure recovery period. (a) Total haemocyte count (THC), (b) plasma protein concentration, (c) cell membrane stability, (d) phagocytic ingestion of zymosan particles, and (e) alkaline phosphatase (ALP) activity mg protein⁻¹. Symbols indicate significant effects due to treatment (⁎) denotes significant difference from the control, (△) denotes significant difference from the low treatment, lowercase letters indicate significant effects due to time, (a) denotes a significant difference from 7 days, (b) denotes a significant difference from 15 days.

tanks were 4.2 × 10⁻⁴ and 2.4 × 10⁻³ mg l⁻¹, respectively, with the high-treatment containing approximately six times higher ∑PAH levels than the low-treatment group. An increase in ∑PAHs was also observed between the 24-h and 7 days sampling periods for both the low- and high-treatment groups (Table 1). Naphthalene (and its alkyl homologues) accounted for a large proportion of the PAH content of the dispersed oil, with concentrations of the dominant PAH, C2-naphthalene, ranging from 0.149 to 0.845 µg l⁻¹ in the low and high treatments after 24 h exposure, up to 0.225 and 0.954 after 7 days (Table 1). Only two other priority PAHs, fluorene and phenanthrene, were detected in the exposure tanks. However, these were considerably lower, with concentrations recorded at 13%
and 22% (phenanthrene) of the naphthalene levels in the high-exposure treatment after 24 h.

PAHs were accumulated in the scallop tissues after 15 days exposure, with C3-naphthalene recorded at the highest concentration of 1439 and 2422 µg kg⁻¹ in scallops from the low and high treatments respectively, reflecting the high levels of naphthalene homologues present in the seawater. A total of nine priority PAHs (as listed by the US EPA) was present in the tissues, of which the tricyclic PAH phenanthrene, was most strongly accumulated with a concentration of 56 and 105 µg kg⁻¹ observed in organisms from the low and high treatments (Table 2).

3.2. Biological endpoints

The number of circulating haemocytes in C. islandica was significantly increased following exposure to dispersed oil (F₂,₄₁ = 7.11, P < 0.01). Total haemocyte counts were significantly higher in organisms from the high-treatment group following 15 days exposure, with 16.9 x 10⁶ cells ml⁻¹ compared to 11.1 x 10⁶ cells ml⁻¹ in the control (Fig. 1a). These increased cell counts did not return to control levels after the 7-day recovery period; THC were still significantly elevated, with 4.3 x 10⁶ more cells ml⁻¹ of haemolymph relative to the control group.

Exposure of C. islandica to dispersed oil resulted in elevated plasma protein, with the highest mean concentration of 2.39 mg ml⁻¹ observed after 7 days in organisms exposed to 0.06 mg ml⁻¹ (Fig. 1b). Plasma protein levels were significantly different (F₄,₉₁ = 3.93, P = 0.01) with protein levels in the haemolymph higher in exposed organisms relative to the control after both 7 and 15 days (Fig. 1b). However, following the post-exposure recovery period, there was no significant difference in plasma protein from both the exposed and control groups with mean concentrations ranging from 1.36 to 1.46 mg ml⁻¹.

Whilst no significant change in cell membrane stability was observed in the control group throughout the exposure period (Fig. 1c), cell membrane stability in exposed scallops was affected by both treatment and exposure time, with a significant interaction between these two factors (F₄,₉₁ = 9.70, P < 0.01). After 7 days exposure, there was no significant difference in cell membrane stability between oil-treated and control scallops. Membrane stability was compromised following 15 days exposure to 0.06 and 0.25 mg I⁻¹ dispersed oil, with the OD of retained neutral red dye significantly decreased to 66% and 40% of the control group respectively (Fig. 1c). This reduction in cell membrane stability was reversed after the post-exposure recovery period, with the OD significantly higher in organisms from the oil-exposed treatments compared to the control group, with a maximum OD of 10.90 mg⁻¹ protein.

Phagocytic activity in the haemocytes of C. islandica was significantly altered during exposure to dispersed oil, with an interaction between treatment and exposure time (F₂,₄₁ = 4.52, P < 0.01). After 7 days, phagocytic ingestion of zymosan in organisms from the oil-exposed treatments was not significantly different from the control group, with phagocytosis ranging from 24.83 x 10⁶ to 28.33 x 10⁶ particles mg⁻¹ protein (Fig. 1d). However, 15 days exposure to 0.06 mg ml⁻¹ oil (high-treatment group) resulted in a significantly reduced phagocytic activity of 16.2 x 10⁶ particles mg⁻¹ protein, only 48% of the level observed in the control group after 15 days. Following the recovery period, phagocytic ingestion in individuals from the high-treatment group (30.6 x 10⁶ particles mg⁻¹ protein) had returned to control levels (Fig. 1d), a significant increase on that observed after 15 days exposure.

A maximum ALP activity of 5.86 U mg⁻¹ protein was recorded in the plasma of oil-exposed scallops after 7 days exposure at 0.06 mg I⁻¹ (Fig. 1e). However, no significant change in ALP activity was observed as a result of an interaction between treatment and exposure time (F₂,₄₁ = 0.59, P = 0.669), and no significant effect of these main factors was detected; F₂,₄₁ = 0.19, P = 0.831 and F₂,₄₁ = 1.29, P = 0.281 for treatment and exposure time respectively.

4. Discussion

An effective immune response is essential in maintaining the health of an organism, and may subsequently affect growth, reproduction and, ultimately, survival (Blaise et al., 2002). The results of this study demonstrated how exposure to sub-lethal concentrations of dispersed oil alters the cellular immune function in the Arctic Scallop C. islandica.

Various contaminants have been reported to exert immunotoxic effects on organisms, including oil (Ordás et al., 2007) and oil-related components such as PAHs (Coles et al., 1994; Wootton et al., 2003a; Prouin et al., 2007). The low levels of PAHs recorded in the seawater of the exposure tanks, relative to the nominal dispersed oil concentration, may reflect the partitioning of these hydrocarbons between the oil droplets and water (Skadsheim, 2004). The adsorption of oil onto both the surfaces of the tank and the organisms themselves will also reduce the PAHs measured in the seawater. This progressive loading of oil onto these surfaces may also account for the increase in PAHs observed during the exposure period, with the adsorbed oil providing a secondary supply of PAHs to the seawater phase, increasing PAH concentrations over time (Skadsheim, 2004).

With PAHs having high octanol-water partition coefficients (log Kₐq > 3.5), they are readily taken up by organisms (Nielsen et al., 1997), and with bivalve molluscs having a limited ability to metabolise PAHs, they can accumulate to high levels in the tissues (Moore et al., 1989). The uptake of PAHs by C. islandica is likely to be the main cause of the low PAH levels observed in the seawater, and is demonstrated by the observed body burden. Evaluating bioaccumulation is an important part in assessing the risk that contaminants pose to marine organisms. Based on the ratio of PAHs in biota and seawater (averaged from the low and high treatments), bioconcentration factors (BCFs) were determined to provide an indication of the potential for bioaccumulation (Dimirov et al., 2002; Arnott and Gabas, 2006). The BCFs of individual priority PAHs ranged from 169 for naphthalene up to 5434 for phenanthrene, a known immunotoxicant (Wootton et al., 2003a).

Body burden analysis also indicated the accumulation of other priority PAHs in the tissues of C. islandica including fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b,k)fluoranthene and benzo(b,k,l)fluoranthene, despite their concentrations in seawater being below the detection limit. As a result, BCFs were unable to be calculated for these higher molecular weight hydrocarbons. However, with PAH hydrophobicity demonstrating a linear relationship with log Kₐq (Baussant, 2004), it can be assumed that these PAHs, some of which are reported immunotoxicants (Grundy et al., 1996a; Prouin et al., 2007; Bado-Nilles et al., 2008), may be strongly accumulated by organisms that lack an efficient detoxification mechanism.

Haemocytes play an important role in the defence mechanisms of the bivalve immune system (Pipe et al., 1999b), with the number of circulating cells often increasing in response to contaminant stress (Coles et al., 1994, 1995) as observed in this study. Whilst increased total haemocyte count following 15 days exposure to 0.25 mg I⁻¹ dispersed oil may be a result of cell migration from the tissues, maintenance of elevated cell counts after the recovery period suggests stimulation of cell production (Pipe et al., 1999). PAHs can cause cytolsis in lysosome-enriched cells such as haemocytes (McCormick-Ray, 1987) with an elevation in haemocyte number indicating compensation for cell lysis. Cell lysis may
also contribute towards the significant increase in plasma protein concentration observed in organisms from the low and high treatments, a possible result of the release of cell contents into the plasma. Alternatively, induction of humoral factors such as lysozyme (Anderson and Beaver, 2001) and pro-phenoloxidase (Xing et al., 2002) may contribute to the increased protein concentration found in the oil-exposed animals.

Cell membrane stability is extremely important in the maintenance and functioning of cellular processes. PAHs can impair cell membrane function (French-McCay, 2004) with reduced cell membrane stability a common result of contaminant-induced cell membrane disruption (Moore, 1985). Within bivalve haemocytes, lysosomes sequester, accumulate and metabolise a range of xenobiotics (Hagger et al., 2005). However, this denaturation process is not effective if the storage capacity is exceeded and may result in damage to the cell (Moore et al., 1984). Overloading of the detoxification process may account for the reduced membrane stability observed in the present study after 15 days exposure to both low and high dispersed oil treatments. Alternatively, the metabolic phase of PAH detoxification can result in the proliferation of reactive oxygen species (ROS) (Winston et al., 1996), directly damaging cell membranes through lipid peroxidation (Gómez-Mendikute and Cajaravuk, 2003). A reduction in cell membrane stability is inversely correlated with lysosomal volume (Moore et al., 2006) and has been reported in marine molluscs following exposure to organic pollutants (Da Ros et al., 2007). If lysosomal enlargement occurred as a result of the reduced cell membrane stability and persisted after the 7-day recovery period, this would provide a larger volume for the neutral red dye to be taken up, accounting for the significant increase in neutral red retention of the oil-exposed recovery scallops compared to the control animals.

Phagocytosis, an essential part of the invertebrate immune response, has been shown to be impaired by numerous organic contaminants including polychlorinated biphenyls (Fourrier et al., 2002) and PAHs (Sami et al., 1993; Grundy et al., 1996a; Bado-Nilles et al., 2008), and was significantly reduced in C. islandica following 15 days exposure to 0.25 mg l⁻¹ dispersed oil. Phagocytic processes are dependent on membrane properties of the haemocytes (Grundy et al., 1996b), therefore, any alterations in the cell membrane may impact phagocytic activity. It has been suggested that disruption of cell membranes is more likely to occur from exposure to lipophilic organic contaminants rather than inorganic metals (Dyrynda et al., 1998), with PAHs (penetrating phospholipid monolayers in model membrane systems) altering membrane fluidity (Nelson et al., 1990). Results from the current study, however, do not demonstrate a coupling of impaired phagocytosis and reduced cell membrane stability, suggesting other mechanisms may also affect the phagocytic process. Haemocyte motility, or changes in membrane recognition, can also impair phagocytic ability (McCormick-Ray, 1987), with PAHs altering the expression of membrane receptors (Sami et al., 1993), which may interfere with the ability to recognise non-self material, essential to the phagocytosis process.

The enzyme alkaline phosphatase participates in the degradation and breakdown of invading non-self material (Liu et al., 2004), and has been recorded in various bivalve species (Xue and Renault, 2000; Xing et al., 2002; Liu et al., 2004; Jing et al., 2006). Previous work has reported increased ALP activity in the oyster Pinctada fucata (Grundy et al., 1996a), and reduced dispersed oil exposure did not significantly alter ALP activity in C. islandica. Previous studies have demonstrated ALP to be a polymorphic enzyme, with variable activity between organisms (Fahselt, 1987; Takada et al., 1990). Such polymorphism has been reported in the coding loci for other enzymes in C. islandica (Fevolden, 1992) and may account for the large variability in ALP activity observed in this species during the present study.

Due to the range on immune parameters that constitute the multifaceted innate immunity of invertebrates, and their inter-linked functions (Pipe et al., 1995b; Livingstone et al., 2000), it is essential to use a suite of endpoints, similar to the approach used in this study, in assessing immunocompetence. Previous studies have proposed the use of an integrated measure to reflect the combined results of different biological endpoints (Auffret et al., 2004, 2006; Gagné et al., 2008), with Auffret et al. (2004) developing an immunotoxicological index based on the cumulative variation of each parameter from a reference value. However, such an approach requires the collection of species-specific baseline data over long-term surveys, something that was beyond the scope of this study.

5. Conclusion

Whilst this study demonstrated an alteration in aspects of immune function caused by exposure to sub-lethal concentrations of dispersed oil, it is not clear whether the overall immunocompetence of the organism was compromised. As the invertebrate immune system consists of a multifaceted defence system, impairment in one aspect of immune function may be compensated for by another defence mechanism. Therefore, further work is required to assess the ability of C. islandica to respond to an immune challenge following contaminant exposure in order to increase the ecological relevance of such results. In addition, alteration in immune function observed in this study also appears to be reversible upon removal of the contaminant stress. However, the impact of long-term continuous exposure and high-level acute exposure is still unknown and may have consequences for disease resistance and hence survival.

At present, environmental risk assessment (ERA) is the widely accepted process on which environmental management decisions are based. The ERA consists of four main steps: hazard identification, dose–response assessment, exposure assessment and risk characterisation. This present study contributes towards the first step in the ERA procedure through identification of the immunotoxic effects of dispersed oil on C. islandica, an essential part of establishing the risks associated with increased oil production in Arctic regions.

Acknowledgements

This study was funded by a University of Plymouth (UK) HEIF 3 research studentship and IRIS-Biomiije (Norway) and ConocoPhillips as part of their Arctic Environmental Research Programme.

References


Paper II

Functional immune response in *Pecten maximus*: Combined effects of a pathogen-associated molecular pattern and PAH exposure

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2010 Fish and Shellfish Immunology 28: 249-252
Short communication

Functional immune response in *Pecten maximus*: Combined effects of a pathogen-associated molecular pattern and PAH exposure

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ARTICLE INFO

Article history:
Received 13 July 2009
Received in revised form
14 October 2009
Accepted 14 October 2009
Available online 23 October 2009

Keywords:
*Pecten maximus*
Scallop
Immune function
Phagocytosis
Lipopolysaccharide
PAMP
Polycyclic aromatic hydrocarbon
Phenanthrene

ABSTRACT

Pathogen-associated molecular patterns (PAMPs) enable recognition of structures present in microorganisms such as lipopolysaccharides (LPS). LPSs are an essential constituent of the outer membrane of Gram-negative bacteria, stimulating the innate immune system of invertebrates. Here, LPSs from *Escherichia coli* (O55:K5) were used to investigate the functional immune response of *Pecten maximus* after stimulation with a PAMP and to determine the combined effect of a phenanthrene exposure and LPS challenge. Organisms were exposed to 200 μg l⁻¹ phenanthrene and after 7 d were injected with either physiological saline (injection controls) or LPS solution, and returned to their respective exposure tanks. Haemolymph was sampled from the scallops 48 h post-injection and immune function was assessed using a combination of cellular biological responses. The LPS challenge significantly altered the immune response in *P. maximus* with increased cell counts and phagocytic activity. An immunosuppressive effect of phenanthrene was also observed in this study; however, exposure to phenanthrene did not significantly impair the organism's ability to respond to a PAMP challenge. The overall level of phagocytosis and cytotoxic capability following the LPS challenge was lower in phenanthrene exposed scallops and may have consequences for disease resistance in this commercially-exploited species.

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1. Introduction

The innate immune system of invertebrates relies upon the recognition of common structures present in microorganisms known as pathogen-associated molecular patterns (PAMPs), which play an important role in initiating an immune response [1]. One such PAMP are lipopolysaccharides (LPS) which constitute an essential part of the outer membrane of Gram-negative bacteria [2] and elicits a strong immune response in bivalves [3]. Whilst previous studies have investigated the immune response in organisms challenged with various PAMPs [2–6], the effect of contaminant exposure on an organism's ability to recognise and respond to a PAMP is unknown.

Polycyclic aromatic hydrocarbons (PAHs) are found throughout the marine environment and have been reported to exert immunotoxic effects in a range of invertebrates [7–9]. Due to their toxicity and ubiquitous distribution in the environment, 16 PAHs have been classed as priority pollutants by the US Environmental Protection Agency. With concentrations in excess of 1000 μg l⁻¹ recorded in seawater samples near areas of crude oil exploration [10], the tricyclic PAH phenanthrene is the most abundant PAH in aquatic ecosystems [11], and is readily bioavailable and toxic to a range of marine invertebrates [12].

The Great Scallop *Pecten maximus* is a commercially-important bivalve for both fisheries and aquaculture, with estimated UK landings of 20,000 t yr⁻¹ worth £30 million [13]. Previous work has indicated the susceptibility of its baseline immune parameters to the PAH phenanthrene, causing alterations in phagocytosis, cell membrane stability and the number of circulating haemocytes [14]. However, it is unclear if such effects will impair an organism's ability to respond to a PAMP challenge. Here we investigated the functional immune response of *P. maximus* after stimulation with LPS, and determined the combined effect of a phenanthrene exposure and LPS challenge.

2. Materials and methods

Diver-collected scallops, *P. maximus*, from Start Bay, Devon, UK (50°15′N 3°37′E) were exposed to 200 μg l⁻¹ phenanthrene (Sigma Aldrich, UK) and a filtered seawater (FSW) control (15 ± 1 °C, 34 PSU) in 61 static tanks (24 tanks in total, 1 animal per tank) for...
a total of 9 days. Phenanthrene was dissolved in dimethyl sulfoxide (DMSO), with the final concentration not exceeding 0.01% of the tank volume, as recommended for aquatic toxicity tests [15]. The DMSO solvent vehicle was also added to the FSW control tanks. The seawater was renewed and spiked with the phenanthrene stock solution every 24 h to maintain water quality and exposure concentration. Phenanthrene levels on day 1 in the exposure tanks were measured at 171 ± 15.6 µg l⁻¹ (GS/MS: HP5890 series II GC, HP5970 MSD Hewlett Packard, USA). No significant difference in the exposure concentration was observed over the exposure period (t-test: t = -0.924, P = 0.365), with 180 ± 18.5 µg l⁻¹ recorded on day 8. After 7 days, 6 scallops from each treatment were subjected to an immune challenge through the injection of 100 µl LPS from Escherichia coli 055:B5 (100 µg ml⁻¹ in physiological saline [0.02 M HEPES, 0.4 M NaCl, 0.1 M MgSO₄, 0.01 M KCl, 0.01 M CaCl₂; pH 7.4]) into the adductor muscle. A further 6 animals from each treatment were injected with 100 µl physiological saline (phys. sal.) to act as controls. Scallop samples were returned to their individual exposure tanks for a further 48 h, before biological assays were conducted. Haemolymph (~0.8 ml) was extracted from the striated region of the adductor muscle using a 21-gauge needle and stored in siliconised Eppendorf tubes until analysis. Using a tiered approach to assess immune function, the first stage is based on the immune apparatus responsible for the immune defence, in this case the haemocytes. Therefore, total haemocyte counts and cell membrane stability were measured in addition to protein concentration [16]. The second tier is centred on mechanisms of immunity, with the determination of phagocytic activity and the ability of the haemocytes to lyse foreign cells (cytotoxic capability), as described in detail by Hannam et al. [17].

Cytotoxicity was expressed as the percentage of lysed target cells (sheep red blood cells). Immune parameters were measured in 6 individuals from each of the four final treatment groups (FSW + phys. sal., FSW + LPS, PAH + phys. sal., PAH + LPS) with results expressed as mean values ±1 standard error. Data sets were checked for homogeneity of variance and percentage data was arcsine square root transformed. Univariate analyses were performed using two-way ANOVA; tests were performed on each immune parameter to determine significant differences due to interactions (exposure treatment × injection challenge) or main factors (exposure treatment and injection challenge). Where differences occurred at, or above, the 95% confidence level, Fisher’s LSD post hoc pairwise comparisons were conducted.

3. Results and discussion

It was well documented that the limited ability of bivalve molluscs to metabolise PAHs often results in accumulation in the tissues; whilst body burdens were not determined in this study, phenanthrene is known to be readily taken up by organisms [12]. Based on similar reductions (75.36% ± 3.67%) in phenanthrene concentration in all the exposure tanks over 24 h, it is assumed that uptake of phenanthrene occurred. This is in agreement with evidence from previous work confirming the accumulation of phenanthrene in the scallop Chlamys islandica [16] and other bivalves including Mytilus edulis [18,19], Mytilus galloprovincialis [20] and Crassostrea virginica [21].

The LPS challenge significantly increased the response of some biological parameters in P. maximus, with a 25% increase in total haemocyte count (THC) and a 41% increase in phagocytic activity (Fig. 1A and D). However, no significant change in plasma protein
concentration was observed (Fig. 1B). Whilst crustaceans show a decrease in cell counts in response to LPS [22], LPS was not found to have any toxic effect on the haemocytes of the mussel *M. edulis* [2]. In addition, the initial decrease in cell counts of crustaceans, changed to elevated THCs 48 h post challenge [22] in accordance with what was observed after 48 h in the present study. This increase in cell counts is likely to reflect the initiation of an immune response to the invading non-self material through migration of haemocytes from the tissues and cell proliferation. This is further evidenced by the parallel increase in phagocytosis following the LPS challenge (Fig. 1D) and has also been reported in the rock oyster *Saccostrea glomerata* in response to PAMPs [4].

The change in haemocyte counts following LPS stimulation was also significantly impacted by phenanthrene exposure (interaction effect: $F_{1.25} = 6.00, P < 0.05$), with PAH-exposed scallops unable to further increase the number of circulating cells after the PAMP challenge (Fig. 1A). Due to the already elevated THC in scallops from the phenanthrene treatment, a common response to contaminant exposure [23], the capacity to further mobilise haemocytes from the tissues in response to the immune challenge is limited.

Many studies have demonstrated the immunomodulating effects of PAHs to various bivalves [17–9.24–26] which is in agreement with results from the present study with phenanthrene exposure significantly reducing cell membrane stability and phagocytic and cytotoxic activity (Fig. 1C–E). This inhibitory effect could be a result of direct effects on the immune capacity of the haemocytes. Pollutants can disrupt cell metabolism and ATP production [27] reducing haemocyte motility and, therefore, phagocytic activity. In addition, phagocytic processes are dependent on the membrane properties of the haemocytes [28] and the cytotoxic capability relies on direct cell-to-cell contact between haemocytes and the target cells [29]. With PAHs reportedly inducing peroxidative injury to cellular membranes of *P. maximus* [14], changes observed in the cell membrane stability are also likely to alter these immune parameters.

Despite the immunosuppressive effect of phenanthrene, it did not significantly affect cell membrane stability or the ability of scallops to illicit a phagocytic or cytotoxic response to the LPS challenge (interaction effect: $F_{1.25} = 0.01, P = 0.955, F_{1.20} = 0.19, P = 0.666$ and $F_{1.20} = 0.01, P = 0.926$ respectively). Whilst the LPS plus PAH-exposed scallops had reduced cell membrane stability, phagocytic and cytotoxic activity compared to LPS unexposed animals (Fig. 1C–E), this is a result of a significant exposure effect, not a decreased ability to respond to the PAMP, since a similar decrease was also observed in scallops not subjected to the LPS challenge. The recognition of PAMPs relies on pattern recognition receptors (PRRs) such as the toll-like receptors reported in the scallops *Cirripeda furren* [30] and *Argopodella marina* [31]. PRRs occur on the surface of haemocytes and therefore alterations in cell membrane integrity resulting from phenanthrene exposure (Fig. 1C) may be expected to interfere with PRRs, however the results from the present study indicate the ability to recognise PAMPs such as LPS is unaffected by phenanthrene exposure.

The results from this study demonstrated the ability of $P$. maximus to recognise the PAMP of LPS and respond through the activation of the innate immune system. Whilst the ability to recognise and respond to a PAMP is not compromised by PAH exposure, the overall level of phagocytic and cytotoxic activity is lowered in phenanthrene exposed scallops due to the immunosuppressive effects of this PAH. Such an effect of contaminant exposure may consequently increase the susceptibility of this commercial species to disease outbreaks.

Acknowledgements

This work was co-funded by a University of Plymouth (UK) HEIF 3 Research Studentship and IRIS-Alvainraje (Norway).

References


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Paper III

Effects of the model PAH phenanthrene on immune function and oxidative stress in the haemolymph of the temperate scallop *Pecten maximus*

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2010 Chemosphere 78: 779-784
Effects of the model PAH phenanthrene on immune function and oxidative stress in the haemolymph of the temperate scallop *Pecten maximus*

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous class of organic contaminants found throughout the marine environment, with the majority of inputs arising from anthropogenic sources (Law et al., 1997). Natural constituents of crude oil at around 19,000 mg kg−1 PAHs are both toxic and biologically persistent (Neff, 2002). PAH concentrations in excess of 600 μg L−1 have been reported in seawater following an accidental blowout of an offshore oil platform (Law, 1978), and PAH levels of 1700 μg L−1 can result from oil spills (Boehm and Page, 2007). Due to their hydrophobicity (log k OW > 4), PAHs are readily accumulated by organisms (Neff and Anderson, 1981), and their toxicity and widespread environmental presence has seen the inclusion of 16 PAHs on the US EPA priority pollutant list (US EPA, 2009). Phenanthrene is an important component of crude oil, with experiments indicating that phenanthrene dominates weathered oil after the volatile naphthalenes have been lost (Carl et al., 1999; Kennedy and Farrell, 2005). With input levels of phenanthrene increasing since the 1990s (Lima et al., 2003), elevated concentrations; up to 1460 μg L−1, have been recorded in seawater samples near areas of crude oil exploration (Aryakura et al., 2005). This low molecular weight PAH is readily bioavailable, exhibiting a greater bioconcentration factor in scallops than other PAHs present in crude oil (Hannam et al., 2005a) and is also highly toxic to marine organisms (Neff and Anderson, 1981). Whilst tissues such as the hepatopancreas, gonad and gill may be targets for PAH toxicity, contaminants reach these organs through the haemolymph circulatory system. This acts as a transfer medium for pollutants and their metabolic products, leaving haemocytes susceptible to potential deleterious effects (Pao et al., 2006).

Adverse effects of PAHs may also result in sublethal alterations of homeostatic mechanisms such as the immune system (Pipe et al., 1999). The complexity and integration with other physiological systems makes the immune system particularly sensitive to environmental contaminants with increasing evidence of immunotoxic properties in a range of xenobiotics including PAHs and crude oil (Galloway and Green, 2005). Bivalves have developed innate defences to recognise and protect against foreign material, with...
cellular immune responses largely centred on the multifunctional haemocytes (Galloway and Goven, 2006). Since growth, disease and survival of an organism is partly determined by the capability of its immune system (Blaise et al., 2002), alteration of immune function is important in assessing the sublethal effects of contaminant exposure (Luegen et al., 2004).

Once a PAH has been taken up by an organism it may be subjected to biotransformation reactions. In bivalve molluscs, PAH metabolism largely occurs through radical oxidation involving reactive oxygen species (ROS) (Stegeman and Lech, 1991) which can be generated at various stages along the metabolic pathway (Livingstone, 1991). ROS are produced continually in living cells, and are essential in maintaining cell function in biological systems. However, an imbalance between formation and neutralisation of these reactive species can induce oxidative damage (Valavanidis et al., 2005).

Recent advances in environmental risk assessment have highlighted the use of molecular, cellular, and physiological endpoints, known as biomarkers, to allow rapid assessment of contaminant effects (Galloway et al., 2006). These sub-organismal changes often occur over relatively short timescales and are often reversible, highlighting their potential as early-warning signals of contaminant-induced stress (Galloway et al., 2002). Bivalve candidates are widely used for immunotoxicology biomarker studies (Garcia et al., 2003; Gagnaire et al., 2004; Prouin et al., 2007; Ordás et al., 2007; Bado-Níñez et al., 2008) due to their worldwide distribution and sedentary, filter feeding behaviour (Pipe et al., 1999). With low metabolic rates, bivalves have a tendency to accumulate pollutants in their tissues (Auffret, 2005); in particular, Pteridinidae scallops reportedly concentrate toxic compounds to a greater extent than other bivalves, including the common sentinel species Mytilus edulis (Young-Lai and Aiken, 1986). The use of scallops in pollution-effect studies has increased over the last decade with PAH, crude oil, pesticide and metal toxicity demonstrated in Chlamys islandica (Camus et al., 2002; Hannam et al., 2005a), Bivalve zicac (Owen et al., 2002) and Adamussium colbecki (Regoli et al., 1998; Bonacci et al., 2004).

The Great Scallop Pecten maximus is commercially exploited by fisheries and aquaculture, with estimated UK landings of 20000 t year−1 worth £30 million (Briggs, 2000). Distributed along European Atlantic coasts from the Iberian Peninsula to northern Norway (Marshall and Wilson), 2008, this temperate species occurs in regions of crude oil production. In addition, the northern limit of P. maximus may be pushed further into Arctic regions as a result of increasing sea temperatures, where a rapid increase in oil exploration activity is predicted (IPCC, 2007), leaving this commercially important species susceptible to PAH exposure. Despite the importance of this species, knowledge of the biological effects of contaminant exposure in P. maximus is severely lacking. This study investigates the sublethal effects of phenanthrene exposure, associated with oil extraction and accidental spills, on the oxidative status and immune function of P. maximus using a suite of biological endpoints.

2. Materials and methods

2.1. Experimental design

Diver-collected Great Scallop, P. maximus, from Start Bay, Devon, UK (50°15'N, 3°37'E) were obtained through Britannia Shellfish Ltd, UK in March 2008. Experimental animals were confirmed to be of similar gonad maturation and at a pre-spawning stage of metamorphosis based on the criteria described by Mason (1961). Scallop were held in 50 L tanks (~30 animals per tank containing 10 μm carbon-filtered, aerated seawater (FSW): (15 ± 1 °C, 34 PSU) for at least two weeks prior to transfer into the exposure system. Water changes were carried out every 2 d and animals were fed twice weekly using the algal concentrate Isochrysis, instant Algae (approx. 2.7 × 106 cells per animal). Phenanthrene was dissolved in dimethyl sulfoxide (DMSO); the final concentration of DMSO did not exceed 0.01% of the total tank volume. Scallop were transferred to the experimental tanks with air exposure limited to <1 min to minimise stress. Organisms were exposed to nominal phenanthrene concentrations of 50, 100 and 200 μg l−1, and a vehicle control in individual 6 L aerated glass tanks with one animal per tank (four treatments, six tanks per treatment (n = 24)). Based on reported observed effects from previous studies, scallop were subjected to each treatment for 7 d, with the seawater renewed and spiked with the PAH stock solution every 24 h to maintain water quality and exposure concentrations.

2.2. Chemical measurements

To confirm the phenanthrene exposure concentration, and any decrease in phenanthrene concentration over 24 h, 1 L water samples were collected from the exposure tanks immediately after the initial phenanthrene spike and 24 h post spike prior to seawater renewal. Samples were collected in 1 L amber Duran bottles and extracted through liquid-liquid extraction with 5 ml cyclohexane. Each extraction was carried out by mixing the sample and solvent through inversion and passed through a 0.45 μm separating funnel. The water phase was drained back into the sampling flask and extracted a further two times. Combined extracts were dried with anhydrous Na2SO4, and concentrated under nitrogen to 1 ml using an 1870 Pierce Reacti-VAP™ tube (Rockford, USA) and analysed using GC/MS (HP5880 series GC, HP5970 MSD Hewlett Packard, USA).

2.3. Biological measurements

Haemolymph from six scallops at each exposure concentration was sampled after 7 d. Haemolymph (0.8 ml) was extracted from the adductor muscle using a 21-gauge needle attached to a 1 ml syringe and transferred to a siliconised Eppendorf tube. Samples for the determination of total cell counts, protein content, cell membrane stability and phagocytosis activity were stored on ice to minimise cell aggregation. Haemolymph samples for oxidative stress analysis were stored at −80 °C until required. All measurements conducted on haemolymph samples were carried out in triplicate.

2.3.1. Total haemocyte count

Immediately after extraction, haemolymph samples were diluted 1 in 4 with Baker's formal calcium (BFC [2% sodium chloride, 1% calcium acetate, 4% formaldehyde]), to fix cells and prevent cell aggregation. Total haemocyte counts (THC) were carried out using an improved Neubauer haemocytometer under 40· magnification.

2.3.2. Protein concentration

 Plasma protein concentration was determined using the method of Bradford (1976) modified for use with microplates. Briefly, diluted haemolymph samples (1.3 physiological saline [0.02 M HEPES, 0.4 M NaCl, 0.1 M MgCl2, 0.01 M KC1, 0.01 M CaCl2, pH 7.4]) were transferred in 5 ml aliquots to a microplate. Five microtitre aliquots of a blank (physiological saline) and 5 μl protein standards (0.2-1.0 mg ml−1 bovine serum albumin) were also added in triplicate Two hundred microtitre dilutions of diluted Brookfield reagent (1.5 distilled water) was added to each well and the absorbance at 595 nm recorded after 20 min incubation at 20 °C.
2.3.3. Cell membrane stability

Haemocyte membrane stability was assessed by measuring retention of Neutral Red (NR) dye (Babich and Borenfreund, 1992) described in detail by Hannam et al. (2009a). Briefly, haemolymph samples (50 µL) were pipetted onto a microplate in triplicate. After 45 min incubation at 4 °C, non-adhered cells were removed by rinsing with physiological saline, before adding 200 µL aliquots of 0.004% NR solution to each well. After 3 h incubation at 20 °C, excess NR solution was removed by rinsing with physiological saline and 200 µL acidified ethanol was added to break down cellular membranes and resolubilise the dye. The absorbance of the neutral red was measured spectrophotometrically at 550 nm and expressed as a function of protein content of the adhered haemocytes.

2.3.4. Phagocytosis

Phagocytic activity of haemocytes was assessed by measuring the uptake of neutral red stained zymosan particles (from Saccharomyces cerevisiae) based on the method of Pipe et al. (1995) as previously described by Hannam et al. (2009a). Briefly, haemolymph samples (50 µL) were incubated at 4 °C for 1 h within a microplate, after which non-adhered cells were removed by rinsing with physiological saline (100 µL × 2), before adding 50 µL of dyed zymosan suspension (50 × 10^6 particles mL^-1). This was incubated for 30 min (20 °C), after which the reaction was halted through the addition of 100 µL BFC. Excess zymosan suspension was removed by rinsing with physiological saline and 100 µL acidified ethanol was added to solubilise the dye before recording the absorbance at 550 nm. Phagocytic uptake of zymosan particles by haemocytes was determined against a standard curve and expressed as a function of protein content of the adhered cells.

2.3.5. Total glutathione

Determination of total glutathione in haemolymph lysate (GSH + GSSG) was based on the cyclic reduction assay of Owens and Belcher (1965). Haemolymph samples were centrifuged at 200 g for 5 min (4 °C), the supernatant was removed and cells resuspended in physiological saline. The haemocytes were lysed through sonication (30% duty cycle, 3 × 15 s; Ultrasonic Processor W-385 (Heat Systems Ultrasonics, USA)) in an ice bath and stored at −80 °C until analysis. Haemolymph lysate samples (80 µL) were thawed on ice before adding 80 µL DTNB solution (10 mM DTNB, 100 mM KH₂PO₄, 5 mM EDTA). Aliquots of 40 µL DTNB-treated samples were transferred to a microplate and 210 µL of glutathione reductase solution (2.06 U mL⁻¹ glutathione reductase, 100 mM KH₂PO₄, 5 mM EDTA; pH 7.5) was added. After allowing samples to equilibrate for 1 min, 60 µL of 1 mM NADPH was added to start the reaction, and the change in absorbance measured kinetically at 405 nm for 10 min. Concentrations of total glutathione were determined against a 40 µM GSH standard, and expressed per mg protein.

2.3.6. Lipid peroxidation

Oxidative damage in the form of haemocyte lipid peroxidation (LPO) was assessed using a modified method of thio barbituric acid reacting substances (TBARS) (Gamejo et al., 1999). Haemolymph samples were thawed on ice and transferred in 40 µL aliquots onto a microplate containing 10 µL BHT (1 mM 2,6-di-tert-butyl-4-methylphenol in absolute ethanol) to prevent further LPO. One hundred microlitre of extraction buffer (20 mM Tris–chloride; 0.15 M KCl; 0.5 M sucrose, 1 mM EDTA; pH 7.6) was added to each well, followed by 50 µL TCA solution (50% w/v trichloroacetic acid) and 75 µL TBA solution (1% w/v thio barbituric acid in 50 mM NaOH). After 60 min incubation at 60 °C, the plate was cooled on ice and the absorbance at 530 nm recorded. Results were measured as malondialdehyde equivalents (MDAₑ) determined against a standard curve using 1,1,3,3-tetraethoxypropane (0–24 µM), and expressed per mg protein.

2.4. Statistical analyses

Biological endpoints were measured in 6 individuals from each treatment with results expressed at mean values ± standard error. Data sets were checked for homogeneity of variance and univariate analysis was performed using one-way ANOVA (Statgraphics 5.1). Post-hoc pairwise comparisons were conducted (Feisher's LSD) to identify where significant differences occurred at or above the 95% confidence level (associated probability <0.05).

3. Results

3.1. Chemical analysis

Phenanthrene concentrations recorded in the exposure tanks after the initial PAH spike were 45.8, 83.2 and 163.2 µg L⁻¹ for the 50, 100 and 200 µg L⁻¹ treatments, respectively (Table 1). These measured phenanthrene concentrations represented between 82% and 92% of the nominal exposure concentrations, with a reduction in this percentage with increasing nominal concentrations. Phenanthrene levels in the exposure tanks had decreased after 24 h, with a 73–75% reduction in the initial phenanthrene concentration across all treatment groups (Table 1). No phenanthrene was detected in the control tanks.

3.2. Biological analyses

No mortalities were recorded from any treatment groups during the exposure, however sublethal effects were observed.

The total number of circulating haemocytes in P. maximus increased following in vivo exposure to 100 and 200 µg L⁻¹ phenanthrene (Fig. 1A; F₂,23 = 3.73, P < 0.05). The lowest total haemocyte count (THC) was recorded in the control group, whilst exposure to 200 µg L⁻¹ increased the average number of haemocytes by 32% (Fig. 1A). Increasing phenanthrene concentrations also produced a trend of increasing plasma protein, with levels ranging from 1.04 mg mL⁻¹ in the control group, up to 1.71 mg mL⁻¹ in organisms exposed to 200 µg L⁻¹ (Fig. 1B). However, there was a large degree of variability in the data, and the measured increase in protein concentrations were not statistically significant (F₂,23 = 1.74, P = 0.191).

A trend of declining cell membrane stability was observed with increasing phenanthrene concentration (Fig. 1C), with exposure to 50, 100 and 200 µg L⁻¹ reducing membrane stability by 11%, 29% and 46% respectively, relative to the control group. However, only exposure to the highest phenanthrene concentration tested, 200 µg L⁻¹, resulted in a significant reduction in cell membrane stability after 7 d (F₂,23 = 4.24, P < 0.05) with an A₅₀ of 3.36 mg⁻¹ protein, compared to the control (6.27 mg⁻¹ protein) and 50 µg L⁻¹ (5.39 mg⁻¹ protein) treatments. The phagocytic activity of P. maximus haemocytes was significantly reduced following exposure to 200 µg L⁻¹ phenanthrene (F₂,23 = 3.20, P < 0.05), with the uptake of zymosan at 13.7 × 10⁵ particles mg⁻¹ protein, just 50% of the levels observed in the control group (Fig. 1D).

Table 1

<table>
<thead>
<tr>
<th>Phenantrene concentration (µg L⁻¹)</th>
<th>Nominal</th>
<th>50.0</th>
<th>100.0</th>
<th>200.0</th>
</tr>
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<tr>
<td>0 h</td>
<td>45.8 (1.52)</td>
<td>83.2 (2.75)</td>
<td>163.9 (3.33)</td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>12.0 (1.65)</td>
<td>20.9 (2.45)</td>
<td>41.6 (4.06)</td>
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</table>
Mean glutathione levels ranged from 13.9 nmol mg⁻¹ protein in haemocyte lysate from the control group to 8.6 nmol mg⁻¹ protein in 200 µg L⁻¹ PAH-exposed organisms (Fig. 1E; F(T,23) = 4.40, P < 0.05). Lipid peroxidation, as determined by TBARS, significantly increased with exposure to phenanthrene (F(T,23) = 3.71, P < 0.05) with maximum LPO, 6.6 nmol MDA, mg⁻¹ protein, observed in the 200 µg L⁻¹ phenanthrene treatment (Fig. 1F).

4. Discussion

The adsorption of phenanthrene onto the surface of the both the organisms and exposure tank will contribute towards the reduction in phenanthrene levels measured in the seawater, and the progressive loading onto these surfaces may act as a secondary supply of phenanthrene to the seawater phase during the course of the exposure (Skadsheim, 2004). In addition, the reduction in phenanthrene concentrations over 24 h is likely to reflect the bioavailability and uptake of this compound; with a log Kow = 4.57 (Skadsheim et al., 2009), phenanthrene is readily taken up by organisms (Nett and Anderson, 1981). In order to maintain phenanthrene exposure levels, despite the large uptake of this PAH by P. maximus, the water was renewed and spiked at regular frequency (every 24 h). The limited ability of bivalve molluscs to metabolise PAHs often results in accumulation in the tissues; whilst body burdens were not determined in this study, evidence from previous work has confirmed the accumulation of phenanthrene in C. islandicus (Hannam et al., 2009a); M. edulis (Law et al., 1999b; Moore et al., 2007); Mytilus galloprovincialis (Valavanidis et al., 2008) and Crassostrea virginica (Edler and Dressier, 1988).

There is considerable evidence of the vulnerability of an organism’s immune system to environmental contaminants (Auffret et al., 2002; Pérez-Cadahia et al., 2004; Trouin et al., 2007; Ordás et al., 2007). An effective immune response is important in the maintenance of organism health and, in bivalves, relies on efficient functioning of the haemocytes (Pipe and Cole, 1995). Haemocytes participate in a variety of functions, including gas exchange, osmoregulation, nutrient digestion and distribution, waste excretion and wound repair. In addition to these roles, haemocytes are also essential for innate immunity (Cheng, 1981; Beninger and Le Pennec, 2006). An increase in the number of circulating haemocytes was reported in P. maximus following phenanthrene exposure in excess of 100 µg L⁻¹, a trend which appears to be a common response to environmental stress (Auffret et al., 2006). Such an increase in THC may be a compensatory response to the reduction in cell membrane integrity also observed in this study. The increase in THC may reflect migration of haemocytes from the tissues or stimulation of cell production (Pipe et al., 1999). Despite the increase in cell count, low phagocytic activity was observed after phenanthrene exposure, suggesting the latter case more likely; following cell proliferation, immature haemocytes may have a reduced phagocytic capability (Hannam et al., 2009b).

Oxidative stress parameters were measured in the haemolymph (Gagné et al., 2006; Pan et al., 2006; Liu, 2008; Kaloyianni et al., 2008) of P. maximus since the cellular immune function relies on the haemocytes that move throughout this medium. Glutathione is regarded as an essential antioxidant defence mechanism in cells, acting as an oxysradical scavenger, removing hydrogen peroxide (H₂O₂) by coupling the reduction to H₂O with the oxidation of glutathione (Halliwell and Gutteridge, 2007). An increase in glutathione may, therefore, indicate up regulation of antioxidant defences, and this has been correlated with increased PAH tissue burdens in the bivalve Perna viridis (Cheung et al., 2001). Conversely, the results from this study indicate a reduction in glutathione following phenanthrene exposure. This is likely to indicate enhanced formation of ROS overwhelming this antioxidant defence system. When mass oxidation of reduced glutathione occurs, oxidised glutathione may be excreted from the cell more rapidly than it can be reconverted to the reduced form catalysed by glutathione reductase, resulting in decreased total glutathione levels (Regoli et al., 1998). Phenanthrene exposure induces a similar decrease in glutathione in the fish Carassius auratus (Yin et al., 2007), and a reduction in glutathione has also been reported in the Antarctic scallop, A. colbecki following metal exposure (Regoli et al., 1998).
Glutathione also plays an important role in detoxification reactions as a key conjugate of electrophilic intermediates (Van der Oost et al., 2003). Metabolised PAH intermediates, in the form of quinones, have a strong affinity for cellular thiols such as glutathione (Xue and Warshawsky, 2005) and previous studies have suggested that phenanthrene metabolites form glutathione conjugates in C. auratus (Yin et al., 2007). Therefore, the reactivity of phenanthrene intermediates to conjugate with the glutathione molecule may also contribute to reduced levels of total glutathione observed in this present study.

Depletion of glutathione is seen as a sign of oxidative stress and is often a predisposing factor in adverse effects of oxidative damage (Ringwood et al., 1998). The targeting of cell membranes by BCS can cause an autocatalytic oxidation process known as lipid peroxidation (LPO) (de Almeida et al., 2007). An increase in LPO was observed in this current study following exposure to the highest phenanthrene concentration tested (200 μg l⁻¹). LPO can perturb membrane structure and function by altering membrane fluidity, compromising membrane integrity, inactivating membrane-bound enzymes and disrupting surface receptor molecules. In addition, the early stages of LPO form lipid hydroperoxides (LOOH) which also participate in redox reactions and can exacerbate peroxidative cell injury. During LPO, aldehydes such as malondialdehyde are formed as by-products, and these can react with DNA bases forming DNA adducts (Halliwell and Gutteridge, 2007). Previous work has demonstrated an increase in LPO as a result of PAH exposure in sea bream Sparus auratus (Kopecka-Pilarczyk and Correia, 2008) and sea bass Dicentrarchus labrax (Abdah et al., 2008) as well as in the bivalves M. galloprovincialis and Mytilus arenaria (Frouin et al., 2007; Kaloyanni et al., 2009).

5. Conclusion

The results from this study demonstrate the disruption of oxidative status resulting in cellular lipid peroxidation, and reduced immune function in the Great Scallop P. maximus exposed to the model PAH, phenanthrene. Compared to other PAHs, phenanthrene is considered relatively less toxic, with a toxic equivalence factor of 0.001 relative to benzo(a)pyrene (Michel and LeCuy, 1992). However, the ubiquitous distribution of phenanthrene in the aquatic environment and its tendency for accumulation within organisms, results in the potential for deleterious effects.

Since a bivalve's ability to mount an efficient immune response is reliant upon the integrity and efficient functioning of haemocytes, PAH-induced stimulation of BCS production, is likely to be a major contributor to the reduction in an organism's immune-compliance. Previous reports have suggested such a link between oxidative stress and altered immune function in vertebrates (Koner et al., 1997, 1998). Here, oxidative stress and immune function was measured together in the haemolymph of contaminant-exposed scallops, providing evidence for overloading of the antioxidant capacity and subsequent immune suppression in bivalve molluscs.

A reduction in the immune function of P. maximus following PAH exposure, as indicated by the results of this study, may increase the susceptibility to bacterial infection. This may have consequences in terms of the health of scallops, and may also have implications for the human consumption of P. maximus.

Acknowledgements

This work was co-funded by a University of Plymouth UK HEFRC research studentship and IRS-Akvanni (Norway).

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