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An ecotoxicological assessment of the impacts of chronic exposure to metals and radionuclides on marine mussels: relating genotoxicity to molecular and organism-level effects

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Abstract

An ecotoxicological assessment of the impacts of chronic exposure to metals and radionuclides on marine mussels: relating genotoxicity to molecular and organism-level effects.

Lorna Jane Dallas

Metals and radionuclides are environmentally relevant contaminants, yet their potential impacts on marine organisms have not been adequately evaluated. This is especially true for exposures of longer duration and/or lower contaminant concentration (i.e. chronic) which are often more representative of real world scenarios. In this context, a suite of biomarkers at different levels of biological organisation were investigated in an ecologically relevant bivalve species, *Mytilus galloprovincialis* after exposure to nickel (a metal), zinc pyrithione (an organometal) and tritiated water (a radionuclide). These contaminants were chosen based on their differing properties, and hence, mechanisms of action. All three contaminants produced genotoxicity (DNA strand breaks, as measured by the comet assay, and induction of micronuclei [MN]). For nickel (> 1800 µg L$^{-1}$) and tritiated water (15 MBq L$^{-1}$), biomarkers at lower levels of biological organisation (i.e. DNA strand breaks, MN, changes in the expression of key stress response genes) were more sensitive than those at higher levels (i.e. clearance rate, attachment, tolerance of anoxia). In particular, exposure to tritiated water for 14 days resulted in DNA damage and molecular alterations without affecting higher level responses. As environmental contaminants could interact with other physical or chemical stressors in a complex environment, further exploration of biological responses revealed modulation by hyperthermia with concomitant changes in the transcriptional expression of key defence genes (*hsp70, hsp90, mt20, p53* and *rad51*). In contrast to nickel and tritiated water, exposure to both 0.2 and 2.0 µM zinc pyrithione caused significant deviation from concurrent controls for every biomarker examined, suggesting that further investigation of the environmental impacts of this contaminant is particularly necessary. Variation in biological responses induced by different contaminants suggests that potential links between levels of organisation should be evaluated on a contaminant-specific basis. The integrated, multiple biomarker approach used in the current study provides a robust methodology for such studies, which could be translated to other ecologically relevant species for proper evaluation of risks to both environmental and human health.
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This thesis has not been produced in isolation. Far from it. I would like to take this opportunity to thank all those who have contributed, whether they realise it or not, and to apologise for any omissions from the following list.

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Pour mes collègues CHRONEXPO. Nous vous remercions de votre soutien et de l’amitié. Désolé pour mon Français limité. Restez en contact!

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Author’s Declaration

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

Work submitted for this research degree at the Plymouth University has not formed part of any other degree either at Plymouth University or at another establishment.

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Relevant scientific seminars and conferences were regularly attended at which work was often presented. Four papers have been accepted for publication in refereed journals.

Signed: ____________________

Date: ____________________

Word count for the main body of this thesis: ~67,540
Publications & Conferences

Publications:


Conference presentations:

2013:

*September: Plymouth, UK.* Joint Annual Meeting of ERIC and SETAC  
Poster presentation:  
Zinc pyrithione ecotoxicity in marine mussels: an integrated study revealing effects at multiple levels of biological organisation.

2012:

*May: Berlin, Germany.* SETAC 44th Annual Meeting  
Poster presentation:  
The effect of sublethal nickel concentrations on *Mytilus galloprovincialis*: A multiple biomarker study.
April: Portsmouth, UK. COGER Annual Meeting
Platform presentation: (Winner - "Best Presentation")
An integrated approach to the assessment of chronic exposure of marine mussels to tritiated water: linking radiation dose with genotoxic, histological and physiological impacts.

2011:
December: Plymouth, UK. ‘Blue Horizons’, 4th Annual PMSEF Annual Conference
Poster presentation:
The effect of sublethal nickel concentrations on *Mytilus galloprovincialis*: A multiple biomarker study.

December: Plymouth, UK. Biogeochemistry Group Annual Meeting
Platform presentation:
The effect of sublethal nickel concentrations on *Mytilus galloprovincialis*: A multiple biomarker study.

July: Stirling, UK. COGER Annual Meeting
Platform presentation:
Assessing the effects of ionising radiation on aquatic invertebrates: An ecotoxicological perspective.

June: Nottingham, UK. UKEMS Annual Meeting
Poster presentation:
Assessing the effects of ionising radiation on aquatic invertebrates: An ecotoxicological perspective.

June: Weymouth, UK. Cefas Annual Student Day
Platform presentation:
Assessing the impacts of chronic exposure to contaminants on marine organisms.

April: Plymouth, UK. ERIC Annual Research Day
Platform presentation:
Assessing the effects of ionising radiation on aquatic invertebrates: An ecotoxicological perspective.
2010:
December: Plymouth, UK. Plymouth University MI Christmas Conference
Poster presentation:
Effects of ionising radiation on aquatic invertebrates: An ecotoxicological perspective.

December: Plymouth, UK. Biogeochemistry Group Annual Meeting
Platform presentation:
Effects of ionising radiation on aquatic invertebrates: An ecotoxicological perspective.
Abbreviations, Symbols and Acronyms

Abbreviations, symbols and acronyms used in the text are defined below, excluding those in equations, which are defined *in situ*.

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<tr>
<th>Abbreviation</th>
<th>Definition/Description</th>
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<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>$^3$H</td>
<td>Tritium</td>
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<tr>
<td>8-OHgua</td>
<td>7,8-dihydro-8-oxo-guanine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Apurinic/apyrimidinic</td>
</tr>
<tr>
<td>ASW</td>
<td>Artificial seawater</td>
</tr>
<tr>
<td>B(a)P</td>
<td>Benzo(a)pyrene</td>
</tr>
<tr>
<td>BAI</td>
<td>Bioeffects assessment index</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>Bq</td>
<td>Becquerel(s)</td>
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<tr>
<td>BRI</td>
<td>Biomarker response index</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CERRIE</td>
<td>Committee examining radiation risks of internal emitters</td>
</tr>
<tr>
<td>CF</td>
<td>Concentration factor</td>
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<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
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<tr>
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<tr>
<td>JNK</td>
<td>cJun-N-terminal kinase</td>
</tr>
<tr>
<td>CMR</td>
<td>Carcinogenic, mutagenic or reproductive</td>
</tr>
<tr>
<td>$C_q$</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CR</td>
<td>Clearance rate</td>
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<tr>
<td>DEFRA</td>
<td>Department of environment, food and rural affairs</td>
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<tr>
<td>DETR</td>
<td>Department for the Environment, Transport and the Regions</td>
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<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpsm</td>
<td>Dorsal parallel shell margin</td>
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<tr>
<td>DSB</td>
<td>Double strand break</td>
</tr>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>------------</td>
</tr>
<tr>
<td>EC</td>
<td>European Commission</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>Effective concentration (for 50% of test organisms)</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>Ethyl methane sulfonate</td>
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<td>Endonuclease III</td>
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<td>ENPs</td>
<td>Engineered nanoparticles</td>
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<td>EQS</td>
<td>Environmental quality standard</td>
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<td>ERA</td>
<td>Environmental risk assessment</td>
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<td>ERICA</td>
<td>Environmental Risk from Ionising Contaminants: Assessment and Management</td>
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<tr>
<td>EU</td>
<td>European Union</td>
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<td>EURATOM</td>
<td>The European Atomic Energy Community</td>
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<tr>
<td>F0</td>
<td>Parental generation</td>
</tr>
<tr>
<td>F1</td>
<td>First filial generation</td>
</tr>
<tr>
<td>F2</td>
<td>Second filial generation</td>
</tr>
<tr>
<td>Fpg</td>
<td>Formamidopyrimidine DNA glycosylase</td>
</tr>
<tr>
<td>g</td>
<td>Standard gravity</td>
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<td>GPX</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray(s)</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HRR</td>
<td>Homologous recombination repair</td>
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<td>Health status index</td>
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<td>Heat shock protein</td>
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<tr>
<td>HTO</td>
<td>Tritiated water</td>
</tr>
<tr>
<td>IAEA</td>
<td>International atomic energy agency</td>
</tr>
<tr>
<td>IBR</td>
<td>Integrated biomarker response</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductively coupled plasma optical emission spectrometry</td>
</tr>
<tr>
<td>ICRP</td>
<td>International commission on radiological protection</td>
</tr>
<tr>
<td>IEF</td>
<td>Iso-electric focusing</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>IPG</td>
<td>Immobilised pH gradient</td>
</tr>
<tr>
<td>IR</td>
<td>Ionising radiation</td>
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<tr>
<td>IRSN</td>
<td>Institut de radioprotection et de sûreté nucléaire</td>
</tr>
<tr>
<td>Kd</td>
<td>Distribution co-efficient</td>
</tr>
<tr>
<td>keV</td>
<td>Kilo electron volt(s)</td>
</tr>
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<td>LC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal concentration (for 50% of test organisms)</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal dose (for 50% of test organisms)</td>
</tr>
<tr>
<td>l/dpsm</td>
<td>Length:dpsm ratio</td>
</tr>
<tr>
<td>LET</td>
<td>Linear energy transfer</td>
</tr>
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<td>l/h</td>
<td>Length:height ratio</td>
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<td>LMPA</td>
<td>Low melting point agarose</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>LSC</td>
<td>Liquid scintillation counting</td>
</tr>
<tr>
<td>LT&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal time (for 50% of test organisms)</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption/ionisation</td>
</tr>
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<td>MBq</td>
<td>Megabecquerel(s)</td>
</tr>
<tr>
<td>µCi</td>
<td>Microcurie(s)</td>
</tr>
<tr>
<td>MDR</td>
<td>Mean diverticular radius</td>
</tr>
<tr>
<td>MET</td>
<td>Mean epithelial thickness</td>
</tr>
<tr>
<td>MeV</td>
<td>Mega electron volts</td>
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<td>MF</td>
<td>Mayflower marina</td>
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<td>Milligray(s)</td>
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<td>Microgray(s)</td>
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<td>MLR</td>
<td>Mean luminal radius</td>
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<td>Moloney Murine Leukemia Virus</td>
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<td>MN</td>
<td>Micronucleus</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<td>MS</td>
<td>Mass spectroscopy</td>
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<td>MTC</td>
<td>Maximum tolerated concentration</td>
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<td>MT</td>
<td>Metallothionein</td>
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<td>Description</td>
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<td>-------------</td>
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<tr>
<td>MXR</td>
<td>Multixenobiotic resistance</td>
</tr>
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<td>n</td>
<td>Number of test organisms in sample (treatment)</td>
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<td>NaPT</td>
<td>Sodium pyrithione</td>
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<td>NMPA</td>
<td>Normal melting point agarose</td>
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<td>NPP</td>
<td>Nuclear power plant</td>
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<tr>
<td>NFRP</td>
<td>Nuclear fuel reprocessing plant</td>
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<tr>
<td>NORM</td>
<td>Naturally occurring radioactive material</td>
</tr>
<tr>
<td>p</td>
<td>Probability</td>
</tr>
<tr>
<td>OBT</td>
<td>Organically-bound tritium</td>
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<td>OD</td>
<td>Optical density</td>
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<td>OSPAR</td>
<td>Convention for the protection of the marine environment of the north-east Atlantic</td>
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<td>p38-MAPK</td>
<td>p38 mitogen-activated protein kinase</td>
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<tr>
<td>PBq</td>
<td>Petabecquerel(s)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Principle component</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PFRRT</td>
<td>Pairwise fixed random reallocation test</td>
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<td>Pyrithione</td>
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<td>qPCR</td>
<td>Quantitative (real-time) PCR</td>
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<tr>
<td>r</td>
<td>Pearson’s product-moment correlation coefficient</td>
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<tr>
<td>R²</td>
<td>Coefficient of determination</td>
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<td>RBE</td>
<td>relative biological effectiveness</td>
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<td>RER</td>
<td>Relative expression ratio</td>
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<td>Ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<td>S</td>
<td>Svedberg units</td>
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<td>SAM</td>
<td>Sterile alpha motif</td>
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<td>SCGE</td>
<td>Single cell gel electrophoresis</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>Abbreviation</td>
<td>Definition</td>
</tr>
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<td>--------------</td>
<td>------------</td>
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<tr>
<td>SoS</td>
<td>Stress on stress</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SI</td>
<td>International system of units</td>
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<td>SSB</td>
<td>Single strand break</td>
</tr>
<tr>
<td>SW</td>
<td>Seawater</td>
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<tr>
<td>TA</td>
<td>Transactivation domain</td>
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<td>Tris-acetate-EDTA</td>
</tr>
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<td>Terabecquerel(s)</td>
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<td>Trebarwith Strand</td>
</tr>
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<td>Tributyltin</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TFWT</td>
<td>Tissue free water tritium</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
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<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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<td>UNSCEAR</td>
<td>UN scientific committee on the effects of atomic radiation</td>
</tr>
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<td>UV</td>
<td>Ultraviolet</td>
</tr>
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<td>V/cm</td>
<td>Volts per centimetre</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>W</td>
<td>West</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>WFD</td>
<td>Water framework directive</td>
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<tr>
<td>ZnPT</td>
<td>Zinc pyrithione</td>
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</table>
Chapter 1

Introduction
1. Introduction

1.1 Environment and human health

There are currently many anthropogenic challenges facing the environment, including habitat destruction, climate change, over-fishing and pollution. As well as threatening the environment itself and - by extension - our resources and potential economic stability, these issues also have the ability to impact human health (Depledge et al. 2013). Such impacts may occur via the food chain (Jha 1998, Parache et al. 2011), directly (e.g. via contaminant exposure) or indirectly via complex routes (e.g. lack of plant biodiversity results in a reduced reservoir of novel pharmaceuticals; Frumkin 2001). As such, it is imperative that human society takes steps to mediate its impact on the natural environment, both for the sake of preserving it intrinsically and for the long-term survival of mankind.

Modern, industrial, human civilisation results in the production, consumption and eventual disposal of large quantities of natural and synthetic contaminants (Hutchinson et al. 2013). These include herbicides, pesticides, fertilisers, plastics, hormones, metals, organic compounds and radioactive materials, and growing human populations have led to an increase in their discharge to the environment. In addition to the effects these contaminants induce in the biota, they can also have significant economic and social consequences. In addition, improvements in analytical techniques have led to the identification of many potentially harmful chemicals that have yet to be fully characterised in terms of their toxicity (i.e. emerging contaminants; Hutchinson et al. 2013). Consequently, the impacts of such anthropogenic contaminants on the environment are a major source of concern for governments, regulatory bodies and the public (Moore et al. 2004, Lyons et al. 2010).

1.2 The impact of anthropogenic contaminants on the aquatic environment

Invertebrates make up approximately 90% of life on Earth, and the aquatic environment covers over 70% of the surface of the planet. In addition to their sheer weight of numbers, aquatic organisms (invertebrates in particular) are keystone species in many ecosystems (Lonsdale et al. 2009). They are an important food source for humans (both commercially and in artisanal fisheries; Barnes & Rawl-
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inson 2009, Ren et al. 2010) and for other commercial fish species (Pedersen et al. 2008) and are frequently used as model organisms for toxicological tests (Depledge 1998). As a result of various processes, including sewage discharge, agricultural run-off, and aerial or fluvial deposition, the aquatic environment is the ultimate recipient of discharged contaminants. Therefore, aquatic biota may be especially vulnerable to contaminant-induced harm.

The potential effects induced by anthropogenic contaminants are highly variable and depend on the species, exposure conditions and specific contaminant. Some of these exposure scenarios have been characterised and are now well-reported in the literature. For example, the disturbance of normal sexual function by endocrine-disrupting chemicals in aquatic animals, such as female dogwhelk (*Nucella lapillus*; Gibbs & Bryan 1986) and male dab (*Limanda limanda*; Stentiford & Feist 2005), is now notorious. Neurotoxic effects and decreased immune function have been reported in marine mussels (*Mytilus edulis*) exposed to organophosphate pesticides (Canty et al. 2007) and the incidence of transmissible sarcoma in clams (*Mya arenaria*) has been linked to organochlorine exposure (Farley et al. 1991). Organic chemicals, such as poly-aromatic hydrocarbons, have been linked to liver neoplasms in brown bullhead catfish (*Ameirus nebulosus*; Baumann & Harshbarger 1995); and inorganic heavy metals have been shown to cause reduced survival and hatching in zebrafish (*Danio rerio*) embryos (Dave & Xiu 1991) among numerous other examples. However, there are yet more examples of situations and contaminants where our knowledge is lacking and where, as a result, the aquatic biota may be subjected to harmful effects. In order to ensure that the environment - particularly aquatic ecosystems - is adequately protected it is essential that these hazards are identified and the risk from such contaminants is accurately quantified.

1.3 Biomarkers and bioindicators

Detection and quantification of contaminants in the environment is not enough to accurately determine their impact on biological systems (Jha et al. 2000). Additionally, where contamination is from a complex mixture of pollutants, accurately and consistently predicting toxicity is also challenging (see also section 1.9; Jha et al. 2000). As a result of these difficulties, it is necessary to identify the effects of contaminants on biological systems, which are the ultimate recipients of toxicant-induced damage (Jha 2008), in addition to quantifying the presence of a pollu-
tand. Thus, in ecotoxicology, the responses of molecular, cellular, physiological and behavioural processes, structures and functions (‘biomarkers’) of ecologically representative organisms (‘bioindicators’) are used to detect ecosystem damage (Moore et al. 2004, Jha 2008).

1.4 Levels of biological organisation

Any ecosystem is composed of successive levels of biological organisation, where the ‘lower’ levels combine to form the ‘higher’ ones. For example, the function of a tissue or organ is determined by its cellular composition. The biological effects of contaminants can occur at one or more levels of biological organisation. It has been concluded that there is no single level at which it is best to examine the effects of contaminants; consequently, ecotoxicological studies which examine effects across multiple levels of biological organisation are recommended (Clements 2000). Furthermore, such a multiple biomarker approach has also been recommended across different trophic levels and using organisms occupying different ecological niches (Brechignac & Doi 2009). This is essential in order to obtain a holistic picture of the impacts of contaminants on aquatic biota and their relevance to ecosystem quality.

Although in ecotoxicology, the primary unit of interest is the ecosystem, community or population (i.e. at an ecological rather than individual level), it is usually necessary to measure biological effects of contaminants at lower levels.
This results from the practical difficulties of measuring effects at higher levels, where endpoints are usually more complex and take longer to occur (Fig. 1.1; Moore et al. 2004). One solution to this problem is to explore the mechanistic links between effects at differing levels of biological organisation, and therefore be able to predict the consequences at the ecosystem level (Attrill & Depledge 1997, Clements 2000, Jha et al. 2000).

Genotoxic responses are of particular interest in this regard, as they are sensitive (i.e. they occur at low levels of exposure) and, as such, may provide potential early warning signals (Anderson & Wild 1994, Jha 2004, 2008). Contaminant exposure may lead to genotoxic effects via a variety of mechanisms, including direct interaction with DNA (either direct strand breakage, as with ionising radiation, or direct binding of contaminants to DNA to form adducts); crosslink formation (preventing replication); generation of reactive oxygen species (ROS, leading to oxidised bases and potential mismatch) and inhibition of DNA repair (leading to accumulation of endogenous damage; Fig. 1.2). Any one of these may result in deleterious mutations or downstream effects, with resulting implications at higher levels of organisation.

Similarly, the advent of ‘omics’ technologies has provided a whole new set of tools for the exploration of contaminant-induced effects at the lower biological levels (i.e. molecular; Snape et al. 2004a). These include the use of microarrays to quantify the expression of multiple genes at once (Neumann & Galvez 2002, Dondero et al. 2011, Venier et al. 2011) and proteomic or metabolomic responses to exposure (Viant et al. 2003, Robertson 2010, Sanchez et al. 2011, Schmidt et al. 2013). Studies using these techniques can provide valuable insight into the
mechanisms behind genotoxic and higher-level effects of contaminants on aquatic species (Snape et al. 2004a). Due to the highly conserved nature of many stress response genes (e.g. Walker et al. 2011), such information is also valuable for the protection of human health. Despite the inherent potential of these techniques, there is limited data on their connection(s) to effects at higher levels of biological organisation (Snape et al. 2004a, Fedorenkova et al. 2010). Consequently, studies which incorporate these technologies into multiple biomarker assessments are necessary to build a foundation on which future environmental risk assessment can be based.

1.5 Chronic versus acute exposure

In exposure scenarios where contaminants are present at low levels, or where contamination has persisted for long periods of time, the impacts of contaminants are not adequately represented by acute toxicity tests. This is often the case in the marine environment, where contaminants are often highly diluted and present at low concentrations. As a result, marine organisms are at particular risk of chronic rather than acute exposure to contaminants. Whilst there is a wealth of information on the potential acute impacts of such contaminants on a range of biota, there is a paucity of such information for chronic exposure (Eggen et al. 2004, Dallas et al. 2012).

1.6 The CHRONEXPO project

As part of INTERREG IVA, the CHRONEXPO project was established as a collaborative project between 5 institutions in the UK and France. Its aim is to investigate chronic toxicity of contaminants to a range of marine invertebrates, with a particular focus on those that are relevant for the English Channel (La Manche) region. Within this project, there is work on the toxicity of metals to worms or cuttlefish (*Nereis virens* and *Sepia officinalis*; University of Portsmouth, Université de Caen Basse Normandie) and of pesticides or radionuclides to oysters (*Crassostrea gigas*; Université de Caen Basse Normandie, Institut de radioprotection et de sûreté nucléaire [IRSN]) in addition to the work presented here. It is expected that the outcomes of this project will add substantially to the limited
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information available on chronic exposure in ecotoxicological studies.

1.7 Stable metal contaminants

Metals and metallic compounds are a ubiquitous part of human civilisation, and have been for thousands of years. Consequently, both essential and non-essential metals are released from a multitude of sources, including mining, industrial applications (e.g. smelting), manufacture of electronics and consumer waste (as landfill or sewage). The marine environment is the ultimate recipient of these discharges, whether directly or indirectly.

Toxic metals exert effects at various levels of biological organisation in aquatic organisms. For example, genotoxicity has been observed in mussels exposed to Cu, Cd, Hg and organic Zn compounds (e.g. Bolognesi et al. 1999, Marcheselli et al. 2011). Intersex in marine snails (e.g. Nucella lapillus) caused by tributyltin is a well known environmental problem. More recently, research into the effects of metal mixtures on mussels has yielded interesting results. In particular, low concentrations (< 200 nM) of common heavy metals (Cu, Cd and Hg) were found to cause genotoxic effects (micronuclei) and alterations in transcriptional patterns of key genes (including those for heat shock proteins, metallothioneins, membrane transporters and detoxification enzymes) when in combination (Varotto et al. 2013).

Despite a wealth of research on metal toxicity, there are still many unanswered questions, particularly with regard to the mechanisms behind their carcinogenic potential. However, it is highly likely that metals exert their toxic effects in a variety of ways (Fig 1.2). These include direct genotoxicity, i.e. interaction with DNA (for example the formation of crosslinks or strand breaks), and indirect effects via the generation of reactive oxygen species, gene silencing (epigenetics) or inhibition of DNA repair enzymes (Hartwig et al. 2002), which may also enhance the effects of other contaminants or stressors (Bolognesi et al. 1999). Speciation is also of utmost importance in assessing the toxicity of chemical contaminants (including metals). In general, the free metal ion is regarded as more toxic, due to its higher bioavailability, and there are numerous studies which indicate that physico-chemical interactions (e.g. the presence of metal binding ligands) can modulate toxicity (e.g. Vercauteren & Blust 1996, Ward & Kramer 2002, Money...
Figure 1.3. Metals and metalloids identified as having carcinogenic potential or as priority substances in the EU Water Framework Directive (EC 2000).

As awareness of the impacts of metals and metallic compounds on the environment has grown, so has regulatory pressure to reduce their discharge. For example, under the EU Water Framework Directive (WFD; EC 2000, 2008, DEFRA 2010) 4 metals (Cd, Hg, Ni and Hg, see Fig 1.3) and one metallic compound (tributyltin [TBT]) have been identified as priority pollutants with defined environmental qualities standards (EQS). Despite this tighter regulation in Western countries, developing nations are increasing production of heavy metals. For example, production of Hg in China increased by 40 % from 2004 - 2007 (He et al. 2011). In addition, legacy pollution (e.g. from long closed mines; David 2003) and accidental release can contribute to intense contamination of the marine environment. For example, the collapse of dams built from mine tailings (such as those at Aznalcóllar, Spain, in 1998 and Baia Borsa, Romania, in 2000) often results in the large scale contamination of aquatic environments with metals (David 2002, Rico et al. 2008). Such spills have concomitant impacts on the biota (Miguel et al. 2013). In addition to acute exposures such as these, it is also important that we adequately characterise the impacts of low-level metal contamination (i.e. chronic duration and/or under the EQS) on marine and aquatic species to ensure long-term protection of human and environmental health.
1.8 Ionising radiation

Ionising radiation (IR) is released during the radioactive decay of an unstable radionuclide to a ‘daughter’ nuclide. This decay emits radiation as either alpha ($\alpha$) particles, beta ($\beta$) particles or gamma ($\gamma$) rays depending on the radionuclide(s) involved. X-rays are also a form of ionising radiation. The types of ionising radiation differ in their ability to interact with biological material, principally because of their differing energy levels, ionising power and ability to penetrate tissue (Fig. 1.4). Exposure of an organism to radionuclides occurs by one of two major pathways: (1) external irradiation, when the source radionuclide is outside the body, e.g. in the air, water or soil surrounding an organism; and (2) internal irradiation, when the source radionuclide is inside the body, after inhalation, ingestion or absorption. Internal irradiation is particularly relevant for aquatic organisms as they are in intimate contact with the water surrounding them and are therefore vulnerable to internalising radionuclides (Dallas et al. 2012).

Nuclear technologies, which generate anthropogenic radionuclides, have a wide range of applications including defence, food preservation, medical diagnostics, cancer treatment, manufacturing and energy production (Valković 2000). It goes without saying that these various applications of nuclear technology have made a very significant contribution to modern civilisation. There have, however, been
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Concerns about potential impacts on health ever since radioactivity was discovered (Valković 2000). Release of radionuclides to the environment occurs from a variety of sources, including medical and defence establishments, scientific institutions and industry. However, the nuclear fuel cycle (which consists of ore mining and metal extraction; enrichment and fuel manufacture; power generation and fuel reprocessing) is the biggest source of artificial radioactivity in the marine environment. In the UK, the largest single contributor situated on UK soil is the Sellafield site on the Cumbrian coast (DEFRA 2005, Environment Agency 2012), while the nuclear fuel reprocessing plant at La Hague, Normandy, also contributes a significant amount of radioactivity to UK waters, particularly to the English Channel (Bailly du Bois & Guegueniat 1999). The locations of the other main sources of radioactivity to the UK marine environment are illustrated in Fig. 1.5. Although, in general, radioactive discharges have declined in the UK over the past 10 years (and the UK Strategy for Radioactive Discharges has specified even lower targets for 2020), discharges of some radionuclides (such as tritium) have shown considerable fluctuation and even increased from 2010 - 2011 (Environment Agency 2012).

Assessment of the risk these artificially generated radionuclides pose to the environment has historically been determined in accordance with dose limits for the protection of humans. The International Commission for Radiological Protection

Figure 1.5. Location of nuclear institutions in the English Channel and Irish Sea region, including those identified as key sources of radioactive contamination (by Bailly du Bois & Guegueniat 1999).
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(ICRP) stated that "if humans are protected from the effects of ionising radiation, then flora and fauna are also adequately protected" (ICRP 1984). Yet this assumption has been questioned, particularly for situations or areas where humans are absent, or where non-human organisms bioaccumulate radionuclides and thus are at risk from elevated internal doses. It is now recognised that "occasionally, individual members of non-human species may be harmed but not to the extent of endangering whole species or creating imbalance between species" (ICRP 1991). As a result, the need to specifically assess the effects of ionising radiation (IR) on the environment, and to protect the non-human biota from adverse effects, has now been recognised (Copplestone et al. 2001, IAEA 2006, ICRP 2007, Pentreath 2009).

A comprehensive review of the literature on the effects of IR on aquatic invertebrates was undertaken as part of this PhD project (Dallas et al. 2012; Appendix A). This research has highlighted that the available information is scarce, fragmented and that there is considerable variation in IR-induced effects within the aquatic invertebrates. It is therefore difficult to generate meaningful estimates for the impact of IR on the aquatic invertebrates as a whole, and purely speculative to make comparisons with other groups. The idea that this group is generally more radioresistant than 'higher' taxonomic groups is potentially inaccurate, as - for certain endpoints - aquatic invertebrates may be just as sensitive to radiation as fish or mammals. It is therefore important that future research fills the knowledge gaps that have been identified, and it is also necessary to consider adopting an integrated approach, incorporating aspects of human and mammalian radiation risk assessment (Copplestone et al. 2010), ecotoxicology and newer technologies, such as ‘omics’. The establishment of robust links between effects at differing levels of biological organisation is essential, as is the incorporation of endpoints that monitor ecosystem level effects (i.e. the ecosystem approach). As the aquatic invertebrates span so diverse a range of organisms, it is essential that future work draws comparisons across phylogenetic groups. Additionally, ionising radiation does not affect the environment in isolation; therefore it is crucial to determine the potential impact of biotic and abiotic factors.

As discussed earlier, ecotoxicological studies on chemical contaminants have previously tended to focus on acute exposure durations. This is also true of the radioecological literature (Dallas et al. 2012). The discharge of radionuclides is permitted as part of the normal operational procedures of nuclear institutions (Brechignac & Doi 2009) and is highly regulated. Although acute contamination
of the environment by radionuclides has occurred (such as that caused by the recent earthquake-tsunami at Japan’s Fukushima Daiichi plant or at the Chernobyl nuclear power plant in 1986), typically the exposure of aquatic organisms to ionising radiation is from legal discharges and is therefore chronic. The pressure on governments to reduce the environmental impact of the nuclear fuel cycle means it is even more likely that, in the future, aquatic organisms will be exposed to radionuclides at chronic, rather than acute levels. For example, the Radioactive Substances Strategy of the OSPAR convention has an “ultimate aim of concentrations in the environment near background values for naturally occurring radioactive substances and close to zero for artificial radioactive substances” (OSPAR Commission 2003). Acute toxicity data can be used to predict chronic toxicity, but this approach is often flawed as the relationships between the two types of toxicity are highly variable (Raimondo et al. 2007). In particular, research in the last 20 years has brought about a major paradigm shift in radiobiology, with the idea that radiation dose-response is non-linear, especially at low doses (Mothersill & Seymour 2013). Although DNA is considered to be an important target for the action of IR (UNSCEAR 2008), the investigation of non-target stress responses (e.g. hypersensitivity) across levels of biological organisation is paramount if we are to fully understand the potential ecological impacts of IR. Finally, it is important to note that most of the existing studies on radiation-induced effects in aquatic biota have used external radiation sources (e.g. $\gamma$ emitters) which are not representative of environmental exposure scenarios (Dallas et al. 2012). In this context it is critical that we investigate how chronic, low dose exposure to environmentally relevant radionuclides affects marine life, to allow for a greater understanding of the impact of IR on these species and for better protection of the environment as a whole.

1.9 Co-exposure to chemical and radionuclide contaminants or physical stressors

Neither chemical nor radionuclide contaminants occur in isolation (Manti & D’Arco 2010). This is particularly the case for aquatic biota, as water bodies are the ultimate recipient of contaminants. This results in exposure of aquatic organisms to complex chemical and/or radionuclide mixtures (Schwarzenbach et al. 2006). Many of these pollutants are present in small amounts (i.e. micropollutants) but
may still have adverse effects (Schwarzenbach et al. 2006). As a result of these concerns, the study of mixture toxicity is becoming especially important in ecotoxicology, in order to elucidate the potential synergistic, antagonistic or additive effects of such combinations (Eggen et al. 2004, Dondero et al. 2011, Hutchinson et al. 2013). However, the interaction of multiple contaminants is not the only confounding factor to be considered in realistic exposure scenarios (Manti & D’Arco 2010). Physical stressors are also important, especially during long-term, chronic exposure scenarios, as variability in natural conditions often fluctuates temporally. Consequently, organisms may also be exposed to temperature and salinity changes, hypoxia or hyperoxia, dessication and changes in pH, all of which may have detrimental effects in their own right as well as potentially compounding those of anthropogenic contaminants (Holmstrup et al. 2010).

1.10  **Mytilus** species in ecotoxicological studies

Marine mussel species of the genus *Mytilus*, particularly *M. edulis* and *M. galloprovincialis*, are often used as bioindicator species in ecotoxicological studies (i.e. as a model organism from which effects can be extrapolated to other species or ecosystems). Both species are widely distributed in the littoral and shallow sublittoral zones of boreal and temperate waters in the Northern and Southern hemispheres (Bayne 1976). Sedentary, filter-feeding, marine invertebrates, such as marine mussels, are useful as bioindicator organisms because of their tendency to accumulate pollutants. The widespread distribution of *Mytilus* species makes them ecologically relevant in an enormous variety of locations, making them well-suited to large-scale studies or international comparisons (Bayne 1976). In addition, *Mytilus* species are easy to collect because of their epifaunal nature; easy to maintain in the laboratory; and relatively tolerant of external factors such as temperature and salinity. This enables them to be used in studies examining the effect of abiotic factors on contaminant toxicity (e.g. Ali & Taylor 2010). Due to their sessile nature and ability to bioaccumulate, mussels have also been a key part of contaminant monitoring programmes for many years. That is, concentrations of chemicals in mussels collected from coastal locations are used as a proxy for contamination of the environment itself. The US ‘Mussel Watch’ programme is probably the best known example of this (Goldberg & Bertine 2000).

In addition to the basic practical advantages of using *Mytilus* species in eco-
toxicological studies, their widespread use has resulted in the development of a wide range of toxicological tests appropriate for these organisms. These include traditional endpoints, such as mortality and behaviour, and more sophisticated assays that examine physiological, biochemical and molecular alterations following exposure to contaminants. Common physiological endpoints used in mussel studies include oxygen consumption, heart rate and feeding or clearance rate. These parameters have variously been used to study the impact of surfactants (Swedmark et al. 1971), metals (Brown & Newell 1972), seasonality (Hagger et al. 2010), culture conditions (Harding et al. 2004) and aromatic hydrocarbons (Scarlett et al. 2008). Additionally, the ‘blood’, or haemolymph, of mussels can be extracted via the posterior adductor muscle and, as such, provides a means of non-destructive sampling (Zaja et al. 2006). As a result of this, and of the many important roles haemocytes play in mussel physiology, many studies use alterations of mussel haemocytes as biomarkers of toxic damage (e.g. Moore & Lowe 1977, Wrisberg et al. 1992, Venier et al. 1997, Pavlica et al. 2000, Tran et al. 2007). The energy metabolism and anti-oxidant defence mechanisms in mussel species are well-studied (Bayne 1976, Fitzpatrick et al. 1997) and enzyme activities are used as endpoints in a wide range of studies (Liu et al. 2010, Brooks et al. 2011, Franzellitti et al. 2011). Assessments of genotoxicity, such as the micronucleus and comet assays, are now well-established in mussels and have been used in studies examining the toxicity of a range of reference chemicals and environmental pollutants. These include hydrogen peroxide (Cheung et al. 2006), methyl methane sulfonate (Canty et al. 2009), metals (Pruski & Dixon 2002, Emmanouil et al. 2007, Dallas et al. 2013), poly-aromatic hydrocarbons (Large et al. 2002), radionuclides (Hagger et al. 2005a, Jha et al. 2005, Jaeschke et al. 2011) amongst numerous other contaminants. Recently, a metabolomic approach has been applied to mussel ecotoxicology (Jones et al. 2008) and Tuffnail et al. (2009) have demonstrated clear differences between the metabolomic signals of control mussels and those exposed to lindane and atrazine. As a result of this large volume of literature, mussels of *Mytilus* species are well-suited to ecotoxicological studies examining endpoints at multiple levels of biological organisation.
Having identified the knowledge gaps outlined above, this thesis aims to move the science forward by addressing the following five major objectives:

(1) Establish the effects of two metal contaminants, nickel (a non-essential inorganic metal) and zinc pyrithione (an organometal anti-fouling compound) on marine mussels, using a variety of endpoints across multiple levels of biological organisation [Chapters 3 and 4]. This approach will also serve to validate our choice of biomarkers (in terms of their sensitivity and reproducibility) for use in subsequent studies on radionuclides;

(2) Establish the effects of chronic exposure to tritiated water on mussels, using a variety of endpoints across multiple levels of biological organisation, and building on previous work in this area [Chapter 5];

(3) Determine the impact of elevated temperature on the effects of chronic exposure to tritiated water in mussels [Chapter 6];

(4) Assess the relative impact of these three contaminants - a radionuclide, a metal and an organometal [Chapter 7];

(5) Determine if there is any correlation between these endpoints, in order to establish the ecological relevance of ‘lower’ level biomarkers (e.g. DNA damage) for these contaminants [Chapters 3, 4, 5 and 6].
Chapter 2

Materials and Methods
2. Materials and Methods

2.1 Chemicals and reagents

Unless otherwise stated all chemicals were purchased from Sigma-Aldrich Ltd (Gillingham, UK) and radionuclides were obtained from American Radiolabeled Chemicals Inc. (Stevenage, UK). Seawater was obtained from the Jennycliff area of Plymouth Sound, stored on site and then filtered (10 $\mu$m) before use. Distilled/ultrapure water was obtained using a Milli-Q RG (Merck Millipore, Billerica, MA, USA) or Elga Purelab Option system (Elga LabWater, Marlow, UK) and had a resistivity of $> 18.2$ M$\Omega$ cm$^{-1}$ at 25 $^\circ$C.

2.2 Selection of exposure concentrations

Defining chronic exposure is subject to many variables. However, in accordance with guidelines for mammalian regulatory genotoxicological tests (Hutchinson et al. 2009) we used maximum tolerated concentrations (MTC; i.e. $\leq$10 % of the LC$_{50}$), obtained experimentally or via the literature, as the basis for selection of exposure concentrations (Canty et al. 2009, Al-Subiai et al. 2011).

2.3 Mussel collection and maintenance

Adult *Mytilus* sp. were collected by hand from Trebarwith Strand, Cornwall (latitude 50 38' 40" N, longitude 4 45' 44" W), packed on ice and transported to the laboratory. Transportation took < 2 h and mussels were kept humid to prevent desiccation. Mussels were transferred to a 75 L aquarium, filled with approximately 55 L of filtered (< 10 $\mu$m), aerated seawater at 15 $^\circ$C. A minimum 2 week depuration period was allowed prior to moving mussels to the exposure vessels. During this holding period, mussels were fed twice weekly with a solution of *Isochrysis galbana* algae (\(\sim 1.05 \times 10^6\) cells mL$^{-1}$; Reed Mariculture, Campbell, CA, USA) and a 100 % water change was performed 24 h after feeding. Unless otherwise stated, during all experiments mussels were fed two hours prior to each water change with 50 $\mu$L L$^{-1}$ of *Isochrysis galbana* at a concentration of approximately $2 \times 10^6$ cells mL$^{-1}$. This combination of feeding, followed closely by a full water change, allowed optimal water quality to be maintained whilst also enabling the mussels’ filtration capacity to be observed (by colour change).
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2.3.1 Verification of mussel species

Hilbish et al. (2002) identified mussels north of St Ives on the Cornish coast as *M. galloprovincialis*. Trebarwith Strand (TBS) lies within this zone. Nonetheless, we considered it prudent to verify the specific identity of our sample mussels.

Mussels (*n* = 100) were collected from the normal sampling location at TBS (~5 m²). For comparison, mussels were also collected from Mayflower Marina, Plymouth (MF; Latitude 50° 21’ 52” N, Longitude 4° 10’ 10” W), a site expected to have a *Mytilus edulis* or hybrid population (Hilbish et al. 2002). Although the best effort was made to size-match samples, mussels at MF had a higher mean length (61.00 ± 7.94 mm) than those at TBS (58.40 ± 3.73 mm), although this difference was not significant (*p* > 0.05).

Morphometric parameters were used to distinguish species, as previously demonstrated by Beaumont et al. (2008). Prior to dissection, a photograph was taken of each mussel’s right valve (Rebel XT Digital SLR, Canon, Reigate, UK). Image analysis software (ImageJ, v1.46r, National Institutes of Health, Bethesda, MA, USA) was used to quantify the following morphometric parameters; length (*l*; along the longest axis), height (*h*; at 90° to *l*) and dorsal parallel shell margin (*dpsm*; parallel distance on the valve edge opposite *l*). Fig. 2.1 illustrates these measurements. From these parameters the ratios length:height (*l/h*) and length:dorsal parallel shell margin (*l/dpsm*) were calculated (Beaumont et al. 2008).

A piece of gill tissue (~25 mg) was dissected from each mussel (*n* = 100), and snap
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frozen in liquid nitrogen. Tissues were then stored at -80 °C until analysis (< 1 week). DNA was extracted from gill tissue using the GenElute Mammalian DNA Miniprep kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer’s instructions. DNA was then quantified (ND-1000, Nanodrop, Wilmington, DE, USA) and 100 - 200 ng used for PCR.

PCR primers were Me 15 and Me 16, encoding a variable region of the Glu-5’ gene (GenBank accession no. D63778), which has been previously validated and used as a diagnostic marker for three of the morphologically similar species within the *Mytilus* genus, *M. trossulus*, *M. edulis* and *M. galloprovincialis* (Inoue et al. 1995, Dias et al. 2008, Kijewski et al. 2011). Amplified fragments produced by PCR using these primers vary in length interspecifically and are 180 bp and 126 bp for the species at TBS (*M. edulis* and *M. galloprovincialis*, respectively; see Fig. 2.2). Hybrid individuals show both bands (Inoue et al. 1995).

PCR was carried out as described by Inoue et al. (1995) and Dias et al. (2008) with some minor modifications. A total reaction volume of 50 µL included 25 µL BioMix Red (Bioline, London, UK), 1 µL of each primer, 2 µL of template DNA and 22 µL molecular grade water. Negative controls were run with each PCR batch and contained 2 µL molecular grade water instead of DNA.

PCR products were run on 2 % agarose gels (w/v in Tris-acetate-EDTA [TAE]),

![Figure 2.2. Primers used to amplify the species-specific, non-repetitive region of the Glu-5' gene in *Mytilus*. (A) Primer sequences and (B) location of primers within the Glu-5' gene (modified from Inoue et al. 1995).](attachment:image.png)
Figure 2.3. Differentiation of *Mytilus* species between Mayflower Marina, Plymouth (MF) and Trebarwith Strand, Cornwall (TBS). Panels show morphometric analyses (A-C), a representative example of Me 15/16 PCR products from each site visualised on 2 % agarose gel (D) and illustrative examples of mussels from either site (E: MF and F: TBS). Significant differences (*p* < 0.05) between sites are illustrated by *.

Mussels collected from the two sites showed different morphometric parameters, with those from TBS having significantly higher l/h (*p* < 0.0001; Fig. 2.3A) and 1/dpsm (*p* < 0.05; Fig. 2.3B) and significantly lower dpsm (*p* < 0.05; Fig. 2.3C). This is consistent with the findings of other authors who have reported clear differentiation between individuals of *M. edulis*, *M. galloprovincialis* and the closely related *M. trossulus* using these parameters (Beaumont et al. 2008, McDonald et al. 1991).

*M. edulis* alleles were only identified at MF, whereas mussels from TBS showed only *M. galloprovincialis* alleles. There were no sampled mussels which showed a hybrid banding pattern. A representative example of Me 15/Me 16 PCR products for mussels from both sites is shown in Fig. 2.3. It can be concluded from this analysis that the mussels used in this study are highly likely to be *M. galloprovincialis*.
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2.4 Water quality testing

During experimental exposure of mussels, water quality parameters (pH, dissolved oxygen [DO], temperature and salinity) were measured before and after water changes using a Hach HQ40D Multi-meter (Hach-Lange, Dusseldorf, Germany) and appropriate probes. Where appropriate ammonia, nitrite and nitrate were measured using a HI 3893 Aquarium Test Kit according to the manufacturer’s instructions (Hanna Instruments, Woonsocket, RI, USA).

2.4.1 Water quality assessment in preparation for chronic exposures

To determine the least stressful method of maintaining mussels over chronic exposure periods, two water quality experiments were conducted. In the first experiment, mussels were acclimatised as in section 2.3 and transferred to six experimental beakers. Three beakers contained mussels at a density of 1.5 mussels L\(^{-1}\), whilst the other three contained mussels at a density of 4.5 mussels L\(^{-1}\), otherwise all beakers were treated identically. Mussels were maintained in these conditions for 72 h and fed daily with *Isochrysis* algae at a density of 20,000 cells mL\(^{-1}\). Water quality parameters were assessed daily (prior to feeding), as
Figure 2.5. (A) Haemolymph extraction from adductor muscle in *Mytilus* spp. (A) Location of musculature (redrawn from Bayne 1976). Adductor muscles hatched, retractor muscles black; (B) Extraction of haemolymph from the posterior adductor muscle.

described in section 2.4. The second experiment was exactly the same as the first, except mussels were only fed on day 3 and an ammonia test and 100 % water change was carried out approximately 2 h after this feed. An additional ammonia test was also performed after this water change. The exposure set up resulted in minimal variation in dissolved oxygen (92.35 ± 0.72 %), pH (7.99 ± 0.18) and salinity (34.56 ± 0.72 psu). Therefore these initial validation tests focused on the build up of nitrogenous waste products.

When mussels were fed daily, ammonia levels at the higher density increased rapidly to 2.5 mg L\(^{-1}\) in the first 24 h and then levelled off. Mussels at the lower density showed a slower increase in ammonia, plateauing at 2 mg L\(^{-1}\) after 48 h (Fig. 2.4). Nitrite and nitrate were below detectable levels at each sampling point independent of mussel density. Consequently nitrite and nitrate were not measured during the second experiment.

When mussels were only fed after 72 h, ammonia levels peaked slightly lower for the higher density group, at 2 mg L\(^{-1}\) after 24 h. This value did not increase, even after feeding. At the lower density, ammonia levels were much lower but increased after feeding. At both densities ammonia levels returned to zero with a 100 % water change (Fig. 2.4) as expected. Based on this data mussels in longer-term, chronic studies were kept at a density of 4.5 L\(^{-1}\) fed every 72 h with a complete water change approximately 2 h later.
2.5 Haemolymph collection

Haemolymph was extracted from the posterior adductor muscle of *M. galloprovincialis* (Fig. 2.5). The valves were separated using a pair of dissecting scissors inserted into the byssal gape, and seawater was allowed to drain completely from the mussel onto absorbent paper. After draining, haemolymph was extracted via a 21 gauge hypodermic needle into a 0.5 mL syringe pre-filled with 0.1 mL physiological saline (20 mM HEPES, 435 mM NaCl, 100 mM MgSO$_4$, 10 mM KCl, 10 mM CaCl$_2$, pH 7.36). Average volume of haemolymph extracted was 0.3 mL. After extraction, the needle was removed from the syringe and haemolymph was transferred to a siliconised eppendorf and stored on ice until use.

2.6 Cell viability assessment with eosin Y

Haemolymph samples were tested for cell viability by dye exclusion assay with eosin Y prior to use (Canty et al. 2009). A 40 µL subsample of haemolymph-saline mixture was transferred to an eppendorf, 2 µL of eosin Y solution (2 mg mL$^{-1}$) was added and the mixture was gently vortex mixed. After mixing, haemocytes were transferred to a slide, a coverslip was applied and the cells were examined under light microscopy (x 40) and scored as either living (clear) or dead (pink colouration). Only haemolymph samples with >90 % viability were used for further tests.

2.7 Clearance rate

The clearance rate (CR) assay was adapted from previous work on mussels (Scarlett et al. 2008, Donkin et al. 2003). It is a simple but efficient method of determining the overall physiological health of mussels (Canty et al. 2007, Scarlett et al. 2008, Widdows 1978, Widdows et al. 1979). Adult mussels (*n* = 9) were individually transferred to beakers containing 350 mL of filtered seawater (15 °C) with constant vortex mixing (Fig. 2.6). After a 10 min acclimation period, 500 µL of *Isochrysis* algal solution was added to produce a final concentration of approximately $2 \times 10^4$ cells mL$^{-1}$. Water samples (20 mL) were taken on addition of the algae ($T_0$), and at the end of the measurement period ($T_1$). Enumeration of algal
cells was achieved using a Beckman Z2 Coulter Particle Size and Count Analyser (Beckman Coulter, Brea, CA, USA). Clearance rate (CR; L h\(^{-1}\)) of mussels was calculated from loss of algal particles, according to the following equation:

\[ CR = \frac{V (\log C_0 - \log C_1)}{t} \]  

where \( V \) is the volume of water (L) and \( C_0 \) and \( C_1 \) are the algal concentrations at \( T_0 \) and \( T_1 \), i.e. the beginning and end of time interval \( t \) (in minutes).

### 2.7.1 Optimisation and validation of clearance rate assay

After acclimation as detailed in section 2.3, 9 mussels (shell length 38.3 ± 1.23 mm) were transferred to beakers and processed through the CR assay as in section 2.7. A series of sampling points were used in order to find the optimum \( t \) for CR measurement. Samples were taken at 0 (\( T_0 \)), 15, 20 30 and 35 min (\( T_1 \)) after first addition of the algal suspension. Although there was no statistical significance between CR calculated over different measurement periods (Fig. 2.7), the 20 min sampling point gave the highest calculated CR (Fig. 2.7). Subsequently all future CR assessments were made with a \( T_1 \) of 20 min.

Mussels are routinely assessed for general health via the clearance rate assay on
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Figure 2.7. Comparison of mean clearance rates (CR) of unexposed *M. galloprovincialis* over different measurement periods (A) and with time since spawning (B). Error bars are ± one SE. Asterisks indicate significant difference (*p* < 0.01) from the pre-spawning value (-1 d), where appropriate.

their first day of laboratory acclimation. One batch of mussels spawned on their second day of depuration. As spawning is known to be an stressful, energetically expensive event, it was hoped that data could be collected to validate the CR assay, using spawning as the stressor. The mussels were returned to their acclimation tank, and clearance rate was measured at 10, 23 and 24 days post-spawning, as detailed above.

Time since spawning had a significant effect on mean clearance rate (*p* < 0.0001) which had decreased at all post-spawning sampling points. At 10 and 23 days post-spawning, this decrease was significant (Fig. 2.7; *p* < 0.01). Whereas by 24 days post-spawning mean clearance rate was decreased in comparison to pre-spawning values, but not significantly so (Fig. 2.7; *p* > 0.05), suggesting that mussels were recovering.

2.8 Micronucleus assay

The micronucleus (MN) assay was first proposed in 1959 (Evans et al. 1959) and then developed and popularised in the 1970s (Countryman & Heddle 1976, Heddle 1973). Micronuclei develop in dividing cells, where chromosome fragments are
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Radiation or contaminant(s)
Centromere
Micronucleus

Figure 2.8. Formation of micronuclei in dividing cells

acentric (lack a centromere) or where whole chromosomes are unable to migrate to the cell spindles. During telophase a separate nuclear envelope forms around these structures, which are smaller than, but morphologically similar to, the main nucleus, creating a ‘micronucleus’ (Fig. 2.8).

The MN assay is simple, does not require extensive technical training and is more rapid than the analysis of chromosomal aberrations (Bolognesi & Hayashi 2011). As the formation of MN is caused by chromosome damage, the MN assay can be used as a reliable and sensitive indicator of genotoxicity (Heddle et al. 1983). Although originally developed for mammalian systems, the MN assay has been adapted for use in aquatic organisms and is now extensively used in aquatic ecotoxicology (Bolognesi & Hayashi 2011, Bolognesi & Fenech 2012). In molluscs, the MN assay is most often performed on haemocytes or gill tissue. The validation of the MN assay in *Mytilus* species started more than 20 years ago and there are now numerous studies reporting dose-dependent increases in MN frequency in response to contaminants (Bolognesi & Hayashi 2011). These genotoxic pollutants include tributyltin (Hagger et al. 2005b), cyclophosphamide, an anti-cancer pharmaceutical (Canty et al. 2009), heavy metals (Bolognesi et al. 1999), EMS (Wrisberg et al. 1992) and HTO (Jha et al. 2006).
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A Cytospin slide centrifuge was used to concentrate 200 1 μl of haemolymph-saline mixture onto a slide pre-coated with poly-l-lysine solution (by spinning at 800 rpm for 2 min). Slides were prepared in duplicate and incubated at 4 °C for 1 h to allow cells to adhere. Samples were fixed by immersing flat in an ice-cold solution of 25 % glacial acetic acid (v/v in methanol) for 10 min. Fixed slides were then drained, covered and allowed to dry overnight at room temperature, before staining with either 20 μL of 20 μg mL$^{-1}$ ethidium bromide or 5 % Giemsa (v/v in Giemsa buffer; Fig. 2.9). Slides were coded and randomised and at least 1000 cells per slide were scored.

Criteria for classification as micronuclei were as follows (Fig. 2.10; Countryman & Heddle 1976):

1. Diameter < $\frac{1}{3}$ of the main nucleus but > $\frac{1}{10}$.

2. No contact with nucleus (absence of chromatid bridge).

3. Colour and texture resembling the nucleus.

4. Spherical cytoplasmic inclusions with sharp contour.
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Figure 2.10. Classification of micronuclei as valid (included in scoring) or invalid (not included in scoring) as determined by the criteria of Countryman and Heddle (1979): (A) Single micronucleus; (B) Pair of micronuclei, one located close to main nucleus; (C) Pair of micronuclei, located close to each other; (D) Binucleate cell; (E) Nuclear ‘bleb’, still attached to main nucleus, and cytoplasmic inclusion with inconsistent colour from main nucleus; (F) non-spherical cytoplasmic inclusion with indistinct border.
2.9 Comet assay

The comet assay (or single cell gel electrophoresis, SCGE) was initially developed in 1984 (Ostling & Johanson) and then modified to detect single strand breaks by Singh et al. (1998). It is routinely used to quantify DNA damage in a variety of disciplines, including human fertility studies (Enciso et al. 2009), human biomonitoring (Valverde & Rojas 2009), fundamental research on DNA repair (Collins 2004), genetic ecotoxicology (Frenzilli et al. 2009, Emmanouil et al. 2006, Jha 2008, Trevisan et al. 2011) and environmental monitoring (AlAmri et al. 2012). A summary of the main steps in any comet assay protocol is given in Fig. 2.11. Cells with elevated levels of DNA damage show the characteristic ‘comets’ due to negatively charged DNA fragments moving away from the nucleoid towards the anode during the electrophoresis step (Fig. 2.12).

DNA strand breaks (measured by the comet assay) may be caused by several factors, including general cytotoxicity and/or excision repair in addition to direct genotoxicity (Hartmann & Speit 1997, Henderson et al. 1998). It is therefore essential to minimise the impacts of these factors in order to accurately determine genotoxic effects. Consequently, cell counts and viability were established (as in section 2.6) prior to performing the comet assay. Only samples with viability > 90% were used. Selection of exposure concentrations at chronic, sub-lethal levels (i.e. at ≤ MTC) was also designed to minimise these effects.

The comet assay was performed as described in Jha et al. (2005) with some minor
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Figure 2.12. *M. galloprovincialis* haemocytes showing increasing DNA damage as measured by the comet assay; (A) Minimal damage, no ‘comet’ tail; (B) Slight damage; (C) Considerable damage; (D) Almost 100 % damage, ‘head’ of comet barely visible.

modifications. Slides were pre-coated with normal melting point agarose (NMPA; 1.5 % in Milli-Q water). Haemolymph-saline suspension (200 µL) was centrifuged at 350 g for 3 min at 4 °C. The resulting pellet was resuspended in 150 µL of low melting point agarose (LMPA; 0.75 % in PBS) and two 75 µL drops were dispensed onto each slide and coverslipped (to produce two replicate microgels). Slides were refrigerated at 4 °C for 1 h to allow gels to set. Coverslips were gently removed and slides transferred to lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 % N-lauryl-sarcosine, 1 % Triton X-100, 10 % DMSO, pH adjusted to 10 with NaOH) and incubated for 1 h at 4 °C. After lysis, slides were transferred to an electrophoresis chamber containing chilled electrophoresis buffer (1 mM EDTA, 0.3 M NaOH, pH 13). DNA was allowed to unwind for 20 min at 4 °C then electrophoresis was carried out for 20 min (∼1 V/cm). Following electrophoresis, slides were transferred to neutralisation buffer (0.4 M Tris, adjusted to pH 7 with HCl) for 10 min, rinsed three times with distilled water and allowed to air dry.

Slides were usually stained and scored within 24 h, and always within 1 week. Each replicate microgel was stained with 20 µL of 20 µg/mL ethidium bromide, and 50 cells per microgel (100 per slide) were scored using an epifluorescence microscope (DMR; Leica Microsystems, Milton Keynes, UK) and imaging system
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Comet assay software packages provide a number of different parameters, including tail length, % tail DNA and tail moment (the product of tail length and % tail DNA). Although some studies in our laboratory have reported tail moment and tail length as the most sensitive to environmental contaminants (Dallas et al. 2013, Cheung et al. 2006), % tail DNA is considered to be the most reliable (Kumaravel and Jha, 2006) and has been successfully validated with in vitro hydrogen peroxide exposure (section 2.9.1 and 2.10.1). Therefore, comet assay results are reported as % tail DNA throughout this thesis.

2.9.1 Validation of the comet assay with hydrogen peroxide

Hydrogen peroxide is a known genotoxic agent and has been successfully used to validate the comet assay and other biomarkers of genetic damage in previous studies (e.g. Henderson et al. 1998, Dallas et al. 2013), therefore it was used to validate our comet assay procedures. Haemolymph was extracted from 5 mussels, as described in section 2.5, pooled to reduce inter-individual variability, and kept on ice until use. Aliquots (300 µL, in triplicate) were transferred to siliconised Eppendorfs (on ice) and spun at 350 g for 2 min at 4 °C. The supernatant was removed and discarded, and 100 µL of hydrogen peroxide (0, 100, 500 or 1000 µM in physiological saline [see section 2.5]) added to the resulting pellet. Samples were incubated with hydrogen peroxide for 1 h at 4 °C in the dark. After incubation, the tubes were spun (as before), the supernatant removed, and the samples processed through the comet assay as in section 2.9.

All hydrogen peroxide concentrations produced a significant increase in percentage tail DNA (Fig. 2.13; \( p < 0.05 \)). A significant dose-dependent increase was observed between 100 and 1000 µM and between 500 and 1000 µM hydrogen peroxide (\( p < 0.001 \)), but the effect at 100 and 500 µM was not significantly different (Fig. 2.13).
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Figure 2.13. Percentage tail DNA in M. galloprovincialis haemocytes following in vitro exposure to hydrogen peroxide. Error bars are ± one SE. Asterisks indicate a significant difference from the control, and mismatched lower case letters indicate significant differences between treatment groups ($p < 0.05$).

2.10 Enzyme-modified comet assay

Oxidative damage of DNA occurs naturally as a result of cellular metabolism, but is increased by exposure to oxidising agents such as hydrogen peroxide, ionising radiation or UV (Collins et al. 1996, 1993, Cooke et al. 2003), contaminants (e.g. metals; Hartwig et al. 2002) or other stressors (e.g. hypoxia; Mustafa et al. 2011). This damage can be strand breaks, apurinic/apyrimidinic (AP) sites, or oxidation of bases (Collins et al., 1996). In addition to detection of strand breaks, the comet assay can be modified to distinguish oxidised DNA bases by the addition of an enzyme incubation step (Collins et al. 1993). The enzymes most commonly used for this are formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (Endo III). Fpg is a DNA repair enzyme which excises oxidised purines, leaving AP sites that are subsequently converted to strand breaks by associated AP endonuclease activity (Collins et al. 1996). This enzyme is particularly useful for the detection of 7,8-dihydro-8-oxo-guanine (8-OHgua), which can cause mutations as a result of mispairing with adenine by DNA polymerases (Collins et al. 1996). Endo III is an AP endonuclease with associated N-glycosylase activity (Kow & Wallace 1987); it nicks DNA at the sites of oxidised pyrimidines and cleaves the resulting AP site (Doetsch et al. 1987, Collins et al. 1993).

The enzyme modification step for the comet assay was as follows. After lysis, slides were washed three times (5 min) in enzyme buffer (40 mM HEPES, 0.1 M
2. Materials and Methods

KCl, 0.5 mM EDTA, 0.2 mg mL\(^{-1}\) BSA, pH 8.0), drained and 50 µl of Fpg, Endo III or enzyme buffer added to each microgel. Slides were then transferred to a humidity chamber and incubated at 37 °C for 45 min. All enzymes were used at a final protein concentration of 1.5 µg mL\(^{-1}\), i.e. one enzyme unit per microgel. Following the enzyme incubation step, the remainder of the comet assay protocol (unwinding, electrophoresis, neutralisation, staining) was carried out as described (section 2.9).

### 2.10.1 Validation of the enzyme-modified comet assay with hydrogen peroxide

Two separate validation experiments were performed for the enzyme-modified comet assay, one with Fpg and one with Endo III. Haemolymph was extracted from 18 mussels (see section 2.5) and pooled. Aliquots of hameolymph-saline mixture (300 µL) were transferred to siliconised eppendorfs, in triplicate, and exposed to various concentrations of hydrogen peroxide (0, 100, or 500 µM in physiological saline) as in section 2.9.1. Two slides were prepared per sample, one enzyme buffer control, and one with Fpg or Endo III. Slides were then processed through the comet assay as described above.
Buffer treated haemocytes showed significantly elevated DNA damage after exposure to 50 and 500 µM hydrogen peroxide in the Fpg validation test, and after 500 µM in the Endo III validation assay (Fig. 2.14; \( p < 0.05 \)). Both Fpg and Endo III treated cells had significantly higher % tail DNA than their equivalent buffer treatments (Fig. 2.14; \( p < 0.05 \)).

2.11 Transcriptional analysis: relative expression of selected genes

Since the advent of ‘omics’ technologies, the field of ecotoxicogenomics (that is, the integration of gene and protein expression into ecotoxicology) has been developing (Neumann & Galvez 2002). This approach has many benefits, including the potential for earlier warning and increased mechanistic understanding of adverse effects (Fedorenkova et al. 2010, Hines et al. 2010). In particular, alterations in transcriptional gene expression (i.e. mRNA levels) have been identified as sensitive biomarkers of stress (e.g. Williams et al. 2003). In aquatic organisms changes in mRNA profiles have been found in response to a diverse range of stressors (e.g. Marie et al. 2006, Venn et al. 2009, Matranga et al. 2010, Poynton et al. 2011) and have been associated with DNA damage (as measured by the comet assay) in the European eel, *Anguilla anguilla*, after PAH exposure (Nogueira et al. 2009). In mussels there are numerous studies examining mRNA profiles in response to contaminants, including hormones (Ciocan et al. 2011), metals (Ciocan & Rotchell 2004, Hines et al. 2010), organic pollutants (Hines et al. 2010, Di et al. 2011) and radionuclides (AlAmri et al. 2012).

Transcription of individual genes (i.e. specific mRNA levels) is quantified using a four step process. Total RNA is extracted from tissue, which is then converted to complementary DNA (cDNA) via reverse transcription. cDNA is then amplified using primers specific to the gene of interest (GoI) and quantified, either by image analysis of band intensity on a gel (e.g. D’Agata et al. 2013) or using a fluorescent reporter in quantitative (real-time) PCR (qPCR), which is now considered the preferred technique (Bustin et al. 2005). This process is illustrated in Fig. 2.15.
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2.11.1 RNA extraction

Total RNA was isolated from gill samples using either the RNAeasy mini kit (Qiagen Ltd, Crawley, UK) or the GenElute RNA MiniPrep kit (Sigma-Aldrich), according to the manufacturer’s instructions. RNA concentration and integrity was determined by spectrophotometry (ND-1000; Nanodrop, Wilmington, DE, USA) and gel electrophoresis. Only samples with an OD\textsubscript{260}:OD\textsubscript{280} ratio > 1.95 and clear, crisp bands were used.

2.11.2 Reverse transcription

Two micrograms of total RNA was used for reverse transcription using M-MLV reverse transcriptase and random primers according to the manufacturer’s instructions (Promega Corporation, Madison, WI, USA).

2.11.3 Quantitative (real-time) PCR

Following cDNA synthesis, qPCR was performed on samples in duplicate. Each 15 \(\mu\)L qPCR reaction contained 7.5 \(\mu\)L SYBR Green Jumpstart Taq ReadyMix, 0.03 \(\mu\)L of forward and reverse primers (100 \(\mu\)M), 4.44 \(\mu\)L of molecular grade water and 3 \(\mu\)L of template cDNA. The qPCR reaction was carried out using an Applied Biosystems Step-One Plus real-time PCR system with StepOne\textsuperscript{TM} Software (v2.2.2; Applied BioSystems). Thermocycling conditions were initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. A dissociation profile (melt curve) was added to verify the purity of PCR products.
2.11.4 Quantification of relative expression

PCR efficiency and fluorescence baseline and thresholds were measured using LinRegPCR software (Ramakers et al. 2003, Ruijter et al. 2009). The relative expression ratio (Pfaffl et al. 2002) of each gene was quantified using REST-MCS (v.2006, available online at www.gene-quantification.de) or REST 2009 software (v2.0.13; Qiagen Ltd, Crawley, UK), from qPCR efficiency and threshold cycle ($C_q$) values, relative to the reference gene and compared back to control samples as a calibrator. $C_q$ values for the reference gene were compared across all samples and treatments to determine if a single or multiple reference genes were needed.

2.12 Proteomics

Proteomics (i.e. the examination of the total protein content of a cell, tissue or organism) is another key component of ecotoxicogenomics (Snape et al. 2004a) and has been used in mussels to study differences in fitness (Diz & Skibinski 2007) and responses to temperature (Tomanek & Zuzow 2010, Fields et al. 2012), as well as contaminant-induced effects (e.g. McDonagh & Sheehan 2006, Letendre et al. 2011, Gomes et al. 2013, Schmidt et al. 2013). Analysis of the proteome involves 3 major steps: (1) separation of the proteins at high resolution (most often by two-dimensional gel electrophoresis [2D-GE]); (2) comparison of the resulting expression profiles; and (3) identification of any differentially expressed proteins (Snape et al. 2004a).

This technique was used for Chapter 4 only, so specific methodological details are included there.

2.13 Histopathology of mussel tissues

The standardized dissection technique developed by the Cefas laboratory, Weymouth, UK (ICES, 2011) was used to ensure maximum comparability between samples, and that all the major mussel tissues were available for analysis. Mussels were excised from their valves whole, and a cross-section of the vital organs
taken using a razor blade (Fig 2.16A-F). After dissection, samples were immediately transferred to Davidson’s seawater fixative (Pioneer Research Chemicals Ltd., Colchester, UK) and allowed to fix for > 24 h. After fixing, samples were placed in 70 % IMS until processing (<1 week). Processing took place in a Leica TP 1020 Automatic Tissue Processor (Leica Microsystems, Wetzlar, Germany). After processing, tissues were embedded in paraffin wax, sectioned (3-6 µm) using a rotary microtome (R2125 RTS, Leica Microsystems), transferred to slides and stained with haematoxylin and eosin.

2.14 Stress on stress response

Anoxia is a typical condition in the natural intertidal habitat of mussels. Consequently, the ability of mussels to survive in air (stress on stress response [SoS]) is regarded as a sensitive index of general stress syndrome in mussels (Viarengo et al. 1995). The SoS response is determined after exposure to the chemical or radionuclide of interest. At the end of the exposure period, exposure vessels were drained and the remaining mussels were blotted dry externally and transferred to open petri dishes. Mussels were maintained in these dishes, without water (but in a humid environment) at 15 °C, and observed daily for mortality. Mortality was
defined as continued opening of the valves and failure to respond when tapped, and was used to calculate LT\textsubscript{50} values (median lethal time for 50 % mortality).

2.15 Analysis of metal content

Inductively coupled plasma – mass spectrometry (ICP-MS) and - optical emission spectrometry (ICP-OES) are routinely used in our laboratory for the analysis of trace metals in aquatic systems (e.g. Federici et al. 2007, Mustafa et al. 2012, Trevisan et al. 2011). Aqueous samples are introduced into the instrument via a nebuliser, producing an ultra-fine mist. This is heated to extreme temperatures (>5000 °C) by a plasma torch, which causes solvent evaporation, vaporisation, atomisation and ionisation of any material contained within the sample. The resulting ions are passed into a mass spectrometer or optical spectrometer and analysed. In general, detection limits of the ICP-MS are more sensitive than similar instruments, including ICP-OES (Nelms 2005), however, selection of the appropriate detection technique was based on multiple factors (including detection limits, analytical range, speed, logistical issues and cost).

2.15.1 Preparation of tissue samples for metal analysis

For analysis of metal content, individual organs (Fig 2.16G) were dissected out of each animal. Each tissue was washed with distilled water, blotted dry and transferred to a pre-weighed and acid-washed (10 % HCl) scintillation vial. Samples were dried to constant weight at 60 °C and re-weighed. Tissue digestion was achieved by addition of 1 mL concentrated nitric acid and incubation for 2 h at 70 °C. Digested tissue samples were diluted to a final volume of 5 mL with Milli-Q water and stored at room temperature in the dark until analysis.

2.15.2 Preparation of water samples for metal analysis

Exposure vessels were mixed thoroughly using a stirrer, before a water sample was removed and transferred to an acid washed scintillation vial, pre-filled with 2 % nitric acid. Water samples were stored at room temperature in the dark until analysis.
2.15.3 ICP-MS or ICP-OES analysis

Samples were analysed using either an X Series II ICP-MS (Thermo Fisher Scientific Inc., Waltham, MA, USA) with PlasmaLab software (Thermo Fisher Scientific Inc., Waltham, MA, USA) or an Varian 725-ES ICP-OES (Agilent Technologies Ltd, Wokingham, UK). In order to monitor instrumental drift, an internal standard of 115-In was added to both tissue and water samples, to a final concentration of $10 \mu g L^{-1}$. Although indium has an atomic mass higher than our two metallic contaminants (Ni and Zn), it was selected based on its minimal occurrence in marine samples and low polyatomic interference with seawater. The limit of detection (LOD; three standard deviations) and limit of quantification (LOQ; ten standard deviations) were determined from 6 replicate analyses of Milli-Q water during each run of the apparatus.

2.16 Liquid scintillation counting for measurement of tritium activity in water

Liquid scintillation counting (LSC) is the preferred method for determining the activity of $\beta$ emitters (Valković 2000). It utilises an organic solvent known as a cocktail, which contains organic phosphors. When $\beta$ radiation enters the cocktail it passes its energy to the phosphor, which emits a pulse of light. This light is detected by photomultiplier tubes and converted to a count of radioactivity (Fig. 2.17).

To determine tritium activity in water, 100 $\mu$L of thoroughly mixed water was added to 5 mL of liquid scintillation cocktail (UltimaGold,Nakayama et al. 2012; Perkin Elmer Inc., Cambridge, UK), further mixed and left in the dark for 2 h prior to counting in a LS 6500 liquid scintillation counter (Beckman Coulter Inc., Brea, CA, USA) to a fixed precision of 5 %.
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2.17 Optimisation of liquid scintillation counting for tritium in mussel tissues

All mussels used for LSC optimisation were collected from TBS and depurated for a minimum of two weeks, as described in section 2.3.

The efficiency of LSC largely depends on two factors - chemical quenching and colour quenching (Valković 2000). Chemical quenching occurs when the components of the cocktail absorb radiation before it interacts with the phosphors. Colour quenching is the prevention of light reaching the detector due to absorbance or scattering caused by the colour of the cocktail-sample solution. Colour quench increases from yellow through brown to red (Herberg 1960).

2.17.1 Effect of colour on activity measurement in unexposed mussel tissues

It became apparent that the preparation and solubilisation of mussel tissues prior to LSC resulted in solutions which varied greatly in colour (Fig. 2.18). Although previous studies have determined the activity of mussel tissue samples after exposure to HTO, they did not consider colour quench from the different solutions (Jaeschke et al. 2011).

To assess the effect of colour on the efficiency of LSC for tritium in mussel tissue
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digests, individual organs (Fig 2.16G) were dissected from 15 unexposed mussels and placed into pre-weighed scintillation vials. Larger tissues (gills and mantle) were chopped into finer pieces to aid solubilisation. Samples were freeze-dried to constant pressure and re-weighed. Samples were then re-hydrated by addition of 100 µL Milli-Q water and incubation at room temperature for 10 min. Soluene-350 (1 mL; Perkin Elmer Inc., Waltham, MA, USA) was added to each vial, and samples were incubated at 50 °C for at least 48 h or until solubilisation had occurred. Following solubilisation, 10 mL of liquid scintillation cocktail (UltimaGold, Perkin Elmer Inc., as above) was added to each vial and the resulting solution was acidified with 100 µL of glacial acetic acid. Samples were transferred to the liquid scintillation counter (LS6500, Beckman Coulter Inc., Brea, CA, USA), left in the dark for 2 h prior to the start of counting, and then counted to a fixed precision of 5%. For comparison with spiked samples (see section 2.17.2) the data here are presented as activity per vial, and not normalised for tissue weight.

After counting, each solution (sample + cocktail + acetic acid) was assessed visually and given a colour code; (0) no colour; (1) straw coloured; (2) yellow and (3) orange/brown; as illustrated in Fig. 2.18.

Average activity increased significantly with increasing colour code ($p < 0.01$) and all colour codes had significantly different activities from each other (Fig. 2.19 A; $p < 0.01$). No significant effect on activity was found in relation to tissue type. Overall, activities were very low (<10 Bq), as expected for unexposed mussels.
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Figure 2.19. Impact of colour on liquid scintillation counting of non-spiked mussel tissue samples. Mean activity concentration (± one SE) of mussel tissues according to colour code (A); proportion (%) of samples per colour code (B); and proportion (%) of each colour code from each tissue type. In panel A, means that do not share a letter are significantly different ($p < 0.01$). No significant differences were observed for data in panels B or C.
2. Materials and Methods

Table 2.1. Parameters used to assess efficiency of liquid scintillation counting when using a Beckman Coulter LS 6500.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
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<tbody>
<tr>
<td>LumEx</td>
<td>The percentage of the total count which is attributable to chemical interaction between sample and cocktail, i.e. chemiluminescence.</td>
</tr>
<tr>
<td>H#</td>
<td>A dimensionless number which indicates the difference between the count distribution of the sample and an external standard radionuclide (usually $^{137}$Cs), i.e. a smaller H# indicates lower sample quench.</td>
</tr>
</tbody>
</table>

Most of the tissues processed were categorised as colour code 0 (32.69 %) or 1 (45.20 %; Fig. 2.19B), which included all gill, muscle, foot and byssus samples (Fig. 2.19 C). Unsurprisingly, digestive gland samples were particularly likely to produce a darker coloured solution, whereas mantle samples were more variable - producing solutions with colour codes 1, 2 or 3 (Fig. 2.19C).

2.17.2 Effect of colour on activity measurement at higher activities

In order to determine the impact of colour on mussel tissue samples of higher activity (i.e. similar to those from exposed mussels), a spiking experiment was performed. Briefly, individual organs were removed from unexposed 6 mussels, as described before. Each organ was transferred to a pre-weighed scintillation vial and freeze-dried before re-weighing. The dry tissues were then processed and counted as described above. After initial counting, the solutions were spiked with 100 $\mu$L of 1 Bq mL$^{-1}$ HTO, resulting in a nominal total activity (per vial) of 0.1 Bq. Sample solutions were then thoroughly vortex mixed and counted again. This procedure was repeated sequentially (with stock solutions of greater activity) to produce nominal total activities of 1.1, 11.1, 111.1, and 1111.1 Bq per vial.

In addition to activity, LumEx and H# (Table 2.1) were also recorded, to ensure counting efficiency was not compromised.

Colour code or treatment had a significant effect on activity ($p < 0.05$). However, in contrast to the earlier results, at higher activities ($> 1.1$ Bq) increasing colour code caused a significant decrease in mean activity (Fig. 2.20 G, J and M). Percentage LumEx increased with colour code and was greatest at lowest activities,
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Figure 2.20. Impact of colour on liquid scintillation counting of mussel tissue samples spiked with 0.1 (A-C), 1.1 (D-F), 11.1 (G-I), 111.1 (J-L) or 1111.1 Bq (M-O). Mean activity per vial (A, D, G, J and M); mean chemiluminescence (B, E, H, K, and N; *LumEx*) and mean spectral index of external standard (C, F, I, L and O; *H#*). Data are means ± one SE.
but also most variable (Fig. 2.20B, E). LumEx peaked at 11.48% in the darkest samples after spiking with 0.1 Bq (Fig. 2.20B). All samples were less than the nominal activity, potentially due to some decay of the stock solution.

### 2.17.3 Effect of bleaching on activity measurement

Although the liquid scintillation counter used in this study does perform corrections for colour quench, we still observed an effect of colour on activity. Therefore it was necessary to bleach sample solutions with hydrogen peroxide prior to counting. It was, however, also necessary to determine the impact of bleaching on our ability to efficiently measure tritium activity, i.e. colour quench and chemiluminescence.

To assess these effects, tissues from 9 unexposed mussels were processed and colour coded as above. Samples were spiked with 250 Bq of HTO and thoroughly mixed. This activity was calculated from the maximum activity reported in mussel tissues after one week’s exposure to 37 MBq L\(^{-1}\) HTO (i.e. \(\sim 6\) MBq L\(^{-1}\); Jaeschke et al. 2011) and a mean tissue weight of 0.04 g. After spiking, colour coded samples were counted as before. They were then bleached by addition of 0.5 mL hydrogen peroxide (8.8 M) and incubated, first at room temperature for 30 min and then at 50 °C for 30 min to degrade the hydrogen peroxide. After cooling to room temperature and 2 h incubation in the dark, counting was performed as before.

Bleaching had a clear impact on the colour of samples. Although the original (before bleaching) colour codes were used for all analyses, samples were also re-categorised in order to assess the effectiveness of the bleaching step. After bleaching, the majority of samples were re-categorised as colour code 0 (81.48%) with the remainder as code 1 (18.52%). Those samples which had not completely lost their colour (i.e. were re-coded as 1 not 0) came exclusively from the groups coded 2 or 3 prior to bleaching.

As before (Fig. 2.20) an increase in colour code resulted in a significant decrease in measured activity \((p < 0.0001)\) before bleaching. Maximal activity was 90.70% of the nominal value (in the 0 colour-coded group) before bleaching and 91.47% (in the 2 colour-coded group) after. These values were not significantly different \((p > 0.05)\). Bleaching significantly increased the measured activity in colour codes
Figure 2.21. Effects of bleaching on liquid scintillation counting of mussel tissue samples spiked with 250 Bq HTO. Mean activity per vial (A); mean chemiluminescence (B; ‘LumEx’) and mean spectral index of external standard (C; ‘H#’). Data are means ± one SE, * significant differences ($p < 0.05$) from the 0 colour-coded mean (*) and from before beaching (§) are indicated.
2. Materials and Methods

Freeze dry dissected tissue → Re-weigh → Rehydrate with 100 µL water → Add 10 mL cocktail → Bleach with 0.5 mL H₂O₂ → Solubilise with 1 mL Soluene-350 → Acidification with 100 µL acetic acid → Incubation in the dark → Counting

Figure 2.22. Final protocol for preparation of mussel tissue samples for liquid scintillation counting, including the addition of a bleaching step to minimise colour quench.

Chemiluminescence (LumEx) and quench (H#) also responded in the same way as in earlier experiments (Fig. 2.20) i.e. increasing with colour code (Fig. 2.21B-C). After bleaching, LumEx significantly increased for all colour codes \( p < 0.0001 \) but peaked at 3.61 % for colour code 1, which is still within recommended guidelines (i.e. < 10 %) and was significantly lower than before bleaching for colour code 3 \( p < 0.0001 \). As expected, colour quench decreased following bleaching, with H# significantly lower than before bleaching for colour codes 1, 2 and 3, although quench was still significantly higher in groups 2 and 3 compared to 0 \( p < 0.0001 \).

Based on these results all subsequent tissue digests were bleached with 0.5 mL hydrogen peroxide prior to LSC. The revised protocol for preparing mussel tissue samples for LSC is illustrated in Fig 2.22.

2.18 Statistical analyses

Statistics were calculated using R (i386, v2.15.2; www.R-project.org) or Minitab (v.16.2.2; Mintab Inc., State College, PA, USA). Significance for all tests was set at \( p < 0.05 \). Data were tested for normality, either by using Kolmogorov-Smirnov tests or by
visually assessing the normality of residuals (for regression models), and appropriate parametric or non-parametric tests were used. In all cases where multiple comparisons were made manually (e.g. multiple t-tests) $p$-values were adjusted using Holm’s sequential Bonferroni method (Holm 1979). All ANOVA or GLM post hoc tests were Tukey’s unless otherwise stated. To avoid overestimation of statistical significance for the comet assay data, % tail DNA values for individual cells were not used for statistical analysis, as recommended by Lovell et al. (1999, 2008). Instead, the median was calculated for each mussel (as data were non-normally distributed). The resulting data sets based on medians was used as the basis for statistical analysis. Significant differences in relative expression were calculated using REST MCS or REST 2009 software (for multiple or single comparisons, respectively) using a pairwise fixed random reallocation test (PFRRT).

Unless otherwise stated, all graphs display data as means ± standard error of the mean (SE) to illustrate differences between groups or treatments, whereas numerical data quoted in the text are means ± SD, to illustrate variability in individual values. For more information on this distinction please see Cumming et al. (2007).

In this chapter, differences in CR before and after spawning were assessed using a repeated measures ANOVA, morphometric differences between species by t-tests (adjusted for multiple comparisons) and effects of colour on LSC by Kruskal-Wallis, followed by multiple Mann-Whitney U-tests with adjustment for multiple comparisons. More details on specific statistical methods for each chapter (i.e. tests used) are included in the relevant sections.
Chapter 3

Acute nickel toxicity in mussels

Published in part as:

3. Acute nickel toxicity in mussels

3.1 Introduction

Nickel (Ni) is a naturally occurring, commercially important metal, used in a variety of industries including coin production, engineering and the manufacture of stainless steel (Eisler 1998). Stainless steel is probably the most commonplace use of Ni, including in food and beverage containers, water pipes and surgical instruments. Other common uses of nickel alloys (which comprise the majority of its everyday uses) include nickel-plating (e.g. jewellery), chrome-plating (with a top layer of Cr), rechargeable batteries (typically Ni-metal-hydride, NiMH) and electrical circuitry (Nickel Institute 2013). Consequently Ni is released into the atmosphere during its mining, smelting and refining, and after disposal of Ni-containing products. As a result, although dissolved Ni concentrations are typically low in the open ocean (0.12 - 0.70 µg L$^{-1}$; Millero 1996), and in rain (< 1 µg L$^{-1}$; Eisler 1998), it is greatly enriched in freshwater environments close to mining establishments. For example, in lakes near the Sudbury nickel refinery, Canada, dissolved concentrations of 180,000 µg L$^{-1}$ have been reported (Eisler 1998). Nickel concentrations in coastal marine waters can also be several orders of magnitude higher than in the open ocean. For example, Ratekevicius et al. (2003) report coastal seawater Ni concentrations of 380 µg L$^{-1}$ in the Cachagua area of Chile. For English estuaries (i.e. those nearest to the CHRONEXPO region of interest) with high anthropogenic inputs, reported Ni concentrations include > 70 µg L$^{-1}$ for the Tyne, Wear and Tees (DETR 1998) and ~ 10 µg L$^{-1}$ for the Humber and Mersey (Comber et al. 1995, Laslett 1995). However, measurements from the English Channel region itself suggests lower dissolved Ni concentrations offshore (< 2.5 µg L$^{-1}$; Laslett 1995). Data for Ni concentrations in coastal, marine areas close to Ni mines are not well reported. For example, it is well accepted that the coral lagoon surrounding the islands of New Caledonia (the third largest producer of Ni globally; Dalvi et al. 2004) has an extremely high heavy metal input (Hédouin et al. 2007); however Ni concentrations are yet to be quantified.

Nickel has been reported to cause a variety of genotoxic lesions, including DNA strand breaks, oxidation of nucleotide bases and DNA-protein crosslinks. It also enhances the formation of DNA lesions induced by other environmental agents such as ultraviolet (UV) radiation (Hartwig et al. 1994). It is generally accepted that Ni causes these lesions via a range of mechanisms, including: (a) the formation of reactive oxygen species (ROS) either directly or indirectly, as the free Ni$^{2+}$
ion interacts with cellular molecules (Kasprzak 1991, Huang et al. 1994); (b) direct interaction with DNA or proteins; and (c) inhibition of DNA repair mechanisms. However, the relative contributions of these mechanisms for observed toxicity is not clear, and there have been only limited studies that have attempted to correlate the expression of key genes with other biological responses following exposure of aquatic organisms to metallic contaminants (e.g. Dondero et al. 2006a).

There is growing regulatory concern about the impact of carcinogenic, mutagenic or reproductive (CMR) toxicants in the aquatic environment (e.g. Fuerhacker 2009). Given its known carcinogenic and mutagenic properties and its input to the environment, Ni and its compounds have been classified as a priority substances in the EU Water Framework Directive (WFD; DEFRA 2010) and in the Priority Substances Directive amending the WFD (EC 2000, 2008). There have been a number of studies determining effects of metals on bivalve molluscs (e.g. Amiard-Triquet et al. 1986, Black et al. 1996, Achard et al. 2004, Cherkasov et al. 2007, Nadella et al. 2009, Al-Subiai et al. 2011). Despite this, there are still only a limited number of studies on the potential toxic effects of Ni in aquatic invertebrates. Recently, though, technological advances have allowed the quantification of Ni-induced cellular and genetic damage in mussels in the field (Rank et al. 2005) and under laboratory conditions (Attig et al. 2010, Millward et al. 2012). Nevertheless, these studies investigated only a biomarker of exposure (i.e. DNA strand breaks as measured by the comet assay) and their scope did not extend to to quantification of any biomarkers of effect (e.g. MN induction). Furthermore, these previous studies did not examine the potential induction of oxidative DNA damage in response to Ni exposure.

### 3.1.1 Objectives and hypotheses

Within the context of the above information, this chapter had the following objectives:

(a) to use an acute Ni exposure to further validate biomarkers for chronic studies;

(b) to determine the genotoxicity of Ni to *M. galloprovincialis*, using modified comet assay to determine oxidative DNA damage and induction of micronuclei to determine clastogenic/aneugenic effects;
(c) to evaluate the transcription profile of key stress genes (pgrp, mt10, mt20, sod, hsp70 and gst) to elucidate potential mechanisms behind observed genotoxicity;

(d) to use an integrated experimental approach to correlate gene expression and genotoxicity with histopathology, i.e. an effect at a higher (more ecologically relevant) level of biological organisation;

(e) to determine tissue-specific Ni accumulation, (which permits comparison of our results with those published previously, e.g. Millward et al. 2012) and assess any relationship between genotoxicity and accumulation, where appropriate.

It was hypothesised that Ni would cause genotoxicity in a concentration-dependent manner, (linked to accumulation of Ni in tissues) and that the responses of stress genes and higher-level biomarkers would also follow this trend.

3.2 Materials and Methods

3.2.1 Experimental design

Two identical exposures were set up during February – March 2011. The first was used to determine DNA strand breaks in mussel haemocytes (using the enzyme modified comet assay) and histopathology of mussel tissues after exposure to Ni. The second allowed us to quantify tissue-specific Ni accumulation, induction of micronuclei in haemocytes, and transcriptional expression of key genes in gill tissue. Fig. 3.1 summarises the design of these experiments graphically.

3.2.2 Mussel exposure conditions

Adult *Mytilus galloprovincialis* (shell length 46.5 ± 2.04 mm) were collected from Trebarwith Strand, transported to the laboratory and maintained as described in section 2.3. After depuration, mussels were transferred to 15 glass beakers containing 2 L filtered (<10 µm) seawater, at a density of 1.5 mussels L⁻¹, and allowed to acclimatise for 48 h prior to exposure. Beakers were randomly assigned to one of 5 treatment groups: 0 (control), 18, 180, 1800 or 3600 µg L⁻¹ Ni (as NiCl₂); with three replicates per treatment (Fig. 3.1). This concentration range was selected
3. Acute nickel toxicity in mussels

Mussels acclimatised in 2 L beakers (1.5 L$^{-1}$) for 5 days and exposed to Ni concentrations of 0, 18, 180, 1800, and 3600 µg L$^{-1}$ for 48 hours. Mussels from 3 beakers per treatment were used.

**Experiment 1**: Extract haemolymph, dissect (standardised cross-section), histopathology, and gene expression.

**Experiment 2**: Extract haemolymph, dissect (small section of gill), micronucleus assay, and tissue samples.

Mussels were depurated for 2 weeks. The experiment included 6 samples per treatment. Figure 3.1. Overall experimental design for the determination of the impacts of acute Ni exposure on *M. galloprovincialis*.

$\text{ICP-MS}$

$n = 6$
3. Acute nickel toxicity in mussels

Based on the published literature and prior research on mussels exposed to Ni at our laboratory (Stuijfzand et al. 1995, Tsangaris et al. 2008, Millward et al. 2012). According to established thermodynamic data, the speciation of inorganic Ni in seawater is as follows; free ion 47 %, hydroxides 1 %, chlorides 34 %, sulphides 4 % and carbonates 14 % (Turner et al. 1981). However, it is important to bear in mind that in seawater Ni is also known to bind strongly to organic ligands (Martino et al. 2004), whose binding characteristics were not quantified during this study. Nickel exposure lasted 5 d, during which mussels were not fed and a complete water change was performed daily. This exposure duration and replacement of water matched that used in earlier studies of mussels exposed to metals and reference genotoxic agents (Bolognesi et al. 1999, Jha et al. 2005, Canty et al. 2009, Al-Subiai et al. 2011).

Water quality parameters were salinity 36.72 ± 0.47, pH 7.85 ± 0.12, dissolved oxygen 90.21 ± 7.48 % and temperature 14.94 ± 0.38 °C for experiment 1. For experiment 2, salinity was 35.6 ± 0.39, pH 7.99 ± 0.05, DO 89.31 ± 4.25 and temperature 15.35 ± 0.68 °C.

3.2.3 Sampling procedures

After 5 d, haemolymph was extracted from each mussel as in section 2.5. Haemolymph was then processed for the modified comet assay or micronucleus assay (see sections 2.8 and 2.10). Following haemolymph extraction, mussels were dissected either into their individual organs for ICP-MS determination of Ni content or cross-sectionally for histopathology. Those mussels dissected for ICP-MS also had a small (~5 mm) section of gill removed (n = 6), preserved in RNALater, and stored at -20 °C for molecular analysis.

3.2.4 ICP-MS measurements to determine tissue-specific accumulation of Ni and water concentrations

Adsorption of Ni to beakers was quantified (by ICP-MS analysis of nitric acid washes) as < 0.1 % of the concentrations added.

Water samples (2.5 mL) were taken immediately following the water change each day and transferred to scintillation vials pre-filled with 22.50 ml of 2 % HNO₃, and
stored at room temperature until analysis. Individually dissected tissues (Fig 2.16 G) were washed, dried and digested as described in section 2.15.1. Samples (water or tissue) were then processed for inductively-coupled plasma mass spectroscopy (ICP-MS) analysis, as detailed in section 2.15.3. The LOD and LOQ for Ni using this apparatus were 0.67 µg L$^{-1}$ and 2.20 µg L$^{-1}$, respectively.

Raw data from ICP-MS measurements was converted to weight of Ni per tissue ($T_{Ni}$) according to equation 3.1. This value was then used in equations 3.2 and 3.3 to calculate the concentration of Ni in tissues ($C_{Ni}$) and the proportion of total Ni ($P_{Ni}$), respectively.

\[
T_{Ni} = (C_{ICP} - B) \times V
\]

where $T_{Ni}$ is the weight of Ni in each tissue (µg); $C_{ICP}$ is the raw ICP-MS data (µg L$^{-1}$); $B$ is the mean Ni concentration of 3 blanks analysed concurrent to the samples (µg L$^{-1}$); and $V$ is the total volume of sample (5 mL).

\[
C_{Ni} = \frac{T_{Ni}}{T_W}
\]

where $C_{Ni}$ is the concentration of Ni in each tissue (µg g$^{-1}$), $T_{Ni}$ is the product of equation 3.1; and $T_W$ is the tissue dry weight (g).

\[
P_{Ni} = \frac{T_{Ni}}{\sum T_{Ni}} \times 100
\]

where $P_{Ni}$ is the proportion (%) of Ni in a tissue, $\sum T_{Ni}$ is the sum of the Ni weights (µg) for all tissues in a given mussel; and $T_{Ni}$ is the product of equation 3.1.

### 3.2.5 Enzyme-modified comet assay to determine oxidative DNA damage

The modified comet assay was performed using the haemocytes of mussels as described in detail in Chapter 2 (section 2.10). The bacterial enzymes used for the determination of oxidative DNA damage were formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (Endo III). Three slides were prepared per
sample, one enzyme buffer control, one with Fpg and one with Endo III. Slides were coded and randomised to ensure scoring was blind.

### 3.2.6 Induction of micronuclei to determine clastogenic and/or aneugenic effects

Haemocytes were concentrated, adhered and fixed as described in section 2.8, before staining with 20 µl of 20 mg L⁻¹ ethidium bromide. Slides were coded and randomised and at least 1000 cells per slide were scored. In addition to the criteria outlined by Countryman and Heddle (1976) and detailed in section 2.8, efforts were made to only score cells of similar size, thereby reducing variability in MN counts due to the different haemocyte cell subpopulations, as previously discussed by Bolognesi and Hayashi (2011).

### 3.2.7 Determination of relative transcriptional expression of selected genes

In filter feeding organisms, such as mussels, the gills are the first point of contact for contaminants. Consequently they have been reported to be a sensitive organ for the assessment of genetic damage (Rank et al. 2005, Bolognesi & Hayashi 2011). For these reasons, and to elucidate downstream effects of potential genetic damage, we selected gill tissue for molecular analysis. Total RNA isolation from gill samples (n = 6), reverse transcription and qPCR were performed as described in detail in Chapter 2 (section 2.11). PCR efficiency and fluorescence baseline and thresholds were measured using LinRegPCR software (Ramakers et al. 2003, Ruijter et al. 2009). The relative expression ratio (RER) of 6 genes associated with xenobiotic defence or oxidative stress (glutathione-s-transferase, two metallothionein isoforms, p-glycoprotein, a heat shock protein and superoxide dismutase) and one housekeeping gene (18S ribosomal RNA; Table 3.1) was quantified using REST 2009 software (Pfaffl et al. 2002), from qPCR efficiency and threshold cycle (C_q) values, relative to the reference gene and compared back to control samples as a calibrator. As C_q values for 18 S rRNA were consistent across all samples and treatments (10.97 ± 0.90) it was used as a single normalising gene (Di et al. 2011).
<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione S-transferase (gst)</td>
<td>AY557404</td>
<td>5'-CCTGAAACCAACCAAGGGTTACAT-3'</td>
<td>5'-TGGACTCCTGGTCTAGCCAACACT-3'</td>
</tr>
<tr>
<td>Metallothionein 10 (mt10)</td>
<td>AJ577130</td>
<td>5'-TGACACTGGCTGCAGCGGT-3'</td>
<td>5'-CACATTTACACGTTAAAGGTCCTGTACAT-3'</td>
</tr>
<tr>
<td>Metallothionein 20 (mt20)</td>
<td>AJ577131</td>
<td>5'-GACGCCTGCAAATGTGCAAGT-3'</td>
<td>5'-TCGGACCAGTGCGGTCACAT-3'</td>
</tr>
<tr>
<td>P-glycoprotein (pgp)</td>
<td>AF159717</td>
<td>5'-AACATCGGTGTGGTTTCTCAGGA-3'</td>
<td>5'-GATCAAAGCTCTGGCAATGGCT-3'</td>
</tr>
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<td>Heat shock protein 70 (hsp70)</td>
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<td>5'-GACGCCTGCAAATGTGCAAGT-3'</td>
<td>5'-TCGGACCAGTGCGGTCACAT-3'</td>
</tr>
<tr>
<td>18S ribosomal RNA (18S)</td>
<td>L33448</td>
<td>5'-GTGGTGCATGGCCGTTCTTAGT-3'</td>
<td>5'-CATCTAAGGGCATCACAGACCTGTT-3'</td>
</tr>
<tr>
<td>Cu/Zn Superoxide dismutase (sod)</td>
<td>AF178174</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1. Genes and primers used for qPCR on mussel tissue samples after exposure to nickel chloride.
3.2.8 Determination of histopathological changes

After dissection, fixation and processing as described in section 2.13, slides ($n = 6$) were systematically examined for histopathological lesions, including presence of parasites (if any), structural damage, inflammation and neoplasia. In order to perform semi-quantitative analysis, 5 fields of view of gill tissue were separately analysed per mussel (i.e. slide) and scored for the presence or absence of structural damage (e.g. loss of inter- or intra-filamental junctions, distortion of filaments, loss of cilia; Fig. 3.5B-C). Incidence of such damage (as a percentage) was calculated from this data (Fig. 3.5A).

3.2.9 Statistical analyses

Statistical analyses were conducted according to the principles detailed in section 2.18. Specifically, accumulation data were non-normally distributed and could not be transformed to normality. Consequently non-parametric tests were used (Kruskal-Wallis, followed by multiple Mann-Whitney U-tests with adjustment for multiple comparisons). Additionally, proportional (percentage) data for tissue-specific Ni accumulation were square root transformed to normality, before analysis using a 2-way ANOVA with Tukey’s pairwise comparisons as post hoc tests. Linear regression analyses were performed for all combinations of biomarkers and $p$-values were adjusted for multiple comparisons. The gene expression parameter used for relationship analysis was $C_q$ normalised for reference gene (i.e. $\Delta C_q = C_{q[GoI]} - C_{q[18S]}$). All other statistical analyses used ANOVA with post hoc tests.

3.3 Results

Measured Ni concentrations showed good agreement with nominal values (data not shown) and were consistently within 20% of the nominal concentration, as recommended by the OECD (1992).
3.3.1 Tissue-specific accumulation of Ni

Nickel accumulation occurred in a concentration-dependent manner, with a clear tissue-specific trend. Byssus accumulated the highest amount of Ni per gram of tissue weight for all treatments (maximum at 18 $\mu$g L$^{-1}$ Ni, 5389 ± 3656 $\mu$g g$^{-1}$) but was highly variable and showed no significant differences across treatments. For these reasons, and to allow an appropriate scale, byssus data have been omitted from Fig. 3.2A. There were significant overall effects of Ni treatment ($p < 0.001$) and concentration-dependent increases in Ni accumulation in every tissue except byssus ($p < 0.05$). Nickel was generally accumulated in the order byssus > digestive gland > gills > foot > muscle > mantle across all treatment groups, although this trend was reversed for foot and muscle in the 1800 $\mu$g L$^{-1}$ Ni group (Fig. 3.2A).

Proportionally, the only tissue to show a treatment effect was digestive gland, which represented a significantly lower proportion of the total Ni at 3600 $\mu$g L$^{-1}$ Ni than in the control (Fig. 3.2B; $p < 0.05$). In terms of within-treatment between-tissue effects, byssus made up a significantly higher proportion of total Ni content than all other tissues at 3600 $\mu$g L$^{-1}$ Ni, and was significantly higher than foot, gills, mantle, and muscle at all other concentrations ($p < 0.05$).

3.3.2 Genotoxicity of Ni to mussel haemocytes

The results of the comet assay indicated that DNA damage (% tail DNA) in mussel haemocytes was significantly affected by exposure to Ni (2-way ANOVA, $p < 0.0001$). All enzyme/buffer treatments at the highest Ni concentration (3600 $\mu$g L$^{-1}$) produced a significant increase in DNA damage compared to the equivalent enzyme/buffer treatment for the control group (Tukey’s pairwise comparisons, $p < 0.005$). Enzyme treated slides showed significantly more DNA damage than their respective controls at 1800 $\mu$g L$^{-1}$, but the corresponding buffer treated slides at this treatment did not (Fig. 3.3A). DNA damage in the controls was relatively low, at ~10% tail DNA, and the highest amount of DNA damage (35.31 % tail DNA) was seen in haemocytes from mussels in the 3600 $\mu$g L$^{-1}$ Ni group following treatment with Endo III. Fpg and Endo III treatments did not produce consistent results across all Ni treatments, and the effect of enzyme and enzyme/Ni interaction was not significant.
3. Acute nickel toxicity in mussels

Figure 3.2. Tissue-specific accumulation of nickel per gram of mussel tissue (dry weight)(A) and as proportion of total Ni per tissue (B) after 5 d exposure. Error bars are ± one SE. To allow for appropriate scaling, byssus measurements (which showed no significant variation) have been removed from (A). Asterisks indicate significant differences from the corresponding control. Means that do not share a lower case letter showed statistically significant differences between nickel treatments for the same tissue ($p < 0.05$).
Data for MN induction was in good agreement with that of the comet assay (Fig. 3.6A). MN (per 1000 cells) could be predicted from % tail DNA in haemocytes using the formula $\text{MN} = (0.26 \times \% \text{ tail DNA}) + 1.78$ (linear regression; $R^2 = 0.78$, $p < 0.05$). Ni concentration had a significant effect on the incidence of MN (Kruskal-Wallis test, $p < 0.05$), although the only dose-dependent differences were between the highest Ni treatment ($3600 \mu g \text{ L}^{-1}$) and the control and $18 \mu g \text{ L}^{-1}$ Ni groups (Fig. 3.3B; Mann-Whitney U tests, $p < 0.05$). Micronuclei induction was highest in the $1800 \mu g \text{ L}^{-1}$ Ni group at 8.66 MN per 1000 cells, although this was not significantly different from the incidence in the $3600 \mu g \text{ L}^{-1}$ Ni group (8.49 MN per 1000 cells). Although Ni concentration could not be determined for the haemocytes themselves, tissue-specific Ni concentrations could be used to predict MN (per 1000 cells) in haemocytes according to the following equations; $\text{MN} = (0.01 \times \text{Ni}) + 4.33$ (Fig. 3.6C) for the digestive gland ($R^2 = 0.17$, $p < 0.05$) and $\text{MN} = (0.07 \times \text{Ni}) + 3.87$ for foot ($R^2 = 0.23$, $p < 0.05$).

### 3.3.3 Alterations in transcriptional expression of selected genes

The efficiency of qPCR was within normal bounds ($18S$, 1.861; $gst$, 1.906; $hsp70$, 1.872; $mt10$, 1.911; $mt20$, 1.844; $pgp$, 1.883; and $sod$, 1.737).

Transcriptional expression of the selected genes in gill tissue samples showed considerable variation (Table 3.2). Despite this, qPCR revealed clear overexpression of the $gst$ and $mt20$ genes after exposure to $3600 \mu g \text{ L}^{-1}$ Ni, with approximately a 2.8 and 2.2-fold increase, respectively (Fig. 3.4). The $pgp$ gene was significantly overexpressed in Ni-exposed mussel gills after exposure to $18 \mu g \text{ L}^{-1}$ Ni (an approximate 19.3-fold increase compared to the control; Fig. 3.4A).

### 3.3.4 Histopathological responses to Ni exposure in the gill

Despite previous work indicating that Ni-exposure renders freshwater mussels (*Dreissena polymorpha*) more susceptible to parasite infection (Minguez et al. 2012), dissection of mussels showed no evidence of parasites (e.g. nematodes) or any neoplastic changes, either in the control or Ni exposed individuals. As with
Figure 3.3. Genotoxic effects in mussel haemocytes after exposure to nickel. (A) DNA strand breaks (% tail DNA); and (B) induction of micronuclei. Error bars are ± one SE. Significant differences from the corresponding control (*) are indicated (p < 0.05).
3. Acute nickel toxicity in mussels

**Figure 3.4.** Relative expression ratios (RER) of six genes (*gst*, *hsp70*, *mt10*, *mt20*, *pgp* and *sod*) in gill tissue of mussels after exposure to nickel for 5 d. Data are presented as RER values, (i.e. normalised for a reference gene [*18S*] and the control treatment) ± 95 % confidence intervals. The dashed grey line indicates no change in expression, values above it indicate upregulation and those below it downregulation. Significant differences (*p* < 0.05) from the control are indicated by *. 

gene expression, gills were chosen for semi-quantitative histopathological analysis as they have been reported to be a sensitive organ, both in terms of metal accumulation and damage. Mussel gills showed a significant increase in the incidence of structural damage from approximately 20% in the controls to approximately 80% in mussels exposed to 3600 µg L\(^{-1}\) Ni (Fig. 3.5; \(p < 0.05\)) but no overall effect of Ni treatment. Linear regression analysis revealed a significant predictive effect of DNA damage in haemocytes (% tail DNA) on incidence of histopathological damage in the gill (damage = (2.02 x % tail DNA) + 24.38; \(R^2 = 0.35, p < 0.05\)), suggesting a relationship between these endpoints (Fig. 3.6B).

3.4 Discussion

3.4.1 Tissue-specific Ni accumulation

Nickel accumulation occurred in the order: byssus > digestive gland > gills > foot > muscle > mantle. Using a \(^{63}\)Ni radiotracer, Millward et al. (2012) also found highest activity concentrations in the byssus. Punt et al. (1998) report that in \(M.\ edulis\) exposed to a much lower concentration of 0.40 µg L\(^{-1}\) \(^{63}\)Ni for 54 h and allowed to depurate for 48 h, Ni accumulation was viscera > byssus > mantle > muscle > gill > foot. Tissue-specific metal accumulation in mussels depends on three processes: (1) absorption across the gastro-intestinal tract, (2) internal redistribution due to metabolic processes, and (3) absorption across exposed body surfaces (Widdows & Donkin 1992). It is possible that during exposure to different concentrations, the balance of these processes is altered. In low Ni exposure (e.g. Punt et al. 1998), the metal accumulates in the viscera (including the digestive gland). At higher Ni exposures the metal is sequestered in the byssus, as reported by us and by Millward et al. (2012). Our observation of a significant decrease in Ni proportion in the digestive gland of mussels exposed to the highest Ni treatment strongly supports this idea. Interestingly, both byssus and digestive gland have also previously been identified as the site of highest accumulation of tritium, a radionuclide (Jha et al. 2005, Jaeschke et al. 2011). Variations in accumulation processes may also explain the fact that we saw a markedly higher accumulation of Ni in the digestive gland than Millward et al. (2012) after exposure of mussels to 180 µg L\(^{-1}\) Ni for 5 d. These authors found levels of approximately 25 µg g\(^{-1}\), in contrast to the same treatment group in the present study, which had a
3. Acute nickel toxicity in mussels

Figure 3.5. Histopathological effects of nickel exposure on mussel gills. (A) incidence of damage (loss of structural integrity) in mussel gills (n=3 mussels, 5 fields of view per mussel). Error bars are ± one SE, and significant differences from the control are indicated (*; p < 0.05); (B) transverse section of gill filaments [F] in a mussel exposed to 0 µg L⁻¹ Ni, showing structural integrity; and (C) transverse section of gill filaments (F) of mussel exposed to 3600 µg L⁻¹ Ni, showing structural deformity [S] and degradation of inter-filament junctions [J]. Scale bars = 100 µm.
3. Acute nickel toxicity in mussels

Figure 3.6. Linear regression analyses of biomarker data for mussels after 5 days nickel exposure. (A) % tail DNA and incidence of MN in haemocytes; (B) % tail DNA in haemocytes and incidence of histopathological damage in gills (C) tissue-specific accumulation of Ni in the digestive gland and incidence of MN in haemocytes; and (D) tissue-specific accumulation of Ni in the foot and incidence of MN in haemocytes. The blue line indicates fitted values and dashed red lines represent 95% confidence limits. Note that (A) shows means for each Ni treatment, whereas all other regressions were achieved using data from individual mussels.
3. Acute nickel toxicity in mussels

mean digestive gland Ni concentration of 154 µg g\(^{-1}\). Tissue-specific Ni accumulation was not quantified for haemocytes, but there were significant correlations between Ni in the digestive gland or foot and induction of MN in haemocytes. The open circulatory system of mussels, whereby tissues or organs are bathed in the haemolymph for gas and nutrient exchange, and the digestive and immune functions of haemocytes (Gosling 1992) may provide an explanation for the link between these effects in different tissues.

Mussels filter large volumes of water through the gills to facilitate both gas exchange and collection of particulate matter. So it is initially surprising that the gills are not the primary site of Ni accumulation. This result is, however, consistent with previous work on endosulfan (Roberts 1972), tritium (Jha et al. 2005, Jaeschke et al. 2011) and \(^{63}\)Ni (Punt et al. 1998), which were also concentrated in tissues other than the gill in *Mytilus* sp. (particularly the digestive gland and byssus). It has been hypothesised that localisation in the digestive gland is due to ingestion of contaminants adsorbed onto food (Roberts 1972, Widdows & Donkin 1992). This is consistent with the findings for \(^{63}\)Ni where it was administered as spiked suspended particulate matter (Punt et al. 1998, Millward et al. 2012). Furthermore, in humans, Ni bioavailability is increased when food is ingested alongside Ni salts (Denkhaus & Salnikow 2002). Although mussels were not fed either during the present study or during experiments using tritium (Jha et al. 2005, Jaeschke et al. 2011), it is possible that contaminants adsorbed or interacted with suspended particulate matter (< 10 µM) already present in the seawater and were subsequently ingested, leading to enhanced accumulation in the digestive gland. The use of ultra-filtered or artificial seawater (with a known particulate content) would help elucidate the mechanism behind selective contaminant accumulation in the digestive gland of mussels.

3.4.2 Genotoxicity

Nickel was only significantly genotoxic to *M. galloprovincialis* haemocytes at the highest dose tested (3600 µg L\(^{-1}\)). Interestingly, even at this dose, there was no significant increase in strand breaks after Fpg or Endo III treatment. This suggests that there was no substantial induction of base oxidation after Ni exposure. Furthermore, the slight downregulation of *sod* at the highest Ni concentration also suggests a lack of oxidative stress. This is in contrast to previous work in
mammalian systems, which have shown enhanced concentrations of ROS in CHO cell cultures exposed to > 650 mg L\(^{-1}\) Ni (Huang et al. 1993, 1994) or oxidised bases in \textit{in vivo} studies of rats exposed to 90 - 180 \(\mu\)mol kg\(^{-1}\) nickel(II)acetate (Kasprzak et al. 1990, 1992). It must, however, be noted that these studies used \textit{i.p} injection or aqueous Ni concentrations considerably higher than those reported here. Similarly, exposure to 1.76 - 17.6 mg L\(^{-1}\) Ni caused an increase in ROS in the freshwater cladoceran flea, \textit{Daphnia magna} (Xie et al. 2007), although only the lowest dose was within the range reported here.

It is possible that modified comet assay on other mussel tissues may produce contrasting results to that of haemocytes. For example, in goldfish (\textit{Carassius auratus}) exposed to 10 – 50 mg L\(^{-1}\) Ni there were tissue-specific differences in the antioxidant response, with liver and spleen having sufficient antioxidant potential to resist Ni-induced oxidative stress, in contrast to the kidney and white muscle, which showed significant antioxidant enzyme induction (superoxide dismutase, glutathione peroxidase and glutathione reductase; Kubrak et al. 2012a,b). Mussel haemocytes are involved in a variety of essential processes, including wound repair, transport and digestion of nutrients and immune/xenobiotic defence (Carballal et al. 1997). Consequently, it is likely that they have high antioxidant potential (Pipe et al. 1993), which may account for the lack of oxidative DNA damage we have observed. To our knowledge, the present study is the first to attempt to quantify oxidative DNA damage in mussels following environmentally relevant Ni exposure; however, future work analysing DNA strand breaks in multiple tissues and using the dichlorofluorescein assay to directly detect ROS might further clarify our results.

Recently, enzymatic repair of oxidative DNA damage has been demonstrated in mussel (\textit{M. edulis}) gill tissue (Emmanouil et al. 2006). This repair could have masked oxidative DNA damage that occurred at an earlier timepoint (i.e. one not sampled in this study). If that is the case, however, it still suggests that - over the longer exposure period used here - mussel haemocytes were able to maintain homeostasis with respect to oxidative DNA damage. Time-course analysis of oxidative DNA damage (sampling earlier and more frequently, perhaps over a longer duration) would confirm if this is the case, although there are limitations in procuring sufficient amount of cells during repeated sampling of the same individual.

It is also possible that alternative forms of Ni-induced damage may act antago-
nistically to produce novel outcomes, thereby confounding modified comet assay results. Although Ni is known to form DNA crosslinks (XLs; Costa et al. 2002), in particular DNA-protein crosslinks (DPXLs; Ciccarelli et al. 1981, Chakrabarti et al. 2001, Chen et al. 2001), which may retard migration of DNA during electrophoresis (i.e. during the comet assay; Merk & Speit 1999) it is unlikely that these structures would be maintained after lysis in 2.5 M NaCl. It has also been suggested that Ni may interfere with incision or post-incision (e.g. ligase) steps of DNA repair processes (e.g. base excision repair [BER]; Hartwig et al. 1994, Hartwig 1998). There is, therefore, potential for any residual Ni (released during lysis) to inhibit the activity of endonucleases in vitro. Cavallo et al. (2003) showed clear evidence for oxidative damage (as measured by the FPG-modified comet assay) in Jurkat cells exposed to 0.17 M Ni for 4 h. This concentration (i.e. 10 µg L\(^{-1}\)) is, however, lower than those reported here, so it is not possible to rule out the potential inhibitory effect of Ni in vitro. Previous research has also indicated that Ni may induce effects by altering cellular metabolism, and consequently mimicking hypoxic conditions, in common with other metallic contaminants (Denkhaus & Salnikow 2002, Kubrak et al. 2011). Our results do not suggest any evidence for this mechanism in Ni-exposed mussels, with respect to DNA strand breaks in haemocytes. Assessment of other oxidative stress markers in different cells or tissues would confirm if this is a cell specific or generic phenomenon (Jha 2008).

Our results support those of previous studies that have found a strong link between DNA strand breakage (as measured by the comet assay) and the induction of micronuclei in both mammals (Vodicka et al. 2001) and aquatic biota (Siu et al. 2004a, Hagger et al. 2005b, Villela et al. 2006, Canty et al. 2009). This suggests a cause-and-effect relationship between DNA strand-breaks and micronuclei formation (Jha 2008). Apart from generation of ROS, the known inhibitory action of Ni on DNA repair enzymes (Hartwig 1998) may provide a causal mechanism for this relationship. However, the genes we have quantified are unlikely to be directly involved in the production or prevention of DNA damage, which may explain the non-correlation of gene expression with our genotoxic endpoints (i.e. comet and MN assays; data not shown).
3.4.3 Transcriptional expression of \textit{mt20} and \textit{pgp} genes

The induction of metallothioneins and expression of \textit{mt} genes in mussels in response to Ni has been subject to some debate (Attig et al. 2010). Although we did not determine the induction of metallothionein proteins, our results indicate that expression of the \textit{mt10} gene remains stable after Ni exposure, whereas \textit{mt20} is upregulated at 3600 µg L\textsuperscript{−1} Ni. This is in contrast to the work of Amiard et al. (2008) who found no correlation between induction of metallothionein proteins and Ni in field- or lab-exposed mussels (10-100 ng individual\textsuperscript{−1}), and Dondero et al. (2011) who reported that \textit{mt10} expression significantly increased following exposure of \textit{M. galloprovincialis} to 22-770 µg L\textsuperscript{−1} Ni for 4 d, but that \textit{mt20} expression remained unchanged. Previously, the discrepancy between these results has been attributed to seasonality (Attig et al. 2010, Dondero et al. 2006a). Since our mussels were collected at the same time of year as those of Dondero et al (2011), this suggests that adaptation to local conditions or climate may also have a role in this variability.

The p-glycoprotein multidrug transporter is induced following exposure to a range of contaminants in aquatic organisms (Eufemia & Epel 2000), and has been identified as part of the multixenobiotic resistance mechanism (MXR), similar to the multidrug resistance mechanism in mammals (Dano 1973, Ling 1997, Higgins 2007). Our results indicate that low-level Ni exposure causes increased transcriptional expression of the \textit{pgp} gene in mussels, and therefore that MXR may play an important role in Ni detoxification. Although the p-glycoprotein response is often simultaneous with increased heat shock protein or \textit{hsp70} expression (Eufemia & Epel 2000, Minier et al. 2000), our results did not indicate any overexpression of the \textit{hsp70} gene. This suggests that, in Ni-exposed mussels, overexpression of \textit{pgp} is part of a general cellular response, distinct from the heat shock response, as in mussels exposed to organic toxins (Eufemia & Epel 2000). Quantification of the expression of heat shock proteins and the relative transcriptional expression of other \textit{hsp} genes after Ni exposure is needed to verify this hypothesis. Interestingly, p-glycoprotein is found localised on the nuclear membrane in rat brain cells (Babakhanian et al. 2007). If similar subcellular localisation occurs in mussel cells, the overexpression of \textit{pgp} we have observed could result in efflux of Ni from the nucleus, resulting in reduced genotoxicity, particularly at low concentrations where expression was significantly upregulated. An increase in MXR protein or transport activity has been reported in mussels exposed to other contaminants.
(PAHs, PCBs, and heavy metals) in the field (Smital et al. 2003, Achard et al. 2004, Minier et al. 2006, Brooks et al. 2009) and increased transcriptional expression of MXR genes has been shown in response to various stressors (e.g. temperature, salinity) under laboratory conditions (Luedeking & Koehler 2004). Recently, Farcy et al. (2011) also reported that oysters exposed to $^{60}$Co, showed significant transcriptional up-regulation of genes for metallothioneins ($MT$; approximately 2-fold increase after 1 week exposure) and multixenobiotic resistance ($MDR1$; approximately 3-fold increase after 6 weeks). Additionally, increased transcriptional expression of $pgp$ itself has been reported in various bivalves in response to other metals (Zn, Franco et al. 2006; Cd, Achard et al. 2004). However, this is the first report of increased transcriptional expression of the $pgp$ gene in Ni-exposed *Mytilus* sp. in a laboratory study.

### 3.4.4 Histopathology of Ni in mussel tissues

Exposure of mussels to 3600 $\mu$g L$^{-1}$ Ni caused a significant increase in the incidence of histopathological damage in the gills. Previous research has also shown histopathological damage in mussels exposed to other contaminants, including engineered nanoparticles (ENPs; Al-Subiai et al. 2011), polyaromatic hydrocarbons (PAHs; Aarab et al. 2008, 2011, Al-Subiai et al. 2011, ) or complex mixtures from the *Prestige* oil spill (Garmendia et al. 2011). In particular, Al-Subiai et al. (2011) reported ciliary hypoplasia in the gill after exposure to C60 fullerenes (ENPs) and fluoranthene at 0.1-1 and 100 $\mu$g L$^{-1}$, respectively. These authors also reported ciliary erosion with necrosis after exposure to a combined treatment of the two contaminants (32 $\mu$g L$^{-1}$ fluoranthene and 0.1 mg L$^{-1}$ C60). At the relatively high Ni concentration used here (3600 $\mu$g L$^{-1}$) the prevalence of damage (~80 %) is much greater than any reported by Al-Subiai et al. For logistical reasons the incidence of histopathology reported herein has not been broken down into specific types of lesion. This fact, along with the different contaminants and concentrations used, most probably explains this difference.

We have also reported a significant correlation between % tail DNA in haemocytes (as measured by the comet assay) and the incidence of histopathological damage in the gill. This appears to be the first report in the literature of such a predictive relationship between DNA damage and a higher level effect in *Mytilus* spp. (see Fig 3.6B). This is very encouraging for the use of the comet assay as an indicator
for detrimental effects of contaminants at higher levels of biological organisation (e.g. at the tissue level).

### 3.4.5 Relative toxicity of Ni

Nickel appears to be less toxic than other metal contaminants. For example, 56 µg L\(^{-1}\) copper caused approximately 50% more DNA strand breaks (as measured by the comet assay) than the highest dose of Ni used here (Al-Subiai et al. 2011). It is important, however, to bear in mind that inter-experimental comparison of comet assay data can be influenced by many factors (Collins et al. 2008). In a previous study, Ni concentrations of 180 µg L\(^{-1}\) resulted in approximately 60% tail DNA (Millward et al. 2012), in contrast to the present work where the same concentration resulted in 15.6 - 18.2% tail DNA. Although the results of the comet assay can be influenced by seasonality (Rank et al. 2005 and see Chapter 5), there are potentially other factors behind this observed variability. These could include biological factors (e.g. age, size, sex etc), technical parameters (e.g. microscopes, scoring software used etc) or physico-chemical influences (e.g. temperature, salinity etc; Jha 2008, Dhawan et al. 2009). As the speciation of Ni was not determined under our experimental conditions, variable concentrations of the toxic form of Ni (i.e. Ni\(^{2+}\)) could also be one such factor.

The relatively low toxicity of Ni is consistent with examination of metal toxicity in embryos of *Mytilus trossolus* (Nadella et al. 2009). Both Cd and Cu were also found to be more toxic than Ni in terms of causing immobility (mortality) to *Daphnia magna* (Xie et al. 2007). Several other biomarkers have also shown detrimental effects only at relatively high Ni concentrations. For example, Attig et al. (2010) reported that glutathione-s-transferase and malondialdehyde increased after exposure of *Mytilus galloprovincialis* to 770 µg L\(^{-1}\) Ni, but showed no effect at 135 µg L\(^{-1}\). Glutathione peroxidase (GPX) activity did not significantly increase in the same study until the Ni concentration reached 3000 µg L\(^{-1}\).

### 3.5 Conclusions

Although toxic concentrations of Ni are several orders of magnitude higher than that in contaminated estuaries (Roux et al. 2001) or in open oceans (Danielsson...
et al. 1985, Millero 1996), they are considerably lower than in Ni-polluted Canadian lakes (Eisler 1998). Furthermore, there is considerable uncertainty about Ni concentrations at high risk coastal locations (e.g. the New Caledonian lagoon).

Based on the data we present here, we consider it prudent that such at-risk sites are accurately assessed, and recommend that remediate action be instigated if Ni concentrations exceed 3000 $\mu$g L$^{-1}$.
Chapter 4

Genotoxic, transcriptomic, proteomic and organism level effects of zinc pyrithione on marine mussels
4. Introduction

Biofouling of boat hulls by sessile aquatic organisms is a major problem for commercial shipping and leisure boating because of increased maintenance and fuel costs. It is estimated that biofouling on one class of warship alone costs the US Navy $56 million per year. The majority of this cost is attributable to decreased fuel efficiency (Schultz et al. 2011). As such, anti-fouling products (i.e. those that prevent adhesion of sessile marine biota) are highly profitable, worth approximately $4.7 billion globally in 2012 (IPPIC 2013). The most typical of these are anti-fouling paints, applied to a boat hull in order to repel adherent species. Unfortunately, the biocidal nature of compounds in these paints has led to detrimental consequences for the aquatic ecosystem, the most well-known of which is probably imposex in marine molluscs as a result of tributyl tin (TBT) exposure. Female gastropod molluscs exposed to TBT concentrations as low as ng L$^{-1}$ start to develop male sexual characteristics, with obvious implications for reproduction and population health (Santillo et al. 2002). The International Maritime Organisation (IMO) banned the application of organotin compounds as anti-foulants in 2003, with complete prohibition in 2008 (IMO 2002). This has resulted in some concern over the alternative anti-fouling products which are now increasing in use (Evans et al. 2000, Price & Readman 2013). Such products include former biocides (e.g. Irgarol 1051), agricultural chemicals (e.g. diuron) and everyday compounds, such as those found in personal care products (e.g. zinc pyrithione). Despite this variety, many such products have since been banned in one or several European countries (Price & Readman 2013). As a result of this selection, many of the remaining products contain zinc pyrithione (ZnPT), which is still permitted in the UK and Denmark, in contrast to Irgarol 1051 and diuron for example. ZnPT is a booster biocide (Thomas 1999), i.e. it is not the primary anti-fouling component.
4. Multi-level effects of zinc pyrithione

Pyrithione is the conjugate base of 2-mercaptopyridine-N-oxide. In ZnPT, two pyrithione ligands are chelated to a Zn$^{2+}$ ion via oxygen and sulphur (Fig. 4.1). ZnPT is a well-known antibacterial and antifungal agent. As such, it has a variety of household and medical uses, including as a treatment for psoriasis, eczema and vitiligo or impregnated into cleaning products e.g. sponges. Probably the most common everyday use of ZnPT is in medicated anti-dandruff shampoos, where it is the only active ingredient (Reeder et al. 2011b). In addition to its use in anti-fouling paints, ZnPT is also used in other outdoor paints where resistance to mould or mildew is desirable.

Within the literature, the anti-fungal and anti-fouling properties of ZnPT are often addressed together, assumed to be via the same mechanism. Despite its long term use as an anti-fungal agent, this mechanism of action is still not well understood (Reeder et al. 2011b). It has been suggested that the anti-dandruff effect of ZnPT is as a result of indirect effects on the metabolism of skin cells (suppression of DNA synthesis; Imokawa & Okamoto 1983). In contrast, several authors have suggested that these effects are directly anti-fungal, caused by either iron starvation or decreased activity of iron-containing proteins as a result of elevated intracellular Cu (ZnPT acts as a Cu ionophore) in yeast (Yasokawa et al. 2010, Reeder et al. 2011a). There are also several reports of genotoxicity and stress response induction (especially heat shock proteins [HSPs] and increased $p53$ expression) in human skin cells exposed to ZnPT (e.g. Rudolf & Cervinka 2011, Lamore et al. 2010, Lamore & Wondrak 2011).

With respect to ZnPT in anti-fouling paints, there is evidence of transchelation of the pyrithione moiety to Cu when it occurs in concentrations equimolar (or higher) than Zn (Grunnet & Dahllof 2005, Holmes & Turner 2009). As antifouling paints often use a cupric oxide as their primary active ingredient (e.g. Katranitsas et al. 2003), co-exposure of non-target organisms to both ZnPT and Cu is likely. Therefore, the toxicity of CuPT is also of great interest with respect to aquatic species. In this context, Bao et al. (2011) reported similar toxicity of CuPT to TBT in a range of marine species, whereas ZnPT was less acutely toxic (i.e. in terms of mortality). For a review of the many different anti-foulant compounds used in the marine environment, please see Guardiola et al. (2012).

Zinc pyrithione has been considered to present less of a hazard to non-target species than some other commonly used booster biocides (e.g. diuron and Irgarol

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<table>
<thead>
<tr>
<th>Organism</th>
<th>Life stage</th>
<th>Taxonomy</th>
<th>Duration</th>
<th>LC$_{50}$ (µg L$^{-1}$)</th>
<th>Ref.</th>
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<td>48 h</td>
<td>&gt; 100.0</td>
<td>Bao et al. 2008</td>
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<td>98.2</td>
<td>Mochida et al. 2006</td>
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<tr>
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<td>Adults</td>
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<td></td>
<td>96 h</td>
<td>3.7 (light)</td>
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<tr>
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<td>Adults</td>
<td>Polycheata</td>
<td>96 h</td>
<td>2.5 (dark)</td>
<td>Marcheselli et al. 2010a</td>
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<td>96 h</td>
<td>3.7 (light)</td>
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<td>Bivalvia, Mytiloida</td>
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<td>14,600</td>
<td>Marcheselli et al. 2010b</td>
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<td>96 h</td>
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<td>7 d</td>
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4. Multi-level effects of zinc pyrithione

1051) because it is thought to degrade quickly under UV light (Price & Readman 2013). There is, however, some debate about the kinetics of ZnPT degradation in seawater (particularly in turbid water with reduced light penetration; Maraldo & Dahllof 2004) and whether or not it adsorbs to sediments (Bones et al. 2006). To the best of the author’s knowledge there are only two papers on concentrations of ZnPT in the environment, either measured or modelled (Madsen et al. 2000, Mackie et al. 2004), so this area requires further research. Nonetheless it is clear that ZnPT has the potential to cause detrimental effects in aquatic species. It is a known teratogen in fish, with EC_{50} values for developmental abnormalities in zebrafish and medaka of 9 and 5 µg L^{-1}, respectively (Goka 1999). Bellas et al. (2005) also reported embryo toxicity from ZnPT in sea urchins (EC_{50} 7.7 nM) and mussels (M. edulis, EC_{50} 8 nM). Growth of diatoms is significantly reduced by only 1.9 µg L^{-1} ZnPT over 96 h (Bao et al. 2008) and treatment of paddy fields with ZnPT-containing anti-dandruff shampoo resulted in decreased growth (and therefore weight to length ratio) in juvenile medaka (Sánchez-Bayo & Goka 2005). Several studies have also reported acute toxicity of ZnPT to aquatic organisms, a summary of which is included in Table 4.1.

Recently Marcheselli et al. (2011) demonstrated ZnPT-induced genotoxicity in marine mussels, using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. After 7 d exposure, adult M. galloprovincialis showed increased DNA fragmentation and apoptosis at both concentrations tested (0.2 and 0.4 µM). These concentrations also caused induction of the heat shock proteins, HSP27, HSP60 and HSP70, and the higher concentration also caused a reduction in anoxic tolerance (SoS). Despite these very interesting results, there are still many questions yet to be addressed with regard to ZnPT toxicity in aquatic biota, including mussels, particularly in terms of the potential mechanisms behind these effects. Furthermore, no study to date has attempted to connect alterations in genomic integrity with changes in protein expression, by looking at intermediate processes such as transcriptional expression of the relevant genes.

4.1.1 Objectives and hypotheses

Given the above information, the current chapter aimed to fulfil the following objectives:

(a) to use the modified comet assay to determine if oxidative DNA damage is
4. Multi-level effects of zinc pyrithione

(a) to evaluate the transcription profile of key stress genes (hsp70, mt10, mt20, pgp and p53) to elucidate potential mechanisms behind any observed genotoxicity and/or changes in protein expression;

(b) to evaluate any proteomic changes in the digestive gland of M. galloprovincialis exposed to ZnPT over a more chronic timescale (14 days);

(c) to use an integrated approach to correlate genotoxic, transcriptomic and proteomic changes with higher (more ecologically relevant) effects (i.e. attachment, CR and SoS).

It was hypothesised that ZnPT would cause genotoxic effects in mussel haemocytes, including significant oxidative damage to nucleotide bases. As such, oxidative stress genes were expected to be upregulated in response to this contaminant. Finally, it was hypothesised that such effects would also be associated with detrimental impacts at higher levels of biological organisation.

4.2 Materials and Methods

4.2.1 Solid phase extraction to determine organic Zn concentration in seawater

ZnPT has been reported to degrade quickly in seawater (Thomas 1999). Consequently, prior to mussel exposure experiments, it was necessary to quantify the degradation of organic Zn (i.e. ZnPT) in seawater under our proposed experimental conditions. Solid phase extraction (SPE) columns have been used to discriminate between hydrophobic and hydrophilic Zn in previous experiments on ZnPT (Holmes & Turner 2009), and were consequently selected for use here. Initial validation experiments were also carried out to address three questions; (1) would using artificial seawater (ASW) provide more accurate results due to potential background Zn in natural seawater (SW)?; (2) could columns be re-used? and (3) at what flow rate was recovery of organic Zn optimal?

All samples in the following experiments were preserved either in 100 % methanol
Octadecyl silane (C18) columns (15 ml; Chromabond, Macherey Nagel GmbH, Düren, Germany) were conditioned prior to use with successive 15 ml washes of 100 % methanol, 50 % methanol (v/v with Milli-Q water) and Milli-Q water (Fig. 4.2). In this initial validation experiment columns were used once and then discarded. Solutions (50 mL) of 16.2 µM ZnPT (the highest concentration used in this study) were made up in either ASW or SW. This was done immediately prior to addition to the columns to minimise any degradation of ZnPT. Three 10 mL aliquots of each solution were reserved for analysis without column processing. A single 15 mL aliquot was applied to a C18 column and a vacuum pump was used to draw liquid through at one of 6 different rates (0.6 - 6 mL min⁻¹). Each column was rinsed with Milli-Q water and then 100 % methanol was used to elute the hydrophobic fraction (addition, rinsing and elution all took place at the same rate; Fig. 4.2). Recovery (%) was determined by comparing the Zn content of unprocessed samples with those from the columns. Six columns were used for each combination of flow rate/water and the results were averaged (n = 6). Results of this experiment suggested minimal difference in recovery between rates of 0.6 - 3 mL min⁻¹ and reduced recovery at ≥ 6 mL min⁻¹ (Fig. 4.5A). Subsequently, a flow rate of 3 ml min⁻¹ was used in all further column experiments, for the best combination of recovery and speed. Artificial seawater showed considerably lower recovery (∼15 %) so was not used in either column tests or mussel exposures.
In the second validation test, a similar protocol was used, but with three concentrations of ZnPT (0.2 and 2.0 in addition to 16.2 \( \mu \text{M} \)) and re-use of each column. After the initial run, columns were successively rinsed with 15 mL of 1% concentrated HCl (v/v with Milli-Q water) and 15 mL Milli-Q water, before reconditioning as described above and then re-use (Fig. 4.2). Three columns were used for each concentration and the results were averaged \((n = 3)\). Although recovery of organic Zn was decreased at the lower concentration, re-use of columns had no impact (Fig. 4.5B).

For the degradation experiment, logistical demands meant only one concentration could be tested. Six tanks were set up, as for the mussel exposures (i.e. at 15\(^\circ\)C with constant aeration and a 12:12 h photoperiod). At time 0 each tank was dosed to give a final concentration of 2 \( \mu \text{M} \) ZnPT. This concentration was selected based on its similar recovery to 16.2 \( \mu \text{M} \) and better environmental relevance. A 100 mL sample was taken from each tank at 0, 6, 12, 24 and 48 h after thorough stirring. Three 10 mL aliquots from this sample were reserved for analysis without column processing and three 15 mL aliquots were passed through C18 columns, rinsed and eluted as described above. Organic Zn content was normalised as a percentage of that for time 0 and results were averaged \((n = 6)\).

### 4.2.2 Mussel collection and maintenance

Adult *Mytilus galloprovincialis* individuals (48.03 \( \pm \) 2.43 mm) were collected from TBS in June 2012. TBS is a remote location with no local marinas and is therefore likely to have very low ZnPT concentrations. Mussels were maintained and depurated as described in section 2.3.

Water quality parameters during the experiments for this chapter were salinity 31.82 \( \pm \) 0.34, pH 8.16 \( \pm \) 0.21, dissolved oxygen 90.02 \( \pm \) 2.84 % and temperature 15.88 \( \pm \) 0.52 \( ^\circ\)C.

### 4.2.3 Range-finding exposure

A range-finding experiment was performed to determine appropriate concentrations for a sublethal 14 day exposure of *M. galloprovincialis* to ZnPT. Mussels were transferred to glass beakers, containing 2 L of filtered seawater (salinity 34)
and allowed to acclimatise for 48 h. We exposed mussels \((n = 18)\) at a density of 4.5 mussels L\(^{-1}\) to six concentrations of either ZnCl\(_2\) (as an inorganic zinc control), sodium pyrithione (NaPT; as a control for the pyrithione ion) or ZnPT (0, 0.2, 0.6, 5.4, 16.2 µM PT\(^-\)). These concentrations were based on the work of Marchiselli et al. (2010b), factoring in our longer exposure time of 14 d. Mortalities (classified as continued opening of the valves and failure of the mussel to respond when tapped) were recorded daily. For comparison with Marchiselli et al. (2010b), 96 h and 7 d LC\(_{50}\) values (median lethal concentration for 50 % of animals) were calculated in addition to the 14 d LC\(_{50}\).

As NaPT contains only one pyrithione ligand (molar mass 149.5 g mol\(^{-1}\)), in contrast to ZnPT which has two (molar mass 317.7 g mol\(^{-1}\)), concentrations for the range-finding exposure were expressed as moles of PT\(^-\). That is, twice as much total NaPT was used as ZnPT. For consistency, concentrations used throughout this study are given in µM. However, as many studies quote ZnPT values in weight per unit volume, a conversion table is provided (Table 4.2).

### 4.2.4 Integrated biomarker experiment

Following the range-finding tests, unused mussels were transferred to pre-washed plastic tanks (1.25 mussels L\(^{-1}\)) containing 20 L of filtered seawater (34 psu) with constant aeration, and were allowed to acclimatise for 48 h prior to exposure. Mussels were exposed to ZnPT at either 0.2 or 2 µM, an inorganic Zn control (2 µM ZnCl\(_2\)) or a seawater control. ZnPT concentrations were selected as either being chronic (\(\sim\) 10 % of the 14 d LC\(_{50}\) i.e. the MTC) or acute (close to the 14 d LC\(_{50}\)).

#### Table 4.2. Conversion of concentrations of zinc pyrithione (ZnPT) from micromolar to weight per unit volume.

<table>
<thead>
<tr>
<th>Concentration of ZnPT (µM)</th>
<th>Concentration of ZnPT (µg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>63.5</td>
</tr>
<tr>
<td>0.6</td>
<td>190.6</td>
</tr>
<tr>
<td>2.0</td>
<td>635.4</td>
</tr>
<tr>
<td>5.4</td>
<td>1,716.0</td>
</tr>
<tr>
<td>16.2</td>
<td>5,146.7</td>
</tr>
</tbody>
</table>
as determined by previous experiments (see section 4.3.2). The exposure lasted for 14 d. As the ZnPT concentration reduced significantly after 48 h (see section 4.3.1), 100 % water changes were performed every other day, with replenishment of the appropriate stock solution at this time.

At each sampling point, haemolymph samples were extracted from nine mussels per treatment (see section 2.5) and stored on ice until use in the comet or micronucleus assay. Following haemolymph extraction, small (5 mm²) sections of gill and digestive gland were removed and stored at -80 °C for molecular or proteomic analysis (see Figure 4.3 for full experimental design).

### 4.2.5 Enzyme-modified comet assay to determine oxidative DNA damage

The modified comet assay was performed using the haemocytes of mussels as described in section 2.10, except that only two slides were prepared per sample, one enzyme buffer control and one with Fpg.

### 4.2.6 Induction of micronuclei

Mussel haemolymph \((n = 9)\) was processed for the micronucleus assay, as in section 2.8. Scoring was according to Countryman and Heddle (1976) for at least 1000 cells on each coded and randomised slide, as previously described in sections 2.8 and 3.2.6.

### 4.2.7 Determination of relative transcriptional expression of selected genes

Total RNA extraction, reverse transcription and qPCR reactions were performed as described in section 2.11. The relative transcription of 5 genes \((\text{pgp, } \text{mt10, } \text{mt20, } \text{hsp70} \text{ and } \text{p53})\) was quantified using REST 2009 software (as described in section 2.11.4; Pfaffl et al. 2002). Two reference genes were also used - \(\text{atub}\) (encodes alpha tubulin; Ciocan et al. 2011) and \(\text{ef1}\) (encodes elongation factor 1; Ciocan et al. 2011). Details of the primers used in this chapter are included in Table 4.3.
4. Multi-level effects of zinc pyrithione

Figure 4.3. Overall experimental design to determine the impacts of zinc pyrithione on mussels at multiple levels of biological organisation.
Table 4.3. Genes and primers used for qPCR on mussel tissue samples after exposure to zinc pyrithione (ZnPT) or zinc chloride (ZnCl\textsubscript{2}):

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metallothionein 10 ((mt10))</td>
<td>AJ577130</td>
<td>5'-TGACACTGGCTGACACGGT-3'</td>
<td>5'-CACATTACACGTTAAAGGTCCTGTACAT-3'</td>
</tr>
<tr>
<td>Metallothionein 20 ((mt20))</td>
<td>AJ577131</td>
<td>5'-GACGCCTGCAAATGTCAGGTTCTCAGGA-3'</td>
<td>5'-TCGGACCAGTGCGGTCAGAT-3'</td>
</tr>
<tr>
<td>P-glycoprotein ((pgp))</td>
<td>AF159717</td>
<td>5'-AACATCGGTGTGGTTTCTCAGGA-3'</td>
<td>5'-GATCAAAGCCTCTGGCAATGCTG-3'</td>
</tr>
<tr>
<td>Heat shock protein 70 ((hsp70))</td>
<td>AF172607</td>
<td>5'-CCCTTTCTCTGCACAAAGACAAGCA-3'</td>
<td>5'-AATCTGTGCATGGCTCCTCAGA-3'</td>
</tr>
<tr>
<td>p53 anti-oncogene ((p53))</td>
<td>AY579472</td>
<td>5'-GAATCTGGGAAAAACATGGA-3'</td>
<td>5'-GATCTCTGGGAAAACATGGA-3'</td>
</tr>
<tr>
<td>Elongation factor 1 ((ef1))</td>
<td>AF063420</td>
<td>5'-CACCCAGGAGTCTTCCAGAGA-3'</td>
<td>5'-GCTGTACCCAGACAGACATCC-3'</td>
</tr>
<tr>
<td>Alpha tubulin ((atub))</td>
<td>DQ174100</td>
<td>5'-TTGCAACCACATCGACACAGA-3'</td>
<td>5'-TGACAGACGGCTCTCAG-3'</td>
</tr>
</tbody>
</table>
4.2.8 Two dimensional gel analysis of protein expression

Samples were prepared for 2 dimensional gel electrophoresis (2D-GE) using methods adapted from those of Schmidt et al. (2013). The digestive gland samples from each of the 3 mussels per exposure tank (see section 4.2.4) were pooled and homogenised with four parts homogenisation buffer (10 mM Tris-HCl, 0.5 M sucrose, 0.15 M KCl, 1 mM EDTA, 1 mM PMSF). After centrifugation at 15,000 $g$ for 1 h at 4 °C, the supernatant was assayed for protein content (Bradford 1976) and stored at -80 °C. This pooling approach was used for two reasons; to provide enough protein material and to reduce the impact of biological outliers and therefore enable robust statistical analysis from fewer replicates (Karp & Lilley 2005).

An 85 µg subsample of total protein was mixed with TCA (final concentration 10 % v/v), and allowed to incubate in the dark for 5 min on ice. After centrifugation at 11,000 $g$ for 3 min at 4 °C, 40 µl of Milli-Q water was added to the resulting pellet and vortexed for 5 min. Following vortexing, 500 µL of ice cold acetone (100 %) was added and the suspension was incubated at -20 °C for > 1 h. Centrifugation (as above) produced a pellet which was incubated at room temperature in the dark for 5 min, to allow any remaining acetone to evaporate. At this point, the sample pellet was resuspended in rehydration buffer (7 M urea, 2 M thiourea, 2 % CHAPS, < 1 % bromophenol blue) with the addition of 1.5 µL DeStreak solution (GE Healthcare, Little Chalfont, UK) and 5 µl IEF ampholyte (Pharmalyte 3-10; GE Healthcare). Rehydration buffer-sample mix (125 µL) was incubated with a 7cm IPG strip (pH 3 - 10; GE Healthcare) overnight, to facilitate sample loading. Iso-electric focusing was performed using a Protean IEF cell (Bio-Rad) and the following programme: 300 V for 3 h, 1,000 V for 6 h, 8,000 V for 3 h followed by 8,000 V for 20 kVh and hold at 500 V.

After IEF, strips were equilibrated firstly by 20 min incubation in equilibration buffer (6.6 M urea, 0.4 M Tris, 0.07 M SDS and 20 % glycerol [v/v]) with 2% DTT (w/v). Then secondly, by 20 min incubation (in the dark) in equilibration buffer (as before) with 2.5 % iodoacetamide (w/v). After transfer of strips to precast 12 % polyacrylamide gels (Mini-PROTEAN TGX, Bio-Rad), electrophoresis was carried out at 90 V for 1 h, followed by 120 V for 30 min, in a running buffer of 0.25 M Tris, 1.92 M Glycine and 0.03 M SDS. Gels were then stained with 0.2 % Coomassie brilliant blue solution (R-250; in 30 % methanol, 10 % acetic acid [v/v]) for 1 h, and destained (40 % methanol, 10 % acetic acid [v/v]) overnight.
4. Multi-level effects of zinc pyrithione

Figure 4.4. 2-dimensional gel electrophoresis (2D-GE) for protein separation. (A) Basic steps in the 2D-GE process; and (B) Illustration of the relationship between iso-electric focusing (IEF) and sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with respect to their ability to separate proteins by isoelectric point (pI; colours) and molecular weight (MW; size/shape), respectively.
Visualisation was achieved using a Gel Doc XR+ system (Bio-Rad).

After determination of spots of interest (see section 4.3.5) the largest examples of each spot were excised from their gels (using sterile razor blades), dried and sent for identification (Mass Spectrometry Unit, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa). Gel bands were subjected to in-gel tryptic digestion. The extracted peptides were then loaded onto a R2 micro column (RP-C18 equivalent) where they were desalted, concentrated and eluted directly onto a MALDI plate using $\alpha$-cyano-4-hydroxycinnamic acid (CHCA) as the matrix solution in 50 % acetonitrile and 5 % formic acid. Mass spectra of the peptides were acquired in positive reflectron MS and MS/MS modes using a MALDI-TOF/TOF MS instrument (4800plus MALDI TOF/TOF analyser) with exclusion list of the trypsin autolysis peaks (842.51, 1045.56, 2211.11 and 2225.12). The resulting MS and MS/MS spectra were analysed in combined mode using the Mascot search engine and the NCBI database restricted to 50 ppm peptide mass tolerance with no taxonomy restrictions.

### 4.2.9 Behavioural responses

Two behavioural responses were quantified – attachment of mussels to the substrate and clearance rate.

All mussels were transferred to tanks individually, i.e. unattached to either other mussels or to the container. During the water changes on days 4, 6 and 12 (i.e. those closest to the sampling days) mussel attachment was recorded. Mussels were classified as attached if they remained adhered (to the tank or to another mussel) when the empty tank was inclined to a 45° angle.

The clearance rate assay was performed as described in section 2.7 ($n = 9$). For logistical reasons, clearance rate measurements were only performed on day 14.

### 4.2.10 Stress on stress response

After the 14 day exposure period, the SoS test was performed on remaining mussels ($n = 25$) as described in section 2.14.
Figure 4.5. Characterisation of organic Zn in seawater using C18 solid phase extraction columns. (A) Recovery of organic Zn from 16.2 \( \mu \)M ZnPT with different column flow rates in artificial (ASW) or natural seawater (SW); (B) Recovery of organic Zn from 0.2 (cream), 2 (green) or 16.2 \( \mu \)M ZnPT (blue) after column reconditioning; and (C) Proportion of organic Zn (as a percentage of time 0) in natural seawater over a 48 h period. Data are means ± one SE (\( n = 3 \) [B] or 6 [A, C]).
4.2.11 Statistical analyses

Data for micronuclei induction were non-normal, but were transformed to normality by square rooting prior to analysis by 2-way ANOVA. LC$_{50}$ values were determined by probit analysis (Randhawa 2009). Survival curves and lethal time to 50% mortality (LT$_{50}$) values were generated using the Kaplan-Meier method (Kaplan & Meier 1958) and were compared using the log rank test. Two-dimensional gels were analysed using Progenesis Samespots software (v4.5.4325.32621; Nonlinear Dynamics Ltd, Newcastle Upon Tyne, UK). Spots were considered significantly altered if $p < 0.05$ and fold change was $\geq 1.5$. All other data were normal and were analysed using ANOVAs.

Correlation matrices were produced for the different biomarkers at days 4, 7 and 14, using Pearson’s correlation coefficients. Principle component analyses (PCA; ‘prcomp’ method in R) were applied for the six biomarkers common to samples from days 4 and 14 (i.e. gene expression of $hsp70$, $mt20$ and $mt10$; % tail DNA [buffer], % tail DNA [Fpg] and MN/1000 cells) and treatment. Although performed at days 4 and 14, proteomic results were for pooled samples and thus were excluded from PCA. As in Chapter 3, the gene expression parameter used for relationship analysis was C$_q$ normalised for reference gene. As two reference genes were used in this study, this normalisation was performed according to the equation:

$$\Delta C_q = GoI - \frac{atub + ef1}{2} \quad (4.1)$$

4.3 Results

4.3.1 Degradation of ZnPT in seawater

The organic Zn concentration of water initially containing 2 $\mu$M ZnPT decreased sharply for the first 12 h and then less so from 12 - 24 h, appearing to plateau between 24 - 48 h (Fig. 4.5C). The final organic Zn concentration after 48 h, was $\sim 30\%$ of its time 0 value. The approximate half life for ZnPT degradation in seawater was 10 h.
4. Multi-level effects of zinc pyrithione

4.3.2 Range-finding exposure

During the initial 96 h, ZnPT produced an LC$_{50}$ of 14.50 ± 1.45 µM. This was more toxic than that of both ZnCl$_2$ and NaPT, neither of which caused 50% mortality at this timepoint. At 7 d, it was possible to calculate an LC$_{50}$ for NaPT (24.58 ± 1.57 µM) but ZnPT was still more toxic (8.94 ± 1.30 µM) Finally, by 14 d, the toxicity of ZnPT and NaPT was similar, with LC$_{50}$s of 2.54 ± 1.32 and 2.97 ± 1.36 µM, respectively.

4.3.3 Genotoxicity of ZnPT to mussel haemocytes

The higher concentration of ZnPT induced DNA strand breaks at all timepoints, although on day 4 the buffer treated group was significantly higher than the day 0 control, but not for the same day control (Fig 4.7). The low concentration of ZnPT showed a comparative delay in its genotoxic effect, causing significantly elevated strand breakage on days 7 (Fpg-treated) and day 14 only. Despite a trend towards elevated DNA strand breakage for Fpg-treated ZnPT samples on days 7 and 14, there was no significant effect of enzyme for any of the Zn compounds. The use of an inorganic Zn control (ZnCl$_2$) allowed us to make comparisons with ZnPT treated mussels, revealing some variability. Significantly elevated % tail DNA was observed for ZnPT treated mussels in comparison to ZnCl$_2$ (ANOVA with Tukey’s post hoc tests, $p<0.05$) on day 4, day 7 and day 14 (Fig 4.7).

Control mussels showed normal levels of micronuclei (∼3 per 1000 cells) across the entire exposure period (Fig. 4.8). Mussel haemocytes treated with 2 µM ZnCl$_2$ showed a slight increase in MN frequency, but this was not significant. In contrast, ZnPT exposure caused a concentration- and time-dependent increase in MN, with no significant effect on day 4 and peaking at 22.42 MN per 1000 cells in the 2 µM ZnPT treated group on day 14.

4.3.4 Transcriptional expression of selected genes

Both $atub$ and $ef1$ were used as reference genes for PFRRT tests within REST 2009 software, as both showed low variability across all samples ($C_q$ values $atub$, 20.45 ± 1.26; $ef1$, 18.01 ± 1.01). Two genes, $p53$ and $pgp$, showed qPCR efficiencies < 1.5 and were consequently excluded from further analysis.
4. Multi-level effects of zinc pyrithione

Figure 4.6. Acute toxicity of zinc chloride (ZnCl$_2$), zinc pyrithione (ZnPT) and sodium pyrithione (NaPT) to mussels over 96 h (A), 7 days (B) and 14 days (C). Toxicity is represented as cumulative mortality (%) of 18 mussels. Concentrations are expressed as µM PT.
Figure 4.7. DNA strand breaks in mussel haemocytes after exposure to zinc chloride \((\text{ZnCl}_2)\) or zinc pyrithione \((\text{ZnPT})\) for 0 (A), 4 (B), 7 (C), or 14 days (D), as measured by the enzyme-modified comet assay (% tail DNA). Error bars are ± one SE. Asterisks indicate significant differences \((p < 0.05)\) from the same-day control, whereas daggers indicate differences from day 0.
Figure 4.8. Induction of micronuclei in mussel haemocytes after exposure to ZnCl$_2$ or ZnPT for 0 (A), 4 (B), 7 (C) or 14 days (D). Error bars are ± one SE. Asterisks indicate significant differences ($p < 0.05$) from the same-day control, whereas daggers indicate differences from day 0.
In gill tissue, qPCR efficiencies were $\text{atub} \ 1.982$, $\text{ef1} \ 1.869$, $\text{hsp70} \ 1.750$, $\text{mt10} \ 1.791$ and $\text{mt20} \ 1.838$. The transcriptional expression of the three target genes was unchanged on day 4, however by day 14 significant upregulation of both $\text{hsp70}$ and $\text{mt20}$ had occurred for the highest ZnPT concentration (Fig. 4.9).

In digestive gland qPCR efficiencies were $\text{atub} \ 1.753$, $\text{ef1} \ 1.867$, $\text{hsp70} \ 1.798$, $\text{mt10} \ 1.830$ and $\text{mt20} \ 1.846$. The digestive gland transcriptional profile of these genes was markedly different from that in the gill. Significant changes in expression were only seen at day 4, where all three target genes showed upregulation for the lower ZnPT concentration but only $\text{hsp70}$ and $\text{mt20}$ did so at the higher concentration (Fig. 4.10).

4.3.5 Protein expression

Digestive gland proteins were generally well separated in defined spots, with minimal streaking or smearing (Fig. 4.11). Due to excess staining it was necessary to exclude the lower 1 cm of each gel from analysis. Overall, 14 spots showed significant up- or down-regulation in response to ZnPT. This included 12 spots that responded significantly after only 4 d exposure (Fig. 4.11A), and a further 2 spots that showed significant differences after 14 d (Fig. 4.11B). Only spots showing significant fold change relative to both the seawater and inorganic Zn controls (at the same ZnPT concentration) were considered to be showing ZnPT-specific effects (239, 355, 412, 607 and 550; Table 4.4). This excluded both spots that were significantly different after 14 d exposure. Of the remaining differentially expressed proteins after 4 d exposure, spots 239 and 607 were significantly upregulated and spot 355 was downregulated at the higher ZnPT concentration, spot 550 was downregulated at the lower ZnPT treatment and spot 412 showed significant downregulation for both ZnPT concentrations. These 5 proteins were excised manually from gels and digested with trypsin (see section 4.2.8).

Of the 5 spots of interest, only two could be positively identified by mass spectroscopy. These were spot 355, whose sequence was significantly homologous to a predicted protein sequence for phosphoenolpyruvate carboxykinase (PEPCK) in barley (GenBank Accession No. BAK02183.1), and spot 550, identified as a small heat shock protein from $M. \text{galloprovincialis}$ (GenBank Accession No. AEP02968.1). Of the remaining spots, one could not be identified due to low protein content (607) and the other two had either no matching sequences or
4. Multi-level effects of zinc pyrithione

![Graphs showing relative expression ratios (RER) of three genes (hsp70, mt10, and mt20) in gill tissue of mussels after exposure to zinc chloride (ZnCl₂) or zinc pyrithione (ZnPT). Data are presented as RER values (i.e., normalised for two reference genes (atub and ef1) and the control treatment) ± 95% confidence intervals. The dashed grey line indicates no change in expression, values above it indicate upregulation and those below it downregulation. Significant differences (PFRRT, \( p < 0.05 \)) from the control are indicated by *.](image_url)

Figure 4.9. Relative expression ratios (RER) of three genes (hsp70, mt10, and mt20) in gill tissue of mussels after exposure to zinc chloride (ZnCl₂) or zinc pyrithione (ZnPT). Data are presented as RER values (i.e., normalised for two reference genes (atub and ef1) and the control treatment) ± 95% confidence intervals. The dashed grey line indicates no change in expression, values above it indicate upregulation and those below it downregulation. Significant differences (PFRRT, \( p < 0.05 \)) from the control are indicated by *.
4. Multi-level effects of zinc pyrithione

Figure 4.10. Relative expression ratios (RER) of three genes (hsp70, mt10 and mt20) in digestive gland tissue of mussels after exposure to zinc chloride (ZnCl$_2$) or zinc pyrithione (ZnPT). Data are presented as RER values (i.e. normalised for two reference genes [atub and ef1] and the control treatment) ± 95% confidence intervals. The dashed grey line indicates no change in expression, values above it indicate upregulation and those below it downregulation. Significant differences (PFRRT, $p < 0.05$) from the control are indicated by *. 

hsp70

mt10

mt20

Day 4

Day 14
extensive post-translational modification.

4.3.6 Behavioural responses

Control mussels showed increasing attachment with time, peaking at 100% attachment for the seawater control and ~98% attachment for the inorganic Zn control (Fig. 4.12). In contrast, mussels exposed to both concentrations of ZnPT had significantly reduced attachment at all time points compared to the controls ($p < 0.0001$). Attachment in ZnPT exposed mussels peaked at 4% for 0.2 $\mu$M at 4 days.

Clearance rate was also seriously affected by exposure to ZnPT at both tested concentrations, causing a reduction from ~1.75 - 2.1 L h$^{-1}$ in the two controls to < 0.3 L h$^{-1}$ (Fig. 4.13).

4.3.7 Stress on stress response

A clear trend was observed in the ability of mussels to tolerate anoxic conditions during the SoS test (Fig. 4.14). Those mussels exposed to either the control or 2 $\mu$M ZnCl$_2$ treatments generally survived well, declining steadily after 8 - 10 days. The LT$_{50}$ values for these two curves were similar (14.00 ± 0.58 and 14.00 ± 0.55 days, respectively). In contrast, mussels treated with either concentration of ZnPT declined rapidly between days 3 - 6 and then more steadily, but reached total mortality at only 11 - 14 days (in comparison to the controls at 16 - 17 days). The LT$_{50}$ values for 0.2 and 2 $\mu$M ZnPT were 6.00 ± 0.40 and 6.00 ± 0.34, respectively. These values were significantly different from the two controls ($p < 0.0001$).

4.3.8 Relationship analyses

Figures 4.15, 4.16 and 4.17 display the correlations obtained at days 4, 7 and 14, respectively. For those biomarkers sampled at all three timepoints (MN/1000 cells and the two comet assay results [buffer and Fpg-treated]) there was a clear temporal trend. After 4 days there were no significant correlations between these three biomarkers of genotoxicity, but by days 7 and 14 significant positive correlations
4. Multi-level effects of zinc pyrithione

Figure 4.11. Annotated image of 2-dimensional protein gels, showing spots differentially expressed in digestive gland after 4 (A) or 14 days (B) exposure to ZnPT. Spots were selected based on an ANOVA $p$-value $< 0.05$ and fold change $\geq 1.5$ when compared to control or ZnCl$_2$ treated mussels.
Table 4.4. Differentially expressed proteins in the digestive gland of *M. galloprovincialis* exposed to 0.2 or 2 µM zinc pyrithione (ZnPT) for 4 days. Non-significant fold changes have been omitted.

<table>
<thead>
<tr>
<th>Spot id</th>
<th>Protein id</th>
<th>Fold change relative to 2 µM ZnCl$_2$</th>
<th>Fold change relative to control</th>
<th>Fold change relative to 2 µM ZnPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>239</td>
<td>phosphoenolpyruvate carboxykinase</td>
<td>-2.91</td>
<td>-1.94</td>
<td>2.10</td>
</tr>
<tr>
<td>355</td>
<td>small heat shock protein 24</td>
<td>+1.78</td>
<td>1.73</td>
<td>+3.91</td>
</tr>
<tr>
<td>412</td>
<td>small heat shock protein 24</td>
<td>-1.68</td>
<td>-2.04</td>
<td>1.91</td>
</tr>
<tr>
<td>550</td>
<td>small heat shock protein 24</td>
<td>+1.59</td>
<td>1.94</td>
<td>+1.78</td>
</tr>
<tr>
<td>607</td>
<td>110</td>
<td>?</td>
<td>?</td>
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</tr>
</tbody>
</table>
Figure 4.12. Number of mussels showing attachment (either to the substrate or other mussels) as a percentage of total mussels, after exposure to zinc chloride (ZnCl₂) or zinc pyrithione (ZnPT) for 4 (A), 6 (B) or 12 days (C). Error bars are ± one SE. Asterisks indicate significant differences (p < 0.05) from the same-day control, whereas daggers indicate differences from the same-day ZnCl₂ treatment. Percentages were calculated as means of three replicate beakers (n = 25).
were apparent for all pairs. For the gene expression data, \textit{hsp70} was significantly correlated with both metallothionein genes at both day 4 and 14. At day 4, there was a significant positive relationship between \textit{mt10} and \textit{mt20}, whereas by day 14 the expression of both these genes was significantly correlated with Fpg-treated \% tail DNA.

Further exploration of the data with PCA revealed clear temporal and treatment-specific trends. The first component (PC1) explained 40.30 and 54.55 \% of the total variance at day 4 and 14, respectively (Fig. 4.18A and 4.19A) and was mostly related to \textit{mt20} activity at day 4, whereas by day 14 there was no single defining biomarker (Fig 4.18B and 4.19B). Treatment-specific clusters were evident at both timepoints, but were more diffuse by day 14, by which time two groupings of biomarkers were apparent - gene expression describing more variability in the control, \textit{ZnCl2} and low ZnPT groups, in contrast to genotoxic parameters and treatment which distinguished the high ZnPT treatment.
4. Multi-level effects of zinc pyrithione

Figure 4.14. Survival of mussels ($n = 25$) in continuous anoxic conditions after exposure to 2 µM zinc chloride (ZnCl$_2$) or zinc pyrithione (ZnPT) for 14 days. Significant differences from the control (*) and ZnCl$_2$ (§) treatments are indicated ($p < 0.0001$).
Figure 4.15. Correlation matrix of 6 biomarkers ($\Delta C_q$ values for $hsp70$, $mt20$ and $mt10$; % tail DNA [buffer]; % tail DNA [Fpg] and MN/1000 cells) in $M.\ galloprovincialis$ exposed to control seawater (cream circles), 2 $\mu$M zinc chloride (green circles) 0.2 $\mu$M zinc pyrithione (magenta circles) or 2 $\mu$M zinc pyrithione (blue circles) for 4 days. Lower diagonal panel shows data plots and upper diagonal panel shows Pearson’s correlation coefficients ($r$) and $p$-values.
4. Multi-level effects of zinc pyrithione

Figure 4.16. Correlation matrix of 3 biomarkers (% tail DNA ([buffer]; % tail DNA [Fpg] and MN/1000 cells) in *M. galloprovincialis* exposed to control seawater (cream circles), 2 µM zinc chloride (green circles) 0.2 µM zinc pyrithione (magenta circles) or 2 µM zinc pyrithione (blue circles) for 7 days. Lower diagonal panel shows data plots and upper diagonal panel shows Pearson’s correlation coefficients ($r$) and $p$-values.
4. Multi-level effects of zinc pyrithione

Day 14

<table>
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</tr>
<tr>
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<td>&lt;0.01</td>
</tr>
<tr>
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<td>&lt;0.01</td>
</tr>
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</tr>
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<td>&lt;0.01</td>
</tr>
<tr>
<td>% tail DNA (F)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.17. Correlation matrix of 6 biomarkers (ΔCq values for hsp70, mt20 and mt10; % tail DNA [buffer]; % tail DNA [Fpg] and MN/1000 cells) in M. galloprovincialis exposed to control seawater (cream circles), 2 µM zinc chloride (green circles) 0.2 µM zinc pyrithione (magenta circles) or 2 µM zinc pyrithione (blue circles) for 14 days. Lower diagonal panel shows data plots and upper diagonal panel shows Pearson’s correlation coefficients (r) and p-values.
Figure 4.18. Principle component analysis of 6 biomarkers (ΔCq values for *hsp70*, *mt20* and *mt10*; % tail DNA [buffer]; % tail DNA [Fpg] and MN/1000 cells) in *M. galloprovincialis* exposed to control seawater, 2 µM zinc chloride, 0.2 µM zinc pyrithione or 2 µM zinc pyrithione for 4 days. (A) Scree plot showing the percentage variation explained by each component; and (B) Biplot showing individual PCA scores (black) and loadings for each biomarker (green).
4. Multi-level effects of zinc pyrithione

Figure 4.19. Principle component analysis of 6 biomarkers (ΔCq values for hsp70, mt20 and mt10; % tail DNA [buffer]; % tail DNA [Fpg] and MN/1000 cells) in M. galloprovincialis exposed to control seawater, 2 µM zinc chloride, 0.2 µM zinc pyrithione or 2 µM zinc pyrithione for 14 days. (A) Scree plot showing the percentage variation explained by each component; and (B) Biplot showing individual PCA scores (black) and loadings for each biomarker (green).
4.4 Discussion

4.4.1 Acute toxicity of ZnPT

The similar level of toxicity induced by ZnPT and NaPT after 14 days exposure suggests that it is the common pyrithione ion which causes the majority of toxic effects. However, the differences between the toxicity of the two compounds at different timepoints suggests a more complicated picture. The fact that ZnPT was more toxic at earlier timepoints suggests that Zn (either bound to PT$^-$ or having dissociated) causes synergistic or additive effects. One potential mechanism behind this might be altered pyrithione accumulation in the presence of Zn. The reverse phase high-performance liquid chromatography (RP-HPLC) method developed by Marcheselli et al. (2010b) for detecting the presence of both ZnPT and PT$^-$ in mussel tissues might provide more information to address this issue.

4.4.2 Genotoxicity of ZnPT to mussel haemocytes

The results presented here confirm the genotoxicity of ZnPT in mussels, as reported by Marcheselli et al. (2011) in gills and digestive gland (using TUNEL assay). This is, however, the first report of such ZnPT-induced genotoxicity in mussel haemocytes. The enzyme modified comet assay has revealed no evidence of base oxidation as the primary mechanism of this ZnPT induced haemocyte genotoxicity. This is in agreement with \textit{in vitro} data from rat cardiomyocytes, where ZnPT has been used to successfully reduce superoxide generation and oxidative injury (measured as both LDH release and cell survival) after reperfusion (Kasi et al. 2011). However, the mammalian literature also yields contradictory results, indicating that ZnPT causes increased susceptibility to ROS-induced damage in rat thymocytes co-exposed to hydrogen peroxide (Oyama et al. 2012). Furthermore, a study on human skin cells reported upregulation of metallothionein genes, which are commonly associated with oxidative stress (Lamore & Wondrak 2011). This last point may be explained by the known metal binding capacity of metallothionein proteins, i.e. they may be induced by the Zn rather than ZnPT. Further research to directly quantify ROS in mussel cells (of different tissues) is recommended to elucidate the mechanisms behind the genotoxic effects of ZnPT.
4. Multi-level effects of zinc pyrithione

4.4.3 Molecular responses to ZnPT exposure

There are limited studies with which to compare our data on the molecular responses of mussel to ZnPT. Whilst many studies have exposed mussels to metals (e.g., Lemoine et al. 2000, Banni et al. 2007, Varotto et al. 2013), such comparisons are not relevant to this specific organometallic compound, and there is limited information available on ZnPT itself, or other pyrithione compounds. To the best of the author’s knowledge this study is the first to report upregulated metallothionein genes in an aquatic organism in response to ZnPT. In fact, it is the first to report any alterations in transcriptional expression of key genes in response to this anti-fouling agent. The data presented here suggest that the key stress response genes, hsp70, mt10 and mt20 are induced in response to ZnPT in mussels. It is encouraging to compare these data with the protein expression results of Marcheselli et al. (2011) and the proteomic responses identified herein, both of which identified heat shock proteins (HSP27, HSP60, HSP70 or HSP24.1) as components of the ZnPT response in *M. galloprovincialis*. Furthermore, recent studies in human skin cells have reported upregulation of HSPs after ZnPT treatment (Lamore et al. 2010, Lamore & Wondrak 2011), suggesting this response might be highly conserved. Although both gill and digestive gland have been identified as key sites of ZnPT accumulation in mussels, different trends in this accumulation were observed between the two tissues with time, in particular increased accumulation in the digestive gland after ≥ 2 days exposure to 1.5 µM ZnPT (Marcheselli et al. 2010b). It remains to be evaluated whether the temporal switch in hsp70 and mt20 expression observed between these tissues here is purely a tissue-specific effect or is linked to these altered accumulation patterns.

PEPCK is a highly conserved enzyme, involved in the gluconeogenesis pathway by which glucose is generated from non-carbohydrate substrates. Under normoxic conditions, phosphoenolpyruvate (PEP) is converted to pyruvate by the enzyme pyruvate kinase (PK), pyruvate is then converted to acetyl coA ready for the TCA cycle. However, under hypoxic conditions, mussels are able to convert PEP to oxaloacetate (OAA), which is reduced to malate and enters the TCA cycle (Mustafa & Hochachaka 1973, Zandee et al. 1985, Luschak et al. 1997). As such, the PEPCK enzyme is a crucial initial step in anaerobic metabolism and energy production (Jokumsen & Fyhn 1982). The transition from PEP metabolism via PK to PEPCK is regulated by pH, with acidity (i.e. from the products of anaerobic metabolism) inhibiting PK and enhancing PEPCK activity (Mustafa &
The significant downregulation of PEPCK protein at 4 days (Table 4.4) provides an interesting insight into another possible mechanism behind the dramatic decreases observed in attachment and anoxia tolerance. If the significant decrease in protein observed here corresponds to decreased PEPCK activity, this could compromise the mussels’ ability to cope with anoxic conditions, as we have seen in the SoS assay. Most previous studies on PEPCK activity in mussels have focused on its response to physical stressors. For example, Anestis et al. (2010b) reported that there was no significant change in adductor mussel or mantle PEPCK activity over 35 days exposure at elevated temperatures (18 - 28 °C). Similarly, recent molecular studies using proteomic (2D-GE) and microarray approaches have revealed significant decreases in PEPCK in response to elevated temperature (24 and 32 °C; Tomanek & Zuzow 2010) and hyposalinity (29.75 psu; Lockwood & Somero 2011). Interestingly, one of these studies also reported decreased expression of HSP24, which is upregulated here. Further investigation into whether the expression of these two proteins is correlated might further elucidate the mechanisms behind ZnPT-induced toxicity and enhance our understanding of enzymatic processes involved.
To the best of the author's knowledge, only one previous study has examined PEPCK levels (either activity or expression) in mussels in response to contaminants. In contrast to the results presented here, Widdows et al. (1982) reported that PEPCK activity increased *M. galloprovincialis* after 140 days exposure to 30 µg L\(^{-1}\) water-accommodated hydrocarbons. This study is therefore the first to demonstrate a negative impact on PEPCK by a contaminant in the marine mussel, an ecologically relevant model species. Concerning the differences between these two responses, there are several factors to consider. Firstly, enzyme activity was not measured in the current work, which instead determined protein expression. Post-translational modification to activate or suppress PEPCK may result in differences between expression and activity. Given that in the current study changes in PEPCK expression appeared by day 4 but were not evident by day 14, it is also possible that temporal shifts in expression/activity patterns might explain these differences. Lastly, it is important to remember that such responses are often contaminant-specific, as illustrated by the different responses to the contaminants in this thesis.

It has been reported that PEPCK activity in hypoxic mussels is significantly lower than that of PK (Anestis et al. 2010a), suggesting that the PK pathway is more important under these conditions. PK was not identified as differentially expressed in the current study, however specific assays (i.e. antibody linked) for the quantity or activity of these two enzymes would confirm whether similar patterns are present in ZnPT-exposed mussels. As kinetic studies of glycolytic enzymes in *M. edulis* have previously shown significant variation in activity between adductor muscle (catabolic tissue) and mantle (anabolic tissue; Churchill & Livingstone 1989), it would also be interesting to explore tissue-specific differences in the expression and/or activity of PEPCK and related enzymes in response to ZnPT. Recently links between PEPCK and hypoxia-inducible factors (HIF) have been demonstrated in mammalian *in vitro* models (Choi et al. 2005). As hypoxia signalling pathways, including transcriptional changes in *HIF-1*, have also been demonstrated in mechanisms of metal-induced carcinogenesis (Galanis et al. 2009), investigation of this enzyme pathway might yield novel insights into the mechanisms of ZnPT-induced toxicity in mussels and other non-target organisms.

In addition to suggesting a possible mechanism for the observed SoS results, there are also links between PEPCK and food consumption. It is well established that starvation induces elevated PEPCK activity (and hence gluconeogenesis) in mammals, and this enzymatic response has also been reported in plaice starved for 4
months (*Pleuronectes platessa*; Moon & Johnston 1980). Consequently, it is especially notable that after 4 days ZnPT exposure mussels with reduced CR (i.e. lower feeding levels) showed decreased PEPCK expression, opposite to the expected outcome. Once again, this suggests that ZnPT has a considerable effect on mussels, at both physiological and behavioural levels, in addition to its well characterised genotoxicity. The potential for changes in the expression of this protein to link to the effects observed at higher levels of organisation further emphasise the need for studies such as this one, which use an integrated approach to examine effects across this spectrum.

### 4.4.4 Responses at higher levels of biological organisation

It is interesting that although the SoS results presented here confirm that of Marcheselli et al. (2011) our LT$_{50}$ values were higher. This indicates that mussels exposed to 2 $\mu$M ZnPT for 14 days in the current experiment survived longer under anoxic conditions than those exposed to 0.4 $\mu$M ZnPT for only 7 days. It is, however, important to consider than in the earlier experiment, control mussels had an LT$_{50}$ of 10.70 days, in contrast to 16.00 days here. This suggests that either the harbour-collected mussels in the earlier study were already compromised, or that there is a considerable difference between the anoxia tolerance of *M. galloprovincialis* collected from the north Cornish coast and the Mediterranean coast of Italy. Studies showing significant variation in LT$_{50}$ values for mussels sampled from different sites and in different seasons (e.g. Hellou & Law 2003, Koukouzika & Dimitriadis 2005) support this latter idea. Only when this natural variability is fully characterised will it be possible to classify mussel populations as having good or bad ecological status based on LT$_{50}$ values. Until such data is available, it is recommended that future studies using this parameter characterise the impact of a contaminant relative to control organisms. On this basis, the mussels used in the current study showed a greater percentage decrease (-62.50 %) than those of the earlier study (-49.53 %), as is to be expected from a longer exposure to ZnPT.

Although the magnitude of the decrease in CR reported here may seem extreme (> 85 % reduction in comparison to the inorganic Zn control), these reduced CR values are comparable to those for mussels exposed to 18 - 56 $\mu$g L$^{-1}$ Cu for 5 d (Al-Subiai et al. 2011), 32 mg L$^{-1}$ methyl methanesulfonate for up to 7 d (Canty
et al. 2009) and 56 µg L\(^{-1}\) benzo(a)pyrene [B(a)P] for 12 d (Di et al. 2011). The decreases reported here are, however, well in excess of those reported for 14 d exposure to branched alkyl benzenes from crude oil (Scarlett et al. 2008) or 3 d exposure to C60 fullerene nanoparticles (Al-Subiai et al. 2012). This indicates the severity of ZnPT’s effect on mussel CR in comparison to several well-known toxicants. This dramatic reduction in CR shown by mussels exposed to both concentrations of ZnPT suggests a possible explanation for the SoS results. Reduced filtration in mussels causes two main effects: decreased gas exchange at the gills and reduced food intake (Bayne 1976). Either of these parameters could have severe consequences for the energy stores of these organisms, resulting in weakened anoxia tolerance. Given that the mussels in the study of Marcheselli et al. (which also showed significant detrimental effects in the SoS test) were not fed, it would appear that reduced gas exchange has the greater impact. Measurement of scope for growth (which incorporates respiration rate and food adsorption efficiency in addition to CR; Widdows 1978) or storage molecules might provide insight into the specific impacts of ZnPT-induced CR decline.

It is unsurprising that ZnPT, a component of anti-fouling paints used to prevent adhesion of biota, causes decreased attachment in mussels. However, it is interesting to note that the role of ZnPT in such paints is primarily as an algaecide or booster biocide, as cupric oxides are usually used to provide the main toxicity against ‘hard fouling organisms’ i.e. barnacles and molluscs (Almeida et al. 2007). There is limited information from the current study to suggest any mechanisms behind this observed effect. As such, we suggest future studies investigate two major pathways - either a generalised stress response resulting in lack of energetic capacity for byssus production (e.g. Babarro et al. 2008) or a specific chemical interaction between ZnPT/PT\(^{-}\) and byssus proteins.

4.4.5 Environmental risk of ZnPT exposure

Given the detrimental effects of ZnPT on mussels at all levels of biological organisation examined in this study, it is perhaps surprising that they are still readily found on the pontoons of marinas across the globe, despite these areas having potentially higher ZnPT/PT\(^{-}\) concentrations than pristine sites. There are, however, several mitigating factors to consider when examining this idea, all of which provide fertile ground for future studies on this contaminant. Firstly, it
is likely that mussels from marinas are adapted to baseline levels of ZnPT. Although this study used mussels from a reference site, there was no opportunity for direct comparison with ZnPT pre-exposed individuals. The mussels used in the work of Marcheselli et al (2010b, 2011) provide such individuals, but cross-continental and inter-experimental comparisons are confounded by many factors. Future work that makes this comparison \textit{in situ} is therefore highly recommended.

Secondly, although marinas might be expected to have higher levels of ZnPT than open ocean, it is likely that the highest levels of this compound will be around boatyards, where old anti-fouling coatings are removed and new ones applied. Hydrophobic Zn has been shown to leach into seawater from chips of anti-foulant paint, which would be expected at such locations (Holmes & Turner 2009). To the best of the author’s knowledge there is no information on the survival of mussels in these locations. Finally, the environmental fate of ZnPT is still a matter of some debate (see Thomas & Brooks 2010 for review). The data presented here for organic Zn degradation in seawater support the idea that ZnPT is photolytically or biologically degraded in a short period of time (e.g. Thomas 1999, Turley et al. 2005). There was, however, a residual amount of organic Zn (∼30 %) after 48 h and our experiment took place in a full 12:12 h photoperiod. Previous studies suggest ZnPT degradation is substantially reduced in darkness (e.g. Marcheselli et al. 2010b) so it is imperative that its degradation in turbid seawater (or shaded by pontoons and other marina hardware) is assessed.

### 4.5 Conclusions

Although the quantification of ZnPT in water and animal tissues is now possible (Thomas 1999, Mackie et al. 2004, Grunnet & Dahllof 2005, Marcheselli et al. 2011) the procedures are complicated and limited data on environmental concentrations are available. Given currently available estimates of 0.002 - 0.1 µM ZnPT in contaminated water (Madsen et al. 2000, Mackie et al. 2004) and the relatively high tolerance of mussels to ZnPT compared to other species (see Table 4.1), the effects demonstrated here present a potential risk to marine biota. However, more research into actual environmental concentrations of ZnPT and on its effects in other non-target species is essential before regulators can accurately perform environmental risk assessment for this anti-fouling agent.
Chapter 5

Chronic exposure to tritiated water: responses in mussels at different levels of biological organisation
5. Chronic exposure to tritiated water

5.1 Introduction

Tritium ($^3$H) is a radioactive isotope of hydrogen, having two neutrons in addition to its one proton. Its $\beta^-$ decay to $^3$He results in the emission of a $\beta$ particle, i.e. a high energy electron with the capacity to ionise matter. Tritium is naturally occurring, produced via the reaction of cosmic rays with atmospheric gases (e.g. N$_2$). It is, however, also artificially produced in the nuclear fuel cycle. This occurs via the capture of neutrons by deuterium or $^3$He, through neutron activation of other elements within nuclear reactors (e.g. $^6$Li, $^7$Li, $^{10}$B) and as a byproduct of the reprocessing of nuclear fuels (e.g. $^{235}$U and $^{239}$Pu). The basic physical properties of tritium are summarised in Table 5.1. Its chemical properties are very similar to non-radioactive isotopes of hydrogen, and it is therefore highly transferable into biotic and abiotic systems (Melintescu & Galeriu 2011).

Tritium has many uses, including in medicine, electronics manufacture, smoke detection, radio-luminescence, and as a tracer in industrial, environmental and biological applications (Singh et al. 2012). It is also used as a neutron initiator and/or booster for nuclear weaponry. Furthermore, nuclear fusion most commonly uses deuterium-tritium fuel, suggesting that demand and production of $^3$H will increase as this technology becomes industrially-viable (Singh et al. 2012, Jean-Baptiste & Fourre 2013).

<table>
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<tr>
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</tr>
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<tr>
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</tr>
<tr>
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</tr>
</tbody>
</table>

$^a$ Where $e^-$ is an electron ($\beta$ particle) and $\bar{\nu}^-$ is an electron-type antineutrino.
Historically, atmospheric testing of nuclear weapons significantly contributed to $^3$H input into the environment ($\sim$234,000 PBq between 1945 and 1980; UNSCEAR 2000). Although the international ban on these tests has dramatically reduced the amount of $^3$H in the environment (Jean-Baptiste & Fourre 2013), large activities of $^3$H are still discharged annually by nuclear power plants (NPPs) and nuclear fuel reprocessing plants (NFRPs). Of the radionuclides released by these institutions, $^3$H is the most abundant and is mainly produced by NFRPs. For example, during the period 2005 - 2008, the two NFRPs discharging into the English Channel/Irish Sea (i.e. Sellafield in the UK and La Hague in France, see Fig 1.5) discharged $\sim$1000 - 10,000 TBq y$^{-1}$ of liquid $^3$H. In contrast, NPPs in the same area discharged $\sim$1 - 10 % of this activity (Adam-Guillermin et al. 2010). Developing nations, such as China (where NPPs discharged 137 TBq y$^{-1}$ of liquid $^3$H on average between 1993 and 2009) are also potential sources of increasing $^3$H input to the environment (Yang et al. 2012). As a result of this development and the re-framing of nuclear energy as a potential mitigator of climate change (Bickerstaff et al. 2008, IAEA 2012), there is potential for tritium discharges to increase in future.

Tritium is also discharged to the aquatic environment as a result of nuclear accidents. Atmospheric $^3$H concentrations around the damaged Fukushima Dai-ichi NPP peaked at $\sim$1.5 x 10$^3$ Bq m$^{-3}$ at immediately after the March 2011 accident (Matsumoto et al. 2013). Although this activity in air rapidly decreased, it is likely that the marine environment was the ultimate recipient. Furthermore, concentrations of $^3$H in seawater pipes around the plant have exceeded 4.5 MBq L$^{-1}$ (TEPCO 2013) and it has been reported that $^3$H discharge to the nearby sea has been of the order of terabecquerels (BBC 2013).

The majority of $^3$H discharges from nuclear institutions are in the form of tritiated water (HTO), i.e. water where one atom of hydrogen is replaced by $^3$H. Due to its chemical similarity to hydrogen, it is also possible for $^3$H to replace hydrogen atoms in molecules, forming organically-bound tritium (OBT). The biological half-lives of these two forms of $^3$H are different, with OBT being more persistent both in humans (Harrison 2009) and non-human biota (Jaeschke et al. 2011, Kim & Korolevych 2013, Kim et al. 2013). Recently, it has also been suggested that $^3$H from HTO rapidly equilibrates with organic compounds in estuarine water, particularly proteinaceous material (Turner et al. 2009) and can also be transformed into OBT within the tissues of marine algae (Jaeschke & Bradshaw 2013). This leads to increased potential for bioaccumulation and biomagnification in higher
5. Chronic exposure to tritiated water

Although the low $\beta$ energy of $^3$H has caused it to be considered a relatively low-risk radionuclide in the past, there is much uncertainty about the relative biological effectiveness (RBE) of $^3$H (Bridges 2008, Dallas et al. 2012, Chen 2013). That is, its relative potential for harm compared to other types of radiation (e.g. $\alpha$ particles or $\gamma$ rays). This is - in part - because the low energy of the $\beta$ particle emitted (on average 5.69 keV, see Table 5.1) coupled with its low penetrative power results in relatively high linear energy transfer (LET, i.e. more energy deposited within a smaller tissue cross-section; HPA 2007).

The uncertainty surrounding tritium also extends to its biological implications in humans, in particular for cancer risk to residents around NPPs or NFRPs (CERRIE 2004, Bridges 2008). A recent report found evidence of increased risk of childhood cancer in areas < 5 km from German NPPs (Kaatsch et al. 2007), however the authors stated that ‘a direct relation to radiation seems implausible’ (Kaatsch et al. 2008). There are also many criticisms of the methods of this and other studies on the epidemiological effects of nuclear institutions (Little & Wakeford 2008, Bridges 2011, Kinlen 2011a,b). Recent epidemiological studies on the risks from tritium in Canada suggested that current dose limits are adequate for radiation protection and that there was no elevation in radiation-sensitive cancers associated with tritium (Thompson et al. 2011, Wanigaratne et al. 2013), however this subject remains controversial. Unsurprisingly, laboratory data from mammalian models are easier to interpret, with rats chronically exposed to tritiated water showing reduced immunity (Kirillova 1990), increased DNA damage and thymus depletion (Rusinova & Turdakova 1988).

Dosimetry for radiological exposure is not as simple as for chemical toxicology, in that it cannot simply be expressed as units of mass of contaminant normalised for body weight (e.g. $\mu$g g$^{-1}$ Ni in Chapter 3). Instead, the energy transferred to the tissue(s) per unit mass is given, i.e. absorbed dose (joules kg$^{-1}$, Gray [Gy]). Dose rate is given as Gy per unit time. In human radioprotection, absorbed dose is used mostly for point measurements (e.g. tumour dose in radiotherapy), whereas equivalent dose is used more generally. Equivalent dose (also in joules kg$^{-1}$, but now given the unit Sieverts [Sv]) takes into account the RBE of the radiation. Due to the scarcity of information (especially in the aquatic environment), equivalent doses are not well characterised for non-human organisms and absorbed dose is used instead (Copplestone et al. 2001). Direct measurement of absorbed dose in...
5. Chronic exposure to tritiated water

A tissue or organism can be logistically difficult, so methods of estimating absorbed dose from activity are usually implemented, especially for assessment of environmental radioactivity. Therefore, one area of particular interest to aquatic invertebrate radiotoxicology is dose estimation. The current study used a dosimetric equation and an environmental risk assessment (ERA) tool to estimate dose to mussels from $^{3}$H.

The first publication of dose rate calculations for aquatic organisms exposed to tritium ($D_{b}$ in rad h$^{-1}$) was in rainbow trout in 1977, using equation 5.1 (Strand et al. 1977):

$$D_{b} = 2.13 \varepsilon \beta C$$

(5.1)

where 2.13 is a simplified conversion factor for $\mu$Ci to Bq and seconds to hours, incorporating the energy of 1 rad (in MeV); $\varepsilon \beta$ is the mean beta energy of tritium (0.00569 MeV); and $C$ is the water activity concentration in $\mu$Ci mL$^{-1}$.

Equation 5.1 has since been modified to give $D_{b}$ in Gy h$^{-1}$ and use values in accordance with the SI system (Jha et al. 2005, Jaeschke et al. 2011):

$$D_{b} = \frac{2.13 \varepsilon \beta C}{3.7 \times 10^{6}}$$

(5.2)

Where $C$ is the water activity concentration in Bq mL$^{-1}$, and $3.7 \times 10^{6}$ is the combined conversion factor for $\mu$Ci to Bq and rad to Gy.

The ERICA tool is a software program designed as part of the EC EURATOM funded ERICA project to enable environmental risk assessment for radioactivity (Brown et al. 2008). It incorporates three tiers (summarised very briefly in Fig. 5.1, please see Brown et al. 2008 for more details), which allow the user to input site-specific information (e.g. for a lake next to a NPP) and assess the potential risk to wildlife. Tier 2 of the ERICA tool allows the user to input water or biota activity concentrations and produce estimated dose rates, which are then labelled as of ‘concern’, ‘potential concern’ or ‘negligible concern’ after comparison to a dose rate screening value (either 10 $\mu$Gy h$^{-1}$, Garnier-Laplace & Gilbin 2006; or 400 $\mu$Gy h$^{-1}$, IAEA 1992, UNSCEAR 1996). It is therefore possible to use this portion of the ERICA tool to estimate dose rates for mussels after exposure to $^{3}$H, provided that water or biota activity concentrations have been measured. In fact,
the ERICA tool has already been used to estimate dose rates for forest wildlife exposed to radiation after the Fukushima Dai-ichi accident (Garnier-Laplace et al. 2011), but to our knowledge there have been no such dose estimates for marine mussels or any other aquatic invertebrate exposed to any radionuclide.

Only 8 of the papers on the effects of ionising radiation on aquatic invertebrates published between 1950 and 2011 studied $^3$H (reviewed in Dallas et al. 2012, Adam-Guillermin et al. 2012). This previous research has demonstrated that $^3$H has detrimental effects on embryonic development in goose barnacles (Abbott & Mix 1979), Pacific oysters (Nelson 1971), brine shrimp (Higuchi et al. 1980), as well as marine mussels *Mytilus edulis* (Hagger et al. 2005a). In addition, egg mortality (or failure to hatch) has also been demonstrated in fresh and salt water invertebrates (Higuchi et al. 1980, Knowles & Greenwood 1997). Tritium has also been reported to induce genotoxicity in *M. edulis* in a variety of ways, including chromosomal aberrations and sister chromatid exchanges in larvae (Hagger et al. 2005a) and DNA strand breakage alongside aneugenic/clastogenic effects in adults (Jha et al. 2005, Jaeschke et al. 2011). These existing studies have, however, restricted their investigation to biomarkers of genotoxicity and/or development.
abnormalities, without addressing the higher level aspects of $^3$H radiotoxicity, (e.g. attachment, CR, histopathology, SoS, see Chapter 2) or accurate dose estimation. Furthermore, previous research on both $^3$H and environmental radioactivity in general has tended to focus on acute studies. This is problematic for two reasons, (1) chronic exposure is more environmentally relevant, especially at low dose rates; and (2) the rate at which a total radiation dose is achieved can have a significant impact on its effects (Van Hook 1971, Woodhead 1993, Copplestone et al. 2001, Dallas et al. 2012). In this context, it is perhaps surprising that there have been no new studies published on the effects of $^3$H in aquatic organisms since 2011, although its accumulation has been investigated (e.g. Jaeschke & Bradshaw 2013). As a result, there remain many unanswered questions about the impact of $^3$H on aquatic invertebrates. In particular, there is a scarcity of information on links between genotoxicity and effects at higher levels of biological organisation (Adam-Guillermin et al. 2012). Furthermore, two recent events have highlighted the need for a more evidence-based assessment of the impacts of tritium on marine biota - the accidental release of $^3$H at Fukushima Dai-ichi, and high concentrations of OBT in Cardiff Bay (Williams et al. 2001, Melintescu & Galeriu 2011).

5.1.1 Objectives and hypotheses

Against the background of the above information, the objectives of this chapter were:

(a) to determine tissue-specific $^3$H accumulation, for comparison of our results with those published previously on mussels exposed to $^3$H (Jha et al. 2005, Jaeschke et al. 2011) and to allow estimation of dose rates;

(b) to estimate dose rates from chronic $^3$H exposure to marine mussels, using and comparing three different methods, and assess any relationship between genotoxicity and dose rate, where appropriate;

(c) to use an integrated experimental approach to correlate genotoxic effects with effects at higher (more ecologically relevant) levels of biological organisation (i.e. attachment, CR, histopathology, SoS);

The hypotheses of this chapter were that tritiated water would cause genotoxic effects in mussel haemocytes and decreases in attachment ability, CR and anoxia tolerance. Similarly, histopathological effects were expected.
5.2 Materials and Methods

5.2.1 Radiation protection

The experiments in this chapter were carried out within Plymouth University’s Consolidated Radioisotope Facility (CORiF) or in controlled spaces, under the guidance of the Radiation Protection Supervisor (RPS) and Radiation Protection Assistant (RPA). All necessary precautions were taken to minimise $^3$H exposure to experimenters and colleagues. Local rules and other radiation protection documents are included in Appendix B.

5.2.2 Experimental design and mussel exposure conditions

Two separate experiments were performed, firstly to determine genotoxicity, histopathology and organism-level effects of tritiated water to mussels and then to assess both genotoxicity and tissue-specific accumulation (and therefore dose). Adult *M. galloprovincialis* individuals were collected from Trebarwith Strand in July 2011 (48.00 ± 2.25 mm) and November 2012 (49.78 ± 2.32 mm) and depurated as in section 2.3.

After depuration, mussels were transferred to the exposure vessels (2 L glass beakers, 4.5 mussels L$^{-1}$, with constant aeration) and allowed to acclimatise for 48 h. HTO was obtained at a concentration of 185 MBq L$^{-1}$ and diluted with filtered seawater to produce a 1 MBq mL$^{-1}$ stock solution. Following acclimatisation, HTO stock was added to beakers to produce concentrations of 1, 5 or 15 MBq L$^{-1}$ (in triplicate). Filtered seawater with no additional HTO was used as a control. Furthermore, 3 additional beakers were treated with 32 mg L$^{-1}$ ethyl methane sulfonate (EMS), a known genotoxic agent, as a positive control. In the latter experiment the exposure treatments were identical except that the intermediate HTO concentration (5 MBq L$^{-1}$) and EMS positive control were omitted for logistical reasons.

The exposure period lasted 14 days, during which mussels were fed every 3 days, with a 100 % water change 2 h after feeding (see Chapter 2). Water samples were taken before and after each water change, and processed for liquid scintillation counting, to verify water activity concentrations. The experimental design for this chapter is illustrated in Fig. 5.2.
Water quality parameters were salinity 34.54 ± 0.14, pH 7.92 ± 0.14, dissolved oxygen 89.92 ± 1.95 % and temperature 15.13 ± 0.20 °C. For November 2012, they were salinity 33.97 ± 0.24, pH 8.01 ± 0.12, dissolved oxygen 90.17 ± 1.86 % and temperature 15.22 ± 0.23 °C.

5.2.3 Sampling procedure

Although the two experiments had different sampling timepoints and some different endpoints, efforts were made to ensure that the initial sampling procedure was identical in all cases. This involved the removal of 9 mussels from the exposure vessels for the clearance rate assay, after which they were either discarded (day 14, July 2011; day 0, November 2012) or put to one side for use in the SoS assessment (day 14, November 2012). This was to ensure that mussels later used for dose determination had not depurated in clean water during CR measurement. Another 9 mussels were then sampled for haemolymph (as described in section 2.5) and dissected either into cross-sections for histopathological processing (July 2011) or into individual organs for determination of activity concentration by LSC (November 2012). Small pieces (~ 5mm) of gill, mantle and digestive gland from these mussels were snap frozen in liquid nitrogen and stored at -80 °C for later RNA extraction and gene expression analysis. The remaining mussels (n = 21; November 2012) were used for the stress on stress assay, as described in section 2.14.

5.2.4 Liquid scintillation counting of water and mussel tissues

Water samples (100 µL) were taken immediately after each water change (in duplicate) and processed for LSC as described in section 2.16. Water activity concentrations were then used for dosimetry calculations (see section 5.2.5 below).

Mussel tissues were processed for LSC as detailed in section 2.16 apart from two minor modifications - the addition of an extra tissue category, ‘other’, for those soft tissues not removed as individual organs (i.e. gills, mantle, digestive gland, adductor muscle, foot and byssus), and the recovery and counting of water after freeze drying. This was to enable quantification of the activity of tissue free water tritium (TFWT) in tissues (Fiévet et al. 2013). Previous research
Figure 5.2. Overall experimental design for determination of the effects of exposure to tritiated water in mussels. Note that in the November 2012 experiment, CR was measured in different mussels from those used for other endpoints.
5. Chronic exposure to tritiated water

into accumulation of tritium in exposed mussels has excluded TFWT by only counting freeze-dried tissues (e.g. Jha et al. 2005, Jaeschke et al. 2011). Although not organically incorporated, TFWT will still contribute to the organism’s dose. Consequently, it is necessary to quantify the activity of TFWT and OBT to enable accurate dose estimation.

5.2.4.1 Calculation of tissue activity concentrations

The measured tissue activity value was normalised for dry weight to give OBT activity concentration (directly comparable to previous studies; e.g.. Jha et al. 2005, Jaeschke et al. 2011). The activity of the dry sample was summed with the mean TFWT activity of the water in it, to produce total activity, which was then normalised for wet weight to give a final total activity concentration per tissue. For clarity these steps are illustrated in Fig. 5.3.

Proportions of total $^3$H contributed by each tissue were calculated according to the equation:

$$P_{^3H} = \frac{T_{^3H}}{\sum T_{^3H}} \times 100$$

(5.3)

where $P_{^3H}$ is the proportion (%) of $^3$H in a tissue, $\sum T_{^3H}$ is the sum of the $^3$H total activities for all tissues in a given mussel; and $T_{^3H}$ is the total activity of $^3$H in the individual tissue.

Concentration factors (CFs) were calculated according to the equation:

$$CF = \frac{A_O}{A_S}$$

(5.4)

where $A_O$ is the total activity concentration in the tissue (or summed for the whole organism; in Bq kg$^{-1}$) and $A_S$ is the mean activity concentration of the seawater for that treatment (in Bq L$^{-1}$). This equation has been used previously to generate CFs for mussels exposed to HTO (Jaeschke et al. 2011) and in the ERICA tool (Hosseini et al. 2008).
5. Chronic exposure to tritiated water

Figure 5.3. Flow chart illustrating steps for calculation of tissue activity for mussels exposed to tritiated water. Blue boxes indicate processes, green circles show outcomes.
5.2.5 Dose calculations

5.2.5.1 Equation method

All equation-based dosimetry calculations performed in this study used equation 5.2, with the addition of a final conversion from Gy h\(^{-1}\) to µGy h\(^{-1}\) where appropriate.

5.2.5.2 ERICA tool methods

Although the ERICA tool contains a model ‘Benthic mollusc’, a custom ‘Mytilus’ model was programmed to ensure accurate geometry for our samples. Parameters for this model were mean measurements of 40 (untreated) mussels collected from TBS in December 2010. Details of these parameters are included in Table 5.2.

Two different ERICA tool methodologies were used to calculate doses to mussels from tritiated water: (1) input of the mean water activity concentration for each treatment, allowing the ERICA tool to predict tissue concentrations; and (2) input of whole organism activity concentrations for each mussel, forcing the ERICA tool to use these values, and calculating a treatment mean from the resulting individual dose rates.

In both cases, the tier 2 assessment module was used within ERICA. Tritium was selected as the isotope of choice, along with the newly created ‘Mytilus’ organism. The distribution co-efficient (\(K_d\)) was set as 0 L kg\(^{-1}\) (as there was no sediment in our experimental set up) and the concentration ratio was set to 1 Bq kg\(^{-1}\) per Bq L\(^{-1}\). This concentration ratio is the same as that selected by the ERICA tool using its default ‘Benthic mollusc’ model, as a result of the use of specific activity model (i.e. no values from observed data; IAEA 2004, Dallas et al. 2012).

5.2.6 Comet assay to determine DNA strand breaks

Haemolymph was kept on ice after extraction and then processed for the comet assay (see section 2.9), without enzyme modification. Slides were coded and randomised to ensure scoring was blind.
5. Chronic exposure to tritiated water

Table 5.2. Parameters used for "Mytilus" model organism in the ERICA tool. Parameters for 'Benthic mollusc (marine)' are also shown for comparison.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mytilus</th>
<th>Benthic mollusc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass (kg)</td>
<td>0.009</td>
<td>0.016</td>
</tr>
<tr>
<td>Height (m)</td>
<td>0.021</td>
<td>0.006</td>
</tr>
<tr>
<td>Width (m)</td>
<td>0.017</td>
<td>0.012</td>
</tr>
<tr>
<td>Length (m)</td>
<td>0.045</td>
<td>0.025</td>
</tr>
<tr>
<td>Occupancy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water-surface</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Water</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Sediment-surface</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Sediment</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kσ</td>
<td>0.467</td>
<td>0.500</td>
</tr>
<tr>
<td>Chlb</td>
<td>0.378</td>
<td>0.500</td>
</tr>
</tbody>
</table>

*a* input by user; *b* calculated by ERICA tool.

Leftover haemolymph (after comet and micronucleus assays) was pelleted, supernatant removed and cells snap frozen and stored at -80 °C for later RNA extraction and gene expression analysis.

5.2.7 Determination of relative transcriptional expression of selected genes

In order to maximise data yield from finite resources, the gene expression component of this experiment was undertaken by another PhD researcher, Dr Yanan Di (Plymouth University, UK). For details please see Di (2012).

5.2.8 Histopathology of mussel digestive gland

After histological preparation as in section 2.13, digestive tubules were examined for signs of epithelial atrophy, a well established tissue-level biomarker of contaminant-induced stress (Couch 1984, Lowe & Clarke 1989, Cajaraville et al. 1992, Garmendia et al. 2011). Quantitative morphological analysis was performed for a total of 90 tubules per treatment (2 per image, 5 images per mussel, 9 mussels
Figure 5.4. Methods for quantifying histopathology in mussel digestive gland after exposure to tritiated water. (A) Diagrammatic representation of a cross-section of a digestive tubule, showing the measurements used to quantify digestive gland epithelial thickness (mean epithelial thickness [MET, equation 5.5], mean luminal radius [MLR, equation 5.6] and mean diverticular radius [MDR, equation 5.7]); and illustrative examples of digestive tubules showing high (B) and low (C) MET.
per treatment). Image analysis software (Image J v1.44p; National Institutes of Health, Bethesda, MA, USA) was used to determine the area and perimeter of the whole tubule ($A_t$ and $P_t$, respectively) and the area and perimeter of the lumen ($A_l$ and $P_l$, respectively; Fig. 5.4). These parameters were then used to calculate mean epithelial thickness (MET), MLR:MET and MET:MDR (where MLR is mean luminal radius and MDR is mean diverticular [tubule] radius), according to the following equations (Cajaraville et al. 1992):

\[
MET = 2 \frac{A_t - A_l}{P_t + P_l} \quad (5.5)
\]

\[
MLR = \sqrt{\frac{A_l}{\pi}} \quad (5.6)
\]

\[
MDR = \sqrt{\frac{A_t}{\pi}} \quad (5.7)
\]

### 5.2.9 Behavioural responses

Two behavioural responses were quantified; attachment of mussels to the substrate (July 2011) and clearance rate (both experiments).

During the water change on day 12 (i.e. closest to the sampling on day 14), mussel attachment was recorded as in Chapter 4 (section 4.2.9).

The clearance rate assay was performed as described in section 2.7 ($n = 9$).

### 5.2.10 Stress on stress and micronucleus assays

The SoS and MN assays were carried out according to the protocols detailed in Chapter 2, without modification, and had $n = 21$ and $n = 9$ mussels per treatment, respectively.
5.2.11 Statistical analyses

Comet data were processed as described in section 2.18. MET and MLR/MET were analysed using the non-parametric Kruskal-Wallis test, followed by Mann-Whitney U-tests to identify pairwise differences (with adjustment for multiple comparisons). MET/MDR was analysed using one-way ANOVA. Survival curves and lethal time to 50 % mortality (LT$_{50}$) values were generated using the Kaplan-Meier method (Kaplan & Meier 1958) and were compared using the log rank test. All other data were normal and were analysed using ANOVAs.

5.3 Results

5.3.1 Tritium in water and mussel tissues

Measurements of tritium in water showed good agreement with nominal values (> 90 % of expected measurements; Table 5.3). Control water samples’ activity was below the LOD.

All control mussels showed very low tritium concentrations. Total activity concentrations were $\sim$0.5 MBq kg$^{-1}$ for 1 MBq L$^{-1}$ and $\sim$4 MBq kg$^{-1}$ after exposure to 15 MBq L$^{-1}$ HTO for 14 days. The OBT activity concentrations were approximately half these values, with some tissue-specific variation (Fig. 5.5). Tritium was accumulated in a concentration and tissue-specific manner ($p < 0.01$). OBT tissue activity concentrations varied in the order foot $>$ digestive gland $>$ byssus $>$ muscle $>$ mantle $>$ other $>$ gills after exposure to 15 MBq L$^{-1}$ HTO for 14 d (Fig. 5.5A). In contrast, at the lower HTO concentration OBT tissue activity concentration decreased from byssus $>$ foot $>$ muscle $>$ digestive gland $>$ mantle $>$ other $>$ gills. Total tissue activity concentration was greatest in digestive gland, foot and gills (Fig. 5.5B) at 15 MBq L$^{-1}$ and then decreased in the order other $>$ muscle $>$ mantle $>$ byssus, although the only significant differences were between mantle and digestive gland, gill or other. At 1 MBq L$^{-1}$ there was little variation in total tissue activity concentration. Proportionally, there was no significant change, with other, mantle and gill tissues having the highest proportion of $^3$H across treatments (Fig. 5.7).

Whole organism CFs varied significantly between the two $^3$H treatments (Fig.
Figure 5.5. Tissue-specific accumulation of tritium in mussels after exposure to tritiated water for 14 days. (A) Organically-bound tritium (OBT) concentration i.e. that remaining after extraction of tissue free water tritium (TFWT) by freeze drying; and (B) total activity concentration, i.e. OBT + TFWT normalised for wet tissue weight. Data are presented as means ± one SE. Significant differences (p < 0.05) from the equivalent tissue control (*) and the equivalent tissue at 15 MBq L⁻¹ HTO (⁺) are indicated.
5. Chronic exposure to tritiated water

Figure 5.6. Concentration factors (CF) for mussels exposed to tritiated water for 14 days. (A) whole organism; and (B) tissue-specific. CFs were calculated from total tissue and water activity concentrations, according to equation 5.4. Error bars are ± one SE. Asterisks indicate significant differences across treatments ($p < 0.01$), and lowercase lettering indicates between-tissue effects (means which do not share a lower case letter are significantly different [$p < 0.01$]).
5. Chronic exposure to tritiated water

Figure 5.7. Tissue-specific proportions of tritium activity in mussel tissues after 14 days exposure to tritiated water.

5.6 A). Each tissue-specific CF was significantly different from the same tissue in the other $^3$H treatment, however only byssus and digestive gland at 1 MBq L$^{-1}$ showed significant between-tissue variation (Fig. 5.6B). Despite the variation, all CFs (whole organism and tissue-specific) were < 0.6.

5.3.2 Dose calculations

As water $^3$H concentrations in the controls were < LOD, dose calculations were only performed for the two HTO treatments.

Table 5.3. Whole organism dose calculations for mussels exposed to tritiated water.

<table>
<thead>
<tr>
<th>Water activity concentration (MBq L$^{-1}$)</th>
<th>Dose rate ($\mu$Gy h$^{-1}$)</th>
<th>Total dose - 14d (mGy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Equation$^b$</td>
<td>ERICA$^c$</td>
</tr>
<tr>
<td>1 0.94 ± 0.10</td>
<td>3.08</td>
<td>7.74</td>
</tr>
<tr>
<td>15 13.79 ± 0.81</td>
<td>45.18</td>
<td>113.51</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SD; $^b$ Strand et al., 1977; $^c$ from mean water activity concentrations; $^d$ from mean whole organism activity concentrations
5.3.2.1 Equation method

Use of the Strand et al. (1977) equation resulted in dose rates of 3.08 and 45.18 $\mu$Gy h$^{-1}$ for 1 and 15 MBq L$^{-1}$, respectively. The corresponding total doses were 2.60 and 38.14 mGy after 14 days (Table 5.3).

5.3.2.2 ERICA tool methods

The two ERICA tool methods gave different dose rates and the amplitude of this difference varied with treatment (Table 5.3). For 1 MBq L$^{-1}$, the water activity method produced dose rates $\sim$1.8 times that of the whole organism activity concentration method, whereas for 15 MBq L$^{-1}$ this difference was $\sim$3.5 times.

5.3.3 Genotoxicity of tritium to mussel haemocytes

Control mussel DNA damage showed no statistical difference across the two experiments (7.52 ± 0.89 and 11.32 ± 0.94 % tail DNA). Despite this, induction of DNA damage showed considerable inter-experimental variation. In July 2011, % tail DNA increased with $^3$H activity, from $\sim$8 % in control mussel haemocytes to $\sim$32 % for 15 MBq L$^{-1}$. Maximum DNA strand breakage was observed in the positive control (EMS) group (Fig. 5.8A). In the winter experiment both $^3$H activity concentrations produced elevated % tail DNA, but were not significantly different from the control. Variation in the data was evidently higher in the November experiment (Fig. 5.8B).

For logistical reasons micronuclei induction was only measured in July 2011, where it showed significant increases at the two higher $^3$H activities along with the positive control (Fig. 5.9). In this experiment, the two biomarkers of genotoxicity were significantly correlated ($R^2 = 0.21$, $p < 0.01$), and MN could be predicted using the formula $\text{MN} = (0.85 \times \% \text{tail DNA}) + 1.24$ (Fig. 5.10).

5.3.4 Histopathological effects on mussel digestive gland

Neither MLR or MDR showed any significant effect after exposure of mussels to HTO for 14 days (data not shown). MET showed significant reductions after 14 days treatment with both 5 MBq L$^{-1}$ HTO and EMS ($p < 0.05$; Fig 5.11.
Figure 5.8. DNA strand breaks (as measured by the comet assay i.e. % tail DNA) in mussel haemocytes after exposure to (A) *in vitro* hydrogen peroxide for 1 h (validation, October 2012); (B) tritiated water for 14 d (July 2011); and (C) tritiated water for 14 d (November 2012). For clarity when discussing the variability in November 2012 results, data are presented as standard Tukey boxplots, i.e. whiskers are $1.5 \times$ IQR and outliers are indicated as points. Asterisks indicate significant differences ($p < 0.05$) from the control.
5. Chronic exposure to tritiated water

Figure 5.9. Induction of micronuclei in mussel haemocytes after exposure to tritiated water for 14 days. Error bars are ± one SE. Asterisks indicate significant ($p < 0.05$) differences from the control, whereas # indicate significant differences ($p < 0.01$) from the other tritium treatments.

Figure 5.10. Linear regression analysis of % tail DNA and incidence of MN in mussel haemocytes after exposure to tritiated water for 14 days. The blue line indicates fitted values and dashed red lines represent 95% confidence limits.
Figure 5.11. Histopathological effects of tritiated water on mussel digestive gland tubules after 14 days exposure. (A) Mean epithelial thickness (MET) and (B) MET normalised by mean diverticular radius (MDR). Error bars are ± one SE. Asterisks indicate significant ($p < 0.05$) differences from the control.
A), but with very small differences from the control, suggesting statistical but perhaps not biological importance. Furthermore, when this parameter was normalised against MDR (i.e. MET:MDR) these significant differences disappeared (Fig 5.11B).

5.3.5 Behavioural responses

Attachment was monitored in July 2011, where only the EMS treated mussels showed any significant reduction (Fig. 5.12A). Clearance rate varied between $\sim 1.4$ and $2.1 \text{ L h}^{-1}$ across the two experiments but showed no significant increase or decrease with any treatment (Fig. 5.12B-C).
5. Chronic exposure to tritiated water

Figure 5.12. Behavioural responses of mussels exposed to tritiated water for 14 days. (A) Number of mussels attached (as a % of total mussels); and clearance rate (CR) of mussels exposed during the July 2011 experiment (B) and November 2012 experiment (C). Error bars are ± one SE. Asterisks indicate significant differences from the control ($p < 0.05$). Percentages are calculated as means of three replicate beakers ($n = 27$).
5.3.6 Stress on stress response

The ability of mussels to withstand anoxia (i.e. aerial exposure) was not significantly impacted by 14 days exposure to HTO at either activity (Fig. 5.13). LT$_{50}$ values for the November 2012 experiment were 17.00 ± 0.81, 17.00 ± 0.76 and 17.00 ± 0.94 days for mussels exposed to control seawater, 1 and 15 MBq L$^{-1}$, respectively.

![Figure 5.13. Survival of mussels (n = 25) in continuous anoxic conditions after exposure to tritiated water for 14 days. No significant differences were observed.](image-url)
5. Chronic exposure to tritiated water

5.4 Discussion

5.4.1 Tissue-specific tritium accumulation

Tissue OBT activity concentrations in this study were the same order of magnitude as those reported by Jaeschke et al. (2011), i.e. ∼1 - 2.5 compared to ∼2.5 - 5.5 MBq kg⁻¹, respectively. Although it is logical to expect accumulated ³H to be similar in mussels exposed to 37 MBq L⁻¹ for 7 d (Jaeschke et al. 2011) and 15 MBq L⁻¹ for 14 days (this study), the kinetics of this process have not been explored. Temporal differences in accumulation patterns may explain the slight discrepancy between these two studies. Furthermore, Jaeschke et al. (2011) showed that depuration of ³H from mussel tissues occurs rapidly after exposure, adding weight to the idea that this process may have high temporal variability. Both this study and the work of Jaeschke et al. (2011) have reported considerably lower activity concentrations than those reported by Jha et al. (2005). Those authors reported that mussels exposed to 37 MBq L⁻¹ HTO had tissue concentrations of ∼5 - 140 MBq kg⁻¹ after only 96 h, and those exposed to 148 MBq L⁻¹ had even higher tissue activity concentrations (∼10 - 275 MBq kg⁻¹) after the same time period.

Although the experimental set up and procedures of the earlier experiment were very similar to our own, there are some parameters which might explain this considerable difference in ³H accumulation. Jha et al. (2005) collected their mussels from an alternative location in South Cornwall, Whitsand Bay, which is nearer to Plymouth, a major Naval city. There is the potential for contaminants and/or disease to have influenced mussel behaviour or physiology such that increased accumulation occurred (Bignell et al. 2011). Clearance rate may be one such factor - the mussels used in the current study had a ‘normal’ CR (i.e. ∼ 2 L h⁻¹; Widows 1978), however there is scope for mussels in previous studies to have filtered water at nearer to maximal rates (2 - 2.5 L h⁻¹; Pascoe et al. 2009), which may influence ³H accumulation. Algal enrichment has been shown to have a significant effect on the bioaccumulation of B(a)P in M. edulis (Okay et al. 2000). Although no such effect has yet been demonstrated for the accumulation of ³H in mussels, it is interesting to note that the feeding regimes are the main methodological difference between the study of Jha et al. (2005) and that reported here (Liquifry during depuration and no feed during exposure, in contrast to Isochrysis galbana during acclimation and feeding every 3 d during exposure).
Our data support the suggestion by Jaeschke et al. (2011) that the CF for HTO is much less than 1, in fact it is nearer to 0.6. The values reported herein are somewhat higher than the average CF of 0.1 from Jaeschke et al. (2011), although this may be explained by the fact that those authors calculated CFs from dry tissue activity concentration, rather than total activity concentration as here. There is very limited information on CFs for tritiated water in mussels, or indeed any other aquatic animals, so our results will be a crucial addition to the field. It is also interesting to note that dose and tissue appear to have significant effects on CF both independently and in interaction with each other. This is an important finding, which should be taken into account for future studies in this area.

5.4.2 Methods of dose estimation

All three methods of estimating dose to mussels from HTO gave differing dose rates. There are many reasons why this might be the case, including the assumptions made about different parameters (e.g. CF, which - as we have demonstrated - can vary considerably from the recommended value of 1). It is especially interesting that the ERICA tool produces differing dose rates, which illustrates that tools such as this need to be used with care and that transparency in the methodological reporting of their use is essential. Given that the whole organism activity concentration method used experimental data from mussels, it is likely that this gives the most accurate dose rate estimates. It is therefore recommended that future researchers exposing mussels to HTO use this novel method where possible. It is also important to note that, as this method produces lower dose rates than that of the Strand et al. (1977) equation at higher water activity concentrations, had it been used in previous studies on HTO and mussels, their dose rates would have been lower. This is particularly notable in the case of Jha et al. (2005) where the lowest dose to cause induction of micronuclei is already close to 10 µGy h$^{-1}$, the screening value suggested by Garnier-Laplace et al. (2010).

For more accurate dose estimation it is also important to consider the proportion of HTO and OBT making up the total $^3$H content of a tissue and/or organism. Due to its longer residency in biological systems, OBT can contribute to a greater dose, however it is also important to consider exchangeable (i.e. removed by washing with tritium-free water) and non-exchangeable forms of OBT. In plants, inclusion of exchangeable OBT in total OBT estimates can cause overestimation.
of dose (Kim & Korolevych 2013). This study did not determine the activity from different types of OBT, so there is still scope to make these dose estimates more accurate.

5.4.3 Genotoxicity of tritium to mussel haemocytes

The July 2011 experiment resulted in dose-dependent induction of both DNA strand breaks (comet assay) and clastogenic/aneugenic events (micronucleus assay). Given the previous data on radionuclides as a genotoxic or clastogenic agents in both mammalian systems (e.g. Vral et al. 2011, Chen 2012) and aquatic organisms (e.g. Hagger et al. 2005a, Jha et al. 2005, Jaeschke et al. 2011, AlAmri et al. 2012), this is not surprising. However, contrary to expectations, the November 2012 experiment showed no statistically significant increase in % tail DNA under identical exposure conditions (except for the two omitted treatments). Although for logistic reasons it was not possible to run a positive control during the November 2012 exposure, each experiment was preceded by an independent validation of the comet assay protocol with H₂O₂, as in Chapter 2 (section 2.9.1). The successful results of this validation (Fig. 5.8A), in combination with the positive control data from July 2011, suggest that the difference between these two experiments was not attributable to methodological problems.

Mussels in this study were wild-caught in two different seasons. Although depurated at a constant temperature in the laboratory for two weeks prior to the exposures, there is still the potential for seasonal effects to have influenced our results. The impact of season may present itself in one of three ways: (1) the organism is differentially sensitive to potential genotoxicants; (2) the baseline level of DNA damage varies (e.g. Pisanelli et al. 2009); or (3) DNA damage repair kinetics vary, resulting in altered levels of damage after the same period of exposure. Background seasonal variation in DNA damage (measured using the comet assay) has previously been demonstrated in digestive gland and haemocytes of *Mytilus* spp. (Shaw et al. 2000, Pisanelli et al. 2009). Although our control mussels showed different variability between the two experiments, the mean values for % tail DNA were not significantly different, indicating that our results are less likely to have been caused by baseline variation. Both parasitic infection and nutritional status are also known to influence genotoxic responses in mammals (Gentile & Deruiter 1981, Delmore 1997) and aquatic organisms (Jha 2008), whilst Se has been shown
5. Chronic exposure to tritiated water

to be protective against Hg-induced DNA damage in mussels, presumably via a dietary route (Tran et al. 2007). As either of these factors may also vary with season, it is possible that they contribute to the altered genotoxic responses to HTO observed here.

Probably the most well characterised of the seasonal factors affecting marine mussels is the reproductive cycle. Typically *Mytilus* sp. in the south-west of England exhibit gametogenesis in winter, leading up to a spawning period peaking in April - June, followed by a period of inactivity and nutrient acquisition in high summer, before gametogenesis restarts (Seed 1971, Bignell et al. 2008, Hagger et al. 2010). In winter, mussels have higher rates of oxygen consumption to support gametogenesis (Bayne 1976), however, in the spring and early summer (i.e. the periods before, during and after spawning) energy reserves are almost entirely depleted (due to exclusive proliferation of gonadal tissue) and oxygen consumption is at an all time low. This could potentially reduce fitness and decrease the ability of mussels to defend themselves against xenobiotics (including radiation). There are several other factors, both biotic and abiotic, which may cause seasonal variation in mussel physiology, including temperature, salinity, and nutritional status (Ciacci et al. 2009).

Numerous biomarker responses have been reported to be affected by season in *Mytilus* spp. (see Table 5.4 for some key examples). In particular, haemocytes of *M. galloprovincialis* (in which DNA damage was assessed in this study) have been shown to exhibit clear seasonal variation in lysosomal membrane stability, phagocytic/soluble lysosome activity and nitric oxide production - key immune parameters which may influence the capacity for xenobiotic or oxidative defense (Novas et al. 2007, Ciacci et al. 2009). In terms of radiation-induced effects, Farcy et al. (2007) examined field-sampled oysters (*Crassostrea gigas*) from exposed and unexposed sections of the French coast, but were unable to find any significant effects of irradiation on transcriptional expression of chaperone and oxidative stress genes. As genotoxicity is a tissue-specific phenomenon (Jha 2008), assessment of DNA damage (i.e. comet assay) in multiple tissues of mussels collected in different seasons and then exposed to a model genotoxicant could potentially determine whether the effect we have observed is tissue-specific.
Table 5.4. Examples of biomarkers at different levels of biological organisation showing clear seasonal variation in field-sampled mussels (*Mytilus* spp.)

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Endpoint</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. galloprovincialis</em></td>
<td>NW Adriatic Sea</td>
<td>DNA strand breaks</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>Pisanelli et al. 2009</td>
</tr>
<tr>
<td><em>M. galloprovincialis</em></td>
<td>N Adriatic Sea</td>
<td>DNA strand breaks</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>Bocchetti et al. 2008</td>
</tr>
<tr>
<td><em>M. edulis</em></td>
<td>NY, USA</td>
<td>HSP70 and MXR protein expression</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Minier et al. 2000</td>
</tr>
<tr>
<td><em>M. edulis</em></td>
<td>SW UK</td>
<td>mu opiate receptor gene expression</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>Mantione et al. 2010</td>
</tr>
<tr>
<td><em>M. galloprovincialis</em></td>
<td>N Adriatic Sea</td>
<td>Lysosomal membrane stability,</td>
<td></td>
<td></td>
<td>+</td>
<td>c</td>
<td>Petrović et al. 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>only at reference site, polluted sites showed high variability</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) + significantly elevated, - significantly reduced; \(^{b}\) only in April; \(^{c}\) only at reference site, polluted sites showed high variability
Table 5.4. (continued)

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Endpoint</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. galloprovincialis</em></td>
<td>N Adriatic Sea</td>
<td>Catalase activity,</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Bocchetti et al. 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutathione peroxidase activity</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetylcholinesterase activity</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. galloprovincialis</em></td>
<td>Spain</td>
<td>Catalase activity</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Cancio et al. 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Palmitoyl-CoA oxidase (AOX) activity</td>
<td></td>
<td>+b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peroxisomal volume density</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. edulis</em></td>
<td>SW UK</td>
<td>Heart rate</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td>Hagger et al. 2010</td>
</tr>
<tr>
<td><em>M. galloprovincialis</em></td>
<td>Aegean Sea</td>
<td>HSP70 and HSP90 protein expression,</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>Ioannou et al. 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycolytic enzyme activity</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mytilus</em> spp.</td>
<td>N Baltic Sea</td>
<td>Free amino acid concentration</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>Kube et al. 2007</td>
</tr>
</tbody>
</table>

+ significantly elevated, - significantly reduced; b only in April; c only at reference site, polluted sites showed high variability
### Table 5.4. (continued)

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Endpoint</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. galloprovincialis</em></td>
<td>N Adriatic Sea</td>
<td>Phagocytotic activity</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>Ciacci et al. 2009</td>
</tr>
<tr>
<td><em>M. edulis</em></td>
<td>NY, USA</td>
<td>Morphine-stimulated NO release,</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>Mantione et al. 2010</td>
</tr>
<tr>
<td><em>M. galloprovincialis</em></td>
<td>NW Spain</td>
<td>Nitric oxide (NO) production</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>Novas et al. 2007</td>
</tr>
</tbody>
</table>

**Immunological effects:**

- *M. galloprovincialis*, N Adriatic Sea: Phagocytotic activity
  - Ref: Ciacci et al. 2009

- *M. edulis*, NY, USA: Morphine-stimulated NO release
  - Ref: Mantione et al. 2010

- *M. galloprovincialis*, NW Spain: Nitric oxide (NO) production
  - Ref: Novas et al. 2007

**Histopathology:**

- *M. galloprovincialis*, SW UK: Adipogranular status
  - Ref: Bignell et al. 2008

- *Mytilus* hybrids: Ancistrum mytili prevalence
  - Ref: Koukouzika et al. 2009

- *M. galloprovincialis*, Aegean Sea: Lysosomal volume density, Lipofuscin, Neutral lipid accumulation
  - Ref: Koukouzika et al. 2009

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\( a \) + significantly elevated, - significantly reduced; \( b \) only in April; \( c \) only at reference site, polluted sites showed high variability
Table 5.4. (continued)

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Endpoint</th>
<th>Spring</th>
<th>Autumn</th>
<th>Winter</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Summer $^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. galloprovincialis</td>
<td>Bay of Biscay</td>
<td>Lysosomal structure</td>
<td>+</td>
<td>-</td>
<td></td>
<td>Marigómez et al. 1996</td>
</tr>
<tr>
<td>M. galloprovincialis</td>
<td></td>
<td>Lipofuscin accumulation,</td>
<td></td>
<td></td>
<td>+</td>
<td>Petrović et al. 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neutral lipid accumulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. edulis</td>
<td>SW UK</td>
<td>Clearance rate,</td>
<td></td>
<td></td>
<td></td>
<td>Hagger et al. 2010</td>
</tr>
<tr>
<td>M. galloprovincialis</td>
<td>N Adriatic Sea</td>
<td>Stress on stress response</td>
<td></td>
<td></td>
<td></td>
<td>Petrović et al. 2004</td>
</tr>
</tbody>
</table>

$^a$ + significantly elevated, - significantly reduced; $^b$ only in April; $^c$ only at reference site, polluted sites showed high variability
If the reproductive cycle is the major influence on seasonal effects in marine mussels, then it is perhaps unsurprising that the majority of significant seasonal effects on biomarkers have been for the spring/summer period (Table 5.4). The data reported here seem to suggest a similar increase in susceptibility to HTO-induced genotoxicity in summer, at least in haemocytes. It is, however, still possible that abiotic factors may have influenced these results. For example, although Farcy et al. (2007) did not see any evidence of radiation-induced transcriptional changes, they did report a strong seasonal variation in mRNA levels, closely associated with water temperature. It is also important to note that although the data presented here do not suggest a statistical increase in DNA strand breakage after HTO exposure, there is considerably more variability in the comet assay data for the 15 MBq L\(^{-1}\) treatment in November 2012. Repeated laboratory exposures of mussels to HTO (perhaps monthly over a yearly cycle) are necessary to confirm these results and fully elucidate the mechanisms behind them. Despite the well known effects of seasonality on biomarkers in field-sampled *Mytilus* spp., there is abundant room for further research on its implications for laboratory exposures using this model species.

Incidence of MN increased in mussels with higher % tail DNA (as indicated by linear regression analysis), although there was some scatter in the data. This variation is unsurprising considering that a multitude of factors (including stage of the cell cycle, repair capacity, baseline DNA damage and type of cell) control whether or not strand breaks become fixed mutations (He et al. 2000). This finding is in agreement with studies in both mammalian and aquatic organisms, which have used correlation or regression to demonstrate a relationship between these two biomarkers of genotoxicity. In rats exposed to various mutagenic pharmaceuticals (e.g. cyclophosphamide, cisplatin etc), % tail DNA, tail moment, and tail length were all able to predict incidence of MN (Mughal et al. 2010). A significant relationship was also found between comet assay data (Olive tail moment) and incidence of MN in rainbow trout cells (RTG-2) after *in vitro* exposure to arsenic compounds (Raisuddin & Jha 2004) and in a freshwater mussel species (*Perna viridis*) after exposure to B(a)P (0 - 30 \(\mu\)g L\(^{-1}\)) for up to 12 days (Siu et al. 2004b). There are also several studies from our own research group, which show very similar trends for DNA damage and incidence of MN in *Mytilus* spp., albeit without quantifying these relationships (e.g. Hagger et al. 2005b, Jha et al. 2005). The comet assay detected genotoxic effects at lower doses than the MN assay. Initially, this appears to be in contrast to the mammalian literature.
which has reported the MN assay as more sensitive (e.g. Valentin-Severin et al. 2003, Kawaguchi et al. 2010). It is, however, important to consider that little is understood about haemocyte turnover kinetics in mussels (Mayrand et al. 2005, Renwrantz et al. 2013) and MN-production is a cell-cycle dependent process (Jha 2008).

### 5.4.4 Responses at higher levels of biological organisation

Interestingly, mussels used in the July 2011 experiment do not show the low summer clearance rate demonstrated by \textit{M. edulis} from the Exe estuary (Hagger et al. 2010), despite being collected in the same time period (June - August). Clearance rate has been shown to be affected by changes in feeding regime over relatively short time periods (Widdows et al. 1979, Denis et al. 1999), so our relatively high CR values are most likely due to the two week depuration and feeding regime before exposure.

It is also interesting to note that the radioactive contaminant used here (HTO) has not produced any significant deviation from the control in terms of epithelial atrophy in the digestive gland. This is in contrast to other contaminants which cause reduced epithelial thickness. For example, Cajaraville et al. (1992) exposed mussels to three types of water accommodated fraction (WAF) from crude oils, all of which resulted in decreased MET or MET:MDR. In addition, WAF from lubricant oils caused decreased MDR (i.e. reduction in overall tubule thickness), suggesting a severely deleterious response. Similarly, mussels exposed to petroleum-derived compounds and/or Cu showed digestive gland atrophy and reduced volume density (Lowe & Clarke 1989). Such digestive gland parameters in mussels are also prone to reduction after environmental change (e.g. salinity or temperature fluctuations; ICES 2011), which suggests they are part of a generalised rather than contaminant-specific response. It is therefore especially interesting that HTO exposure for 14 days does not illicit this response, suggesting that either it isn’t stressful for the mussels or that repair mechanisms are sufficient to cope within this relatively short time period.

The literature review undertaken as part of this project has already highlighted the paucity of data on higher level effects of radiation exposure in aquatic invertebrates (Dallas et al. 2012). The novel approach used in this study, which selected endpoints in order to span many levels of biological organisation (genetic, tissue,
Chronic exposure to tritiated water physiological and whole organism) has not revealed any significant effects of HTO exposure other than genotoxicity (i.e. in CR, histopathology, attachment or SoS). Previous studies on IR-exposed aquatic invertebrates do not provide much help in interpreting this result. Several of these studies have examined potential changes in behaviour. Blue crabs showed profound changes in activity (including complete catatonia) after exposure to 40 - 640 Gy of γ radiation and freshwater snails ceased crawling when exposed to 10 Gy of X-rays (Bonham & Palumbo 1951, Engel 1967), but these doses were orders of magnitude higher than those reported here. A marine invertebrate, the goose barnacle, altered its moulting behaviour after longterm exposure to low doses of HTO (< 0.01 µGy h⁻¹; Abbott & Mix 1979), but the phylogenetic differences between Mollusca and Crustacea prohibit direct comparison, i.e. mussels do not moult. In addition, it is well accepted that early life stages (such as the goose barnacle embryo-larvae used) are more susceptible to radiation-induced effects than adult organisms (Perlowagara-Szumlewicz 1964, Ravera 1966).

Future research in this area may benefit by selecting endpoints with similar range (of biological levels) but which are more focused on radiation-specific effects. However, given the limited data availability, selection of such endpoints might be problematic. Review of the previous literature on radiation-induced effects in aquatic invertebrates (Dallas et al. 2012) suggests that oxygen consumption is the most sensitive endpoint (Alonzo et al. 2006, 2008) and would therefore be a good choice for measurement in any such study. It may also be beneficial to consider radiation-sensitive endpoints from other, more well studied groups, in particular from the mammalian literature. For example, metallothionein proteins (MTs) have been shown to have extraordinary potential to scavenge hydroxyl radicals \textit{in vitro} (Thornalley & Vašák 1985). As indirect damage via ionisation of water molecules (producing hydroxyl and superoxide radicals among many other ROS) is a primary cause of radiation-induced damage, it is likely that MTs have the potential to be radioprotective. Indeed, induction of MTs after radiation exposure is now well reported in mammalian systems (Cai et al. 2000, Cai & Cherian 2003, Fujiwara & Satoh 2013). Molecular chaperones, in particular \textit{HSP70}, have also been found to have a radioprotective effect (Calini et al. 2003). Measurement of transcriptional or proteomic expression of these and other oxidative defence molecules after radiation exposure may therefore yield interesting results in mussels. The parallel study examining transcriptional gene expression in mussels from our July 2011 experiment found significant upregulation of the cell cycle control
5. Chronic exposure to tritiated water

gene, *p53*, the radiation-specific double-strand repair gene, *rad51* and the onco-
gene, *ras*, particularly for the 1 and 15 MBq L$^{-1}$ treatments, but unfortunately
did not determine mRNA levels for any MT or HSP genes (Di 2012). The modified
comet assay (which detects oxidative base damage, as in Chapter 3) may also be
a valuable tool for detecting oxidative damage in mussels exposed to HTO.

Irrespective of radiation-induced damage, it is interesting to note that this study
is the first to report a detrimental effect on mussel attachment by the known
mutagen, EMS. Byssogenesis and attachment force have been shown to decrease
in juvenile *M. galloprovincialis* after a period of starvation, leading the authors
to suggest that there is an energetic trade off between soft tissues and byssus,
particularly under times of stress (Babarro et al. 2008). Adult mussels show no
such decrease in attachment qualities after starvation for a week (Babarro et al.
2008, Babarro & Reiriz 2010) but were significantly compromised by spawning, a
well known energetically expensive event (Babarro & Reiriz 2010). In this context,
it is possible that EMS-induced toxicity was energetically demanding enough to
reduce attachment ability. It is interesting, however, that EMS-exposed mussels
did not show a change in CR, and were not filtering at maximal reported rates,
as might be expected when resources are a limiting factor. Furthermore, HTO-
exposed mussels did not show any reduction in attachment ability, suggesting it
may be an EMS-specific (i.e. chemical) effect rather than altered energetics due
to a general stress response. The mussel byssus is a complex organ composed
of several adhesive and structural proteins (Brown 1952), and given that EMS
is a known alkylating agent there is the potential for direct alkylation of byssal
proteins to interfere with adhesion.

5.5 Conclusions

In order to avoid effects of seasonality, it was initially intended to conduct the
different experiments for this study at the same time of year, however the scope
and timescale of the project did not allow this, hence season must be treated as
a confounding factor when interpreting our results. Despite this, it is interesting
to note that this study both confirms the potential of $^3$H as a genotoxic agent in
mussel haemocytes (as in previous studies; Jaeschke et al. 2011, Jha et al. 2005)
and suggests that radiosensitivity may be yet another parameter that is affected
by seasonality in this representative marine invertebrate.
Chapter 6

Molecular and genotoxic effects in *M. galloprovincialis* exposed to tritiated water at an elevated temperature
6. Hyperthermia and tritiated water

6.1 Introduction

Chemical or radioactive contaminants do not occur in isolation; organisms are also exposed to fluctuations in biological, biotic and physico-chemical factors, including competition, other contaminants, parasite load, temperature, salinity and dissolved oxygen (Manti & D’Arco 2010). Alterations in these parameters can influence spontaneous or contaminant-induced damage. For example, low salinity has been shown to cause reduced antipredatory responses in green mussels (*Perna viridis*), especially in conjunction with hypoxia (Wang et al. 2013), and alterations from ‘normal’ salinity (20 psu) caused increased mortality in response to Cd in mysids (*Neomysis integer*; Wildgust & Jones 1998). Parasitic infection is known to have a promutagenic effect in mice exposed to aflatoxin B1 (Gentile & Deruiter 1981) and to affect both physiology and behaviour in oysters (Chambon et al. 2007). Similarly nutritional status has been shown to have significant impacts on aquatic species exposed to a range of contaminants (for review, see Lanno et al. 1989). Furthermore, the potential influence of the seasonal gametogenesis cycle on tritium-induced genotoxicity in mussel haemocytes has already been discussed in Chapter 5. As a result of these impacts, assessment of the effects of multiple stressors on biomarkers is a subject of increasing interest in both ecotoxicology and radioecology (Altenburger et al. 2012, Dallas et al. 2012). Despite this, the potential synergistic, antagonistic or additive effects of abiotic stressors with radiological contaminants have not been well characterised in aquatic invertebrates.

Temperature is an abiotic factor of particular concern when it comes to assessing the potential detrimental impacts of tritium exposure in marine species, as cooling water from nuclear institutions is one of the major sources of $^3$H to the aquatic environment. Thermal discharge from nuclear facilities is considered to be one of the most important environmental issues surrounding these establishments, second only to the release of radionuclides (Kokaji 1995). Typically NPPs or NFRPs are located close to the coast, to enable use of economical ‘once-through’ cooling systems, whereby water is drawn from the environment (sea or estuary), used for cooling, then returned to the source having increased in temperature. The amount of water needed for cooling varies with plant operation, but average values of 3 m$^3$ min$^{-1}$ megawatt$^{-1}$ have been quoted (Poornima et al. 2005). Discharged water is typically 8 - 12 °C above intake in temperate areas such as the English Channel (up to a maximum of $\sim$30 °C; Bamber 1995), but thermal plumes (with temperatures elevated by $> 1$ °C) can extend up to 10 km (Tang et al. 2003).
As a result, animals close to discharge pipes can be simultaneously exposed to radioactivity and heat. This is especially true for sessile aquatic invertebrates (e.g. mussels), as they are unable to respond behaviourally to temperature increases (i.e. by moving to cooler areas).

The IAEA first described a ‘timely need’ for research into thermal discharges from NPP/NFRPs in the 1970s (IAEA 1974) and Woodhead (1971) recommended that ‘as many ecological parameters as possible’ be studied to understand the ‘ecological effects of natural and man-made variations’ on the effects of ionizing radiation. Despite this, the majority of studies examining the thermal effects of nuclear effluents do so without any radioactive contaminant and take an ecological rather than ecotoxicological standpoint, i.e. they have assessed changes in community structure of fish (Hillman et al. 1977, Teixeira et al. 2009) or phytoplankton (Poornima et al. 2005). Furthermore, climate change is one of the biggest issues facing environmental protection today. With sea surface temperatures forecast to rise by 0.5 - 3.5 °C in the next 30 - 100 years (IPCC 2007), determining the interaction of elevated temperature with radiological exposure has never been more relevant (both in terms of altered effects and reduced capacity of aquatic organisms to cope with nuclear thermal pollution; Bamber 1995).

In general, it has been agreed that radiation-induced damage is amplified at higher temperatures because metabolic rate is increased (Bacq & Alexander 1961, Blaylock 1974, Gumrich et al. 1986). It is therefore of particular interest to investigate the impact of radiation/heat co-exposure on poikilotherms, where metabolic rate is a direct consequence of external temperature (Buschini et al. 2003). When investigating the effect of irradiation on the morphology of developing pinfish (*Lagodon rhomboides*), White (1969) noted that radiation, salinity and temperature produced significant interactions. It has been well reported that an increase in temperature can cause an increase in radiosensitivity in fish (Angelovic et al. 1969, Blaylock & Mitchell 1969, Egami 1969). The available data for mortality in aquatic invertebrates is consistent with this. For snails (*Physa heterostropha*) a rearing temperature of 30 °C decreased lifespan after irradiation by two thirds compared to 10 °C (Ravera 1967), but only at the highest dose studied (1100 Gy). The hatchability of *Artemia salina* eggs was negatively affected by 60Co gamma radiation at higher temperatures (Iwasaki 1959), and irradiated individuals kept at 32 °C survived for a quarter of the time compared to those at 15 °C (Suyama & Iwasaki 1976).
For factors other than mortality, determining the interaction of radiation with temperature in aquatic invertebrates is not as straightforward, although an increase in radiation damage at higher temperatures has been demonstrated in some instances. Oyster (Crassostrea gigas) embryos exposed to waterborne tritium and $^{65}$Zn and reared at 28 °C developed faster but had an increased frequency of abnormalities and decreased survival, compared to those reared at 20 and 24 °C (Nelson 1971). In aquatic snails of the genus Physa, both Ravera (1967) and Cooley (1973) reported that higher temperatures caused a more severe effect on fecundity. Engel et al. (1971) concluded that the effect of irradiation on ionic regulation in Callinectes sapidus was influenced by the crabs’ environment before and after irradiation. It was suggested that temperature had more of an effect than salinity, however the exact impact of temperature was difficult to determine due to complex interactions.

To date, there is no literature on the effects of radiation and hyperthermia in
mussels, either at molecular or higher levels of biological organisation. There is, however, a growing body of work on molluscs exposed to elevated temperatures alone or in combination with chemical contaminants (e.g. Bayne 1976, Anestis et al. 2007, Mubiana & Blust 2007, Baines & Fisher 2008). Species differences and thermal history are important to consider in such studies. For example, a maximum temperature of 27 °C for adult survival has been reported in *M. edulis*, in contrast to 70% survival of *M. galloprovincialis* over 30 d at 28 °C; Bayne 1976, Anestis et al. 2007). It is also necessary to consider length of exposure as, for example, *M. edulis* can acclimatise (oxygen consumption returns to ‘normal’ levels) to temperatures between 5 and 20 °C within 14 d (Bayne 1976), although this is also likely to vary with species and thermal history. In terms of chemical contaminants, the bioaccumulation of non-essential metals (Cd and Pb) in *M. edulis* increased at higher temperatures (Mubiana & Blust 2007) and biokinetic modelling predicted increased accumulation of dietary Ag, Am and Zn in the same species at low temperatures (2 °C; Baines & Fisher 2008). Furthermore, the toxicity of Cu to developing *M. trossulus* embryos increased at temperatures > 15 °C (Yaroslavtseva & Sergeeva 2007).

### Table 6.1. Summary of the major characteristics and functions of proteins encoded by genes targeted in this study (Hofmann et al. 2002).

<table>
<thead>
<tr>
<th>Protein(s)</th>
<th>Characteristics</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metallothioneins</td>
<td>Low molecular weight, cysteine rich</td>
<td>Metal binding, radical capture</td>
</tr>
<tr>
<td>Heat shock proteins</td>
<td>Six highly conserved classes, based on molecular weight (HSP33, 60, 70, 90, 100 and the small HSPs)</td>
<td>Intra-cellular chaperones (assist with protein folding, prevent aggregation, aid in transport), antigen binding and presentation, vascular relaxation</td>
</tr>
<tr>
<td>p53</td>
<td>393 amino acids(^a), seven domains(^a), including transcription-activation, proline rich and DNA-binding</td>
<td>Cell cycle regulation (tumour suppression), DNA repair, initiation of apoptosis</td>
</tr>
<tr>
<td>RAD51</td>
<td>339 amino acids(^a), ATP-dependent DNA binding activity, DNA-dependent ATPase activity</td>
<td>DNA repair (homologous recombination)</td>
</tr>
</tbody>
</table>

\(^a\) in humans.
Molecular biomarkers of heat stress in *Mytilus* spp. are useful for elucidating mechanisms behind temperature effects. Due to the highly conserved nature of many fundamental mechanisms, molecular approaches also provide synergy between models of environmental and human health (Dixon et al. 2002). This type of approach has revealed complex effects of hyperthermia in mussels, such as higher filtration rates required by *M. galloprovincialis* for optimal gas exchange in warmer, more hypoxic water, discovered as a result of increased tubular protein production (Anestis et al. 2007). In particular, molecular chaperones, such as heat shock proteins (HSPs), are induced by elevated temperature. These stress proteins are transcriptionally activated via heat shock factors, phosphorylation of stress-activated p38 mitogen-activated protein kinase (p38 MAPK) and cJun-N-terminal kinases (JNKs; Fig. 6.1), and function to stabilise the folding of other cellular proteins or aid in refolding of damaged proteins (Table 6.1; Hofmann et al. 2002). During stress, HSP70 also interacts with HSC70 (a constitutively expressed heat shock cognate) preventing it from disassembling and unfolding damaged proteins for translocation across membranes (i.e. preventing their export; Chapple et al. 1997). Increased expression of *hsp70* and/or *hsp90* genes has been reported in *M. chilensis* exposed to elevated temperatures in the field (Núñez-Acuña et al. 2012) and in *M. galloprovincialis* and *M. trossulus* at 24, 28, 32 and 35 °C in the laboratory (Franzellitti & Fabbri 2005, Lockwood et al. 2010). Increased expression of the related proteins, HSP70 and HSP72, has also been reported in the first 48 h of laboratory exposures to elevated temperature (Chapple et al. 1997) and in response to seasonal temperature fluctuations in the field (Ioannou et al. 2009).

Production of potentially damaging ROS may be increased at higher temperatures (Lushchak 2011). It is therefore unsurprising that low molecular weight metallothionein proteins which scavenge ROS, such as MT20, have also been shown to increase in mussels after heat shock, peaking after 2 h at 30 °C (Gourgou et al. 2010). It is well established that radiation generates ROS in biological samples (Riley 1994). Consequently, antioxidant defence genes, such as those for metallothioneins or superoxide dismutase (SOD), may be upregulated during radiation exposure. For example, in human carcinoma cells (MCF-7) overexpression of Mn-SOD has a radioprotective effect, although this was attributed to upregulation of radiation-adaptive responses (via a downstream cascade) in addition to direct antioxidant potential (Guo et al. 2003). The importance of metallothionein proteins in radioprotective effects has also been explored recently using double MT
knockout mice (i.e. mice with non-functional \textit{MT-I} and \textit{MT-II} genes). Such animals show increased carcinogenesis when exposed to contaminants, including X-rays as well as chemical toxicants such as cisplatin and B(a)P (Fujiwara & Satoh 2013).

The \textit{rad51} gene encodes a protein which is involved in the initial stages of homologous recombination repair (HRR) following double strand breaks (DSBs, Table 6.1; Masson & West 2001, Mladenov et al. 2013). After resection of the exposed 3’ ends and attachment of replication protein A (RPA), RAD51 is recruited and forms a protein-nucleic acid filament. This nucleoprotein is essential for locating and identifying suitably similar sequences for ‘strand invasion’, the next step in repair of DSBs by HRR (Fig. 6.2). Strand invasion requires the displacement of one strand of the homologous DNA molecule, forming a D-loop, after which DNA polymerase extends the broken strand using the homologous template. At this stage, the final steps of HRR occur typically by one of two pathways; double strand break repair (DSBR) or synthesis dependent strand annealing (SDSA; Mladenov et al. 2013). As DSBs are indicative of physical damage to DNA (i.e. radiation rather than chemical contaminants), expression of the \textit{rad51} gene is thought to be diagnostic of radiation exposure and has been shown to be upregulated in mussels exposed to radiation, including HTO (AlAmri et al. 2012, Di 2012). There is, however, no data on the response of this gene or its related protein to elevated temperature in mussels.

The tumour suppressor gene, \textit{p53}, is another interesting candidate for investigation in elevated temperature/radiation exposures, because of both its own anti-oncogenic functions and its interactions with other genes. Wild-type p53 protein is often regarded as ‘the guardian of the genome’ and its gene is the most frequently mutated in human cancers (Greenblatt et al. 1994). p53 exerts these effects via cell cycle regulation (i.e. cells are arrested at the G1 checkpoint until DNA is repaired; Yin et al. 1992), initiation of apoptosis and regulation of DNA repair (Table 6.1). It is well known that p53 activates certain pathways of DNA repair, such as base excision repair (BER; Zhou et al. 2001), but it also has a key role in repression of other processes. For example, p53 binds to and inhibits RAD51 and the related protein RAD54, thereby inhibiting HRR, which if uncontrolled is oncogenic (Buchhop et al. 1997, Arias-Lopez et al. 2006). Interestingly, the inhibition of RAD51 and HRR by p53 has been shown to occur via transcriptional and non-transcriptional mechanisms (Bertrand et al. 2004, Arias-Lopez et al. 2006), illustrating the complexity of these interactions. To add further weight to its in-
1. Double strand breakage
2. Resection
3. RAD51 attachment and nucleoprotein formation
4. D-loop formation
5. Extension
6. SDSA or DSBR

Figure 6.2. Simplified diagram illustrating the initial steps of homologous recombination repair following double strand breaks, including resection, binding of replication protein A (RPA) to the exposed 3' ends, recruitment of RAD51 to form nucleoproteins (RAD51 focii), D-loop formation on the homologous strand and extension by DNA polymerase (DP), before either double strand break repair (DSBR) or synthesis-dependent strand annealing (SDSA). Adapted from Mladenov et al. (2013).
clusion in this study, *p53* was previously shown to be upregulated during HTO exposure (July 2011 experiment of Chapter 5; Di 2012) and has been potentially linked to *hsp90* expression in *Mytilus* sp. (Pantzartz et al. 2010).

### 6.1.1 Objectives and hypotheses

Against the backdrop of the above information, this study was designed to fulfil the following aims and objectives:

(a) to use tissue-specific accumulation of $^3$H in mussels to determine the effects of elevated temperature on radiation dose (using the ERICA tool for dose estimation);

(b) to assess the impact of elevated temperature on the genotoxicity of HTO to mussel haemocytes, using the modified comet assay to determine oxidative DNA damage;

(c) to evaluate the transcription profile of key radiation and heat shock genes (*hsc70, hsp70, hsp90, mt20, p53* and *rad51*) to elucidate potential mechanisms behind temperature-effects;

The hypothesis for the current study was that hyperthermia would increase the radiation dose and genotoxicity of tritiated water exposure in mussels. It was also hypothesised that such enhanced effects would necessitate the upregulation of stress response genes.

### 6.2 Materials and methods

#### 6.2.1 Radiation protection

The experiments in this chapter were carried out within Plymouth University’s Consolidated Radioisotope Facility (CORiF) or in controlled spaces, under the guidance of the Radiation Protection Supervisor (RBS) and Radiation Protection Assistant (RPA). All necessary precautions were taken to minimise $^3$H exposure to experimenters and colleagues. Local rules and other radiation protection documents are included in Appendix B.
6. Hyperthermia and tritiated water

Mussels acclimatised in 2 L beakers (4.5 L⁻¹)

48h

In vivo exposure 4 beakers treatment

Mussels depurated 2 weeks

25°C

Control 15 MBq L⁻¹ HTO

15 MBq L⁻¹ HTO 40 µg L⁻¹ Cu

15°C

Gene expression

n = 9

0 h

1 h

12 h

3 d

7 d

Accumulation

Gene expression

Comet assay

n = 9

Figure 6.3. Experimental design for exposure of mussels to tritiated water at an elevated temperature.
6.2.2 Experimental design and mussel exposure conditions

Adult mussels (50.40 ± 0.36 mm) were collected from Trebarwith Strand in April 2013, transported to the lab and depurated as described in section 2.3.

After depuration mussels were transferred to 20 glass beakers with filtered seawater (<10 µm) at a density of 4.5 mussels L\(^{-1}\) and allowed to acclimatise for 48 h, as in previous experiments. Beakers were randomly allocated to one of 5 treatment groups - a seawater control at 15 °C, a seawater control at 25 °C, 15 MBq L\(^{-1}\) HTO at 15 °C, 15 MBq L\(^{-1}\) HTO at 25 °C, or a positive control (40 µg L\(^{-1}\) CuSO\(_4\); D’Agata et al. 2013). HTO was obtained and stock solutions prepared as in Chapter 5. The 15 MBq L\(^{-1}\) activity concentration was selected as it was the only concentration to consistently show genotoxic effects in previous experiments (see Chapter 5).

Mussels were exposed to these conditions for 7 d. As in previous experiments, mussels were fed every 72 h (i.e. on day 0 and day 3) with a 100 % water change 2 h afterwards (see Chapter 2). The 7 d exposure duration was based on previous work with mussels exposed to tritiated water (Jaeschke et al. 2011) or chemical genotoxins (methane methyl sulfonate and cyclophosphamide; Canty et al. 2009). This shorter overall exposure also allowed logistically for greater temporal resolution on day 1 (which is particularly important to produce a time-course for gene expression) whilst still encompassing a moderately chronic overall timescale.

Water quality parameters during this experiment were salinity 34.27 ± 0.31, pH 8.33 ± 0.38 and dissolved oxygen 91.45 ± 3.74 %. Measured temperatures were close to nominal values, at 15.34 ± 0.51 and 15.40 ± 0.37 °C for the 15 °C control and HTO treatments, respectively, 25.61 ± 0.43 and 25.58 ± 0.32 °C, respectively, for the control and HTO-treated groups at the higher temperature and 15.37 ± 0.48 °C for the Cu positive control group.

6.2.3 Sampling procedures

Sampling took place after 0, 1, 12, 72, and 168 h exposure. At the 12, 72 and 168 h time points, 9 mussels (i.e one beaker) had their haemolymph extracted (as described in section 2.5), and were then dissected into their individual organs for LSC (gills, mantle, digestive gland, adductor muscle, foot and byssus). As
in Chapter 5, remaining tissues were pooled and classified as ‘other’ to ensure complete counting. Haemolymph samples were stored on ice until use in the enzyme-modified comet assay. During dissection of mussels, small (∼5mm²) pieces of gill were also removed, weighed and flash frozen in liquid nitrogen. These gill samples were stored at -80 °C for later RNA extraction and gene expression analysis. Additionally, at the 0 and 1 h time points 9 mussels (i.e. one beaker) were sampled for gene expression only. Gill was selected for transcriptional analysis as it has previously been shown to exhibit the greatest induction of HSPs in response to heat stress in *M. edulis* (Chapple et al. 1997). The experimental design for this chapter is illustrated in Fig. 6.3.

6.2.4 Liquid scintillation counting of water and mussel tissues

Water samples (100 µL) were taken daily and processed for LSC as described in section 2.16. Mussel tissues were processed for LSC according to the processes outlined in Chapters 2 and 5. Concentration factors (CF) were calculated as in Chapter 5, according to equation 5.4. As previous experiments had not indicated any change in the proportional distribution of $^3$H between tissues (Chapter 5, section 5.3.1), this analysis was not carried out.

6.2.5 Dose calculations

Dose calculations were performed with the ERICA tool, using total tissue activity concentrations (as in Chapter 5, see section 5.2.5.2). Statistical differences between dose rates for different temperatures and timepoints was investigated using a two-way ANOVA and Tukey’s post hoc tests. As whole organism total activity concentration, dose rate and total dose are mathematically related (i.e. by the ERICA tool algorithms and by a factor of time, respectively) significance is only reported for total dose, but is equivalent between the three parameters.
6.2.6 Enzyme-modified comet assay to determine oxidative DNA damage

Haemolymph samples were stored on ice and 200 µL used for the enzyme-modified comet assay as in section 2.10, except only two slides were produced per sample - one with the buffer control, and one with Fpg. Slides were coded and randomised to ensure scoring was blind.

6.2.7 Determination of relative transcriptional expression of selected genes

Extraction of total RNA, reverse transcription and qPCR were performed on these samples as described in Chapter 2 (section 2.11), except that the target genes were selected based on the literature on heat shock and/or radiation exposure, as described above. For these reasons, the target genes were \textit{hsc70}, \textit{hsp70}, \textit{hsp90}, \textit{mt20}, \textit{p53} and \textit{rad51}. As before, \textit{atub} and \textit{ef1} were also used as reference genes. Details of the primers used in this Chapter are included in Table 6.2.

Quantification of relative expression was performed as described in section 2.11.4, using REST2009 software (Pfaffl et al. 2002).

6.2.8 Statistical Analyses

Comet data were processed as described in section 2.18. Linear regression analyses were performed for all combinations of gene/ timepoint with % tail DNA and \(p\)-values were adjusted for multiple comparisons (Holm’s sequential Bonferroni adjustment). As in previous chapters, the gene expression parameter used for relationship analysis was \(C_q\) normalised for reference gene (i.e. \(\Delta C_q = C_q[\text{GoI}] - C_q[\text{ef1}]\)). All other data were normal and were analysed using ANOVA.
Table 6.2. Genes and primers used for qPCR on mussels exposed to tritiated water and elevated temperature.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha tubulin ((atub)^a)</td>
<td>DQ174100</td>
<td>5'-TTGCAACCCATCAAGACCAAAG-3'</td>
<td>5'GTCAGACGGGCTCTCCTGT-3'</td>
</tr>
<tr>
<td>Elongation factor 1 ((eif1)^a)</td>
<td>AF063420</td>
<td>5'-CACCACGAGTCTCTCCCAGA-3'</td>
<td>5'-GCTGTCACCACAGGACCAATCC-3'</td>
</tr>
<tr>
<td>Heat shock chaperone 70 ((hsc70)^b)</td>
<td>AF172607</td>
<td>5'-GGGTGGGTAACATTTTGATG-3'</td>
<td>5'-GCCGTTGAAAAAGTCCCTGAA-3'</td>
</tr>
<tr>
<td>Heat shock protein 90 ((hsp90)^c)</td>
<td>AJ625655</td>
<td>5'-TCAGTGATGATCCTAGATTAGCA-3'</td>
<td>5'-CGTTTCCTCCTCCTCCATGTAAC-3'</td>
</tr>
<tr>
<td>Heat shock protein 70 ((hsp70)^d)</td>
<td>AF172607</td>
<td>5'-CCCTTTTCCTCAGACAAAGCA-3'</td>
<td>5'-AATGTCCTCTCATGTTGCTTC-3'</td>
</tr>
<tr>
<td>Metallothionein 20 ((mt20)^d)</td>
<td>AJ577131</td>
<td>5'-GACGCCCTGCAAATGTCGAAG-3'</td>
<td>5'-TCGGACCAGTGCGGTACAT-3'</td>
</tr>
<tr>
<td>p53 anti-oncogene ((p53)^e)</td>
<td>DQ158079</td>
<td>5'-CACAATCTCTCTACTAACTGTCAGA-3'</td>
<td>5'-TCCGCTCCTCCTACATGAC-3'</td>
</tr>
<tr>
<td>rad51 ((rad51)^f)</td>
<td>FJ518826</td>
<td>5'-TGGCATTGAGACTGGGTCAA-3'</td>
<td>5'-CCTTCACCTCACCACCATATC-3'</td>
</tr>
</tbody>
</table>

\(^{a}\) Ciocan et al. 2011; \(^{b}\) Franzellitti & Fabbri 2005; \(^{c}\) Banni et al. 2011; \(^{d}\) Cefas (unpublished); \(^{e}\) Ciacci et al. 2011; \(^{f}\) AlAmri et al. 2012
Figure 6.4. Time-dependent tritium accumulation in mussel tissues after exposure to tritiated water (15 MBq L$^{-1}$) at 15 °C. (A) Organically-bound tritium (OBT) concentration i.e. that remaining after extraction of tissue free water tritium (TFWT) by freeze drying; and (B) total activity concentration, i.e. OBT + TFWT normalised for wet tissue weight. Data are presented as means ± one SE. Significant difference from byssus at the same timepoint is indicated by #, whereas differences from the equivalent tissue and timepoint at 25 °C are indicated by *. Different lowercase letters indicate significant differences between the same tissue and temperature at different timepoints ($p < 0.05$).
Figure 6.5. Time-dependent tritium accumulation in mussel tissues after exposure to tritiated water 15 MBq L\(^{-1}\)) at 25 °C. (A) Organically-bound tritium (OBT) concentration i.e. that remaining after extraction of tissue free water tritium (TFWT) by freeze drying; and (B) total activity concentration, i.e. OBT + TFWT normalised for wet tissue weight. Data are presented as means ± one SE. Significant differences from byssus at the same timepoint are indicated by #, whereas differences from the equivalent tissue and timepoint at 15 °C are indicated by *. Different lowercase letters indicate significant differences between the same tissue and temperature at different timepoints (\(p < 0.05\)).
Figure 6.6. Concentration factors (CF) for mussels exposed to tritiated water (15 MBq L\(^{-1}\)) for 7 days, at either 15 or 25 °C. (A) Whole organism; and (B) tissue-specific. CFs were calculated from total tissue and water activity concentrations, according to equation 5.4. Error bars are ± one SE. Asterisks indicate significant differences from the same tissue at 25 °C, whereas time-dependent effects are indicated by lowercase lettering. Means which do not share a letter are significantly different (\(p < 0.05\)).
6.3 Results

6.3.1 Tritium in water and mussel tissues

Tritium activity concentrations in water showed good agreement with nominal values (> 90% of expected; Table 6.3). Control water samples’ activity was below the LOD.

In general, the same trend as in Chapter 5 was observed for accumulation - total activity concentration was approximately two to three times that of OBT activity concentration (at both 15 and 25 °C). One notable exception from this rule was byssus, where OBT activity concentration was higher than total activity concentration. Temporally, total activity concentrations were slightly higher in the mussels exposed at 15 °C after 12 h, but only significantly so for byssus ($p < 0.05$).

Byssus had the highest concentration of $^3$H (in terms of OBT and total) for all timepoints at both temperatures. The order in which other tissues accumulated $^3$H varied with time and temperature, but in general digestive gland, gill and foot showed higher concentrations than mantle, muscle and other (Figs. 6.4 and 6.5).

At 15 °C there was no variation in activity concentration with time, whereas for 25 °C byssus activity concentration increased from 12 to 72 h (OBT and total) and then decreased again (OBT only; Fig. 6.5).

Concentration factors for $^3$H in mussel tissues varied significantly according to treatment, tissue and temperature. In terms of the whole organism CFs, there were significant differences between mussels at 15 and 25 °C at all three timepoints, but the magnitude and order of this differences varied across time. At 12 h, mussels at 15 °C had higher CFs than those at 25 °C, whereas at 72 and 168 h the reverse was true (Fig. 6.6 A). In contrast, temperature-dependent tissue-specific differences in CF were only significant at 12 h, where byssus, digestive gland, foot and other showed increased accumulation relative to 25 °C. The only time-dependent tissue effects were for foot, gill and mantle between 12 and 72 h at 25 °C (Fig. 6.6 B).

6.3.2 Dose calculations

Dose rates estimated using the ERICA tool ranged from 10.94 to 18.72 μGy h$^{-1}$ giving total doses between 0.13 and 2.75 mGy. There was significant variation in
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Table 6.3. Whole organism dose estimates for mussels exposed to tritiated water at either 15 or 25 °C, using the ERICA tool and whole organism total activity concentrations (means ± one SE, n = 9).

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time (h)</th>
<th>Water activity concentration (MBq L$^{-1}$)$^a$</th>
<th>Dose rate (µGy h$^{-1}$)</th>
<th>Total dose (mGy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 °C</td>
<td>12</td>
<td>14.56</td>
<td>18.49 ± 0.16</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>14.00 ± 0.36</td>
<td>15.58 ± 0.18</td>
<td>1.12 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>14.32 ± 0.65</td>
<td>15.13 ± 0.19</td>
<td>2.54 ± 0.03</td>
</tr>
<tr>
<td>25 °C</td>
<td>12</td>
<td>14.13</td>
<td>10.94 ± 0.08</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>14.21 ± 0.45</td>
<td>18.72 ± 0.10</td>
<td>1.35 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>14.28 ± 0.67</td>
<td>16.35 ± 0.15</td>
<td>2.75 ± 0.03</td>
</tr>
</tbody>
</table>

$^a$ Means ± one SE (from daily water samples, n = number of days exposure). Asterisks indicate significant differences from 25 °C. † indicates significant differences from the previous timepoint at the same temperature ($p < 0.001$).

At 15 °C there was significant induction of DNA strand breaks at every timepoint for the Cu positive controls. Although % tail DNA was elevated for HTO exposed mussel haemocytes at each timepoint, it was only significantly so after 168 h ($p < 0.05$; Fig. 6.7A) and did not show any significant evidence of oxidative base damage (i.e. in Fpg-treated slides). At 25 °C there was significant induction of strand breakage at each time point in the Fpg-treated HTO exposed samples ($p < 0.05$), but only in comparison to the Fpg-treated controls, not the equivalent HTO buffer treatments. Significant induction of DNA damage in the buffer treated HTO-exposed mussel haemocytes was observed faster than at 15 °C - after 72 and 168 h (Fig. 6.7B).

6.3.4 Transcriptional expression of selected genes

PCR efficiencies were atub 1.499, ef1 1.863, hsc70 1.869, hsp70 1.756, hsp90 1.665, mt20 1.804, p53 1.760 and rad51 1.736. As the efficiency of atub was considerably lower than that of the other genes, it was discarded and ef1 ($C_q$ variability: 18.95
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Figure 6.7. DNA strand breaks (as measured by the comet assay i.e. % tail DNA) in mussel haemocytes after exposure to (A) in vitro hydrogen peroxide for 1 h (validation, April 2013); (B) tritiated water (15 MBq L$^{-1}$) or copper (40 µg L$^{-1}$) for 7 days at 15 °C; or (C) tritiated water (15 MBq L$^{-1}$) for 7 days at 25 °C. Significant differences from the equivalent control (at the same timepoint) are indicated by * and from the equivalent buffer treatment by § ($p < 0.05$).
± 0.80) was used as a single normalising gene.

In the early stages of exposure (1 - 12 h) expression patterns were very similar in the HTO and Cu treatments at 15 °C, before diverging at 72 h (for all six genes of interest). Both treatments showed a dramatic increase in the transcription of hsc70 at 12 h ($p < 0.0001$). The 15 °C treatment also induced significant upregulation of hsp90 and mt20 after 1 h ($p < 0.0001$), but these effects were gone by 12 h. After 72 h, expression of all genes (except hsc70) was significantly upregulated for the 15 °C HTO treatment compared to the control, and for hsp70, hsp90 and mt20 in comparison to 1 h. Both Cu and HTO (15 °C) also showed significant downregulation after 168 h, although this varied by gene (see Fig. 6.8 A for details). In contrast, there was much less variation in the transcriptional expression of the six target genes in the 25 °C HTO treatment. Downregulation of rad51, hsc70, mt20, and hsp70 was significant at 1, 12, 72 and 168 h, respectively ($p < 0.05$). Statistical analysis also revealed significant decreases in expression compared to the 15 °C HTO treatment at 12 h (hsc70), 72 h (hsp70, hsp90, mt20, and p53) and 168 h (hsp90 and p53; $p < 0.01$).

Linear regression analyses revealed significant correlations between DNA damage and the two genes associated with DNA repair (p53 and rad51), although the shape of these relationships varied with time (Fig. 6.9). After 72 h, only p53 could be predicted from DNA damage, showing a negative correlation according to the formula $P53 = (-0.12 \times \% \text{ tail DNA}) + 12.79$ ($R^2 = 0.21$, $p < 0.05$). By 168 h, p53 was positively correlated with % tail DNA, $P53 = (0.19 \times \% \text{ tail DNA}) + 3.49$ ($R^2 = 0.22$, $p < 0.05$), as was rad51, $RAD = (0.22 \times \% \text{ tail DNA}) + 10.85$ ($R^2 = 0.23$, $p < 0.01$).

### 6.4 Discussion

This study has illustrated the interactive effects of temperature and time on tritium accumulation and dose in mussels, which is especially pertinent in the context of rising sea temperatures and thermal pollution from nuclear institutions. It has also revealed a temporal shift in tritiated water induced genotoxicity, which occurs faster at higher temperatures, in line with our understanding of radiosensitivity and metabolism in ectotherms. Furthermore, changes in the transcriptional expression of key genes quantified here (in particular hsp70, hs90, mt20, p53 and
Figure 6.8. Relative expression ratios (RER) of six genes (*hsc70*, *hsp70*, *hsp90*, *mt20*, *p53* and *rad51*) in gill tissue after exposure to control seawater at 25 °C (A); 15 MBq L⁻¹ HTO at 15 °C (B); 15 MBq L⁻¹ HTO at 25 °C (C) or 40 µg L⁻¹ Cu at 15 °C (D) over 7 days. Data are presented as RER values (i.e. normalised for the reference gene [ef1] and the 15 °C control) ± 95 % confidence intervals. The dashed grey line indicates no change in expression, values above it indicate upregulation, and those below it downregulation. Significant differences (calculated using REST 2009 software; \( p < 0.05 \)) from the equivalent temperature control (*), 15 °C HTO treatment (§) and 1 h timepoint (#) are also illustrated.
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Figure 6.9. Linear regression analyses of DNA strand breakage and gene expression after exposure to control seawater and tritiated water (15 MBq L\(^{-1}\)) at 15 or 25 \(^{\circ}\)C. (A) % tail DNA and \(p53\) expression at 72 h; (B) % tail DNA and \(p53\) expression at 168 h; and (C) % tail DNA and \(rad51\) expression at 168 h. Data are median % tail DNA (of 100 cells) and \(\Delta C_q\) values (\(C_q\) [GoI] - \(C_q\) [ef1]) for individual mussels across all treatments (control seawater, 15 MBq \(\text{L}^{-1}\) HTO and \(40 \mu\text{g L}^{-1}\) Cu). All % tail DNA values are for haemocytes and are buffer-treated (not Fpg). Transcription of \(p53\) or \(rad51\) is as \(\Delta C_q\), i.e. normalised to the housekeeping gene, \(ef1\). The blue line indicates fitted values and dashed red lines represent 95% confidence limits.
suggest that this early genotoxicity may be the result of compromised defence mechanisms.

6.4.1 Tissue-specific tritium accumulation and dose estimation

The concentration of OBT in mussel byssus after exposure to 15 MBq L\(^{-1}\) during this experiment was similar to that of mussels exposed to the same activity concentration in the November 2012 experiment of Chapter 5. The current study showed a slight decrease in OBT concentrations with time (albeit not significantly), which is also reflected in the slightly lower byssus value after 14 days of exposure in the earlier work (see Chapter 5, section 5.3.1). The other tissues were somewhat lower in their OBT concentrations at all timepoints, though this may just be a function of shorter exposure duration (i.e. 12, 72 or 168 h instead of 14 d) and corresponding lower total doses. Interestingly, the tissue-specific pattern of \(^3\text{H}\) accumulation was different between the current experiment and that carried out in November 2012, with the current data indicating a preference for accumulation in the byssus that was not previously apparent. Given the different sampling times in the two studies, it is difficult to say whether this is a true difference, or simply temporal variation (i.e. by 14 days exposure the enhanced byssus accumulation may have decreased).

It is likely that the initial spike in whole organism CFs at 15 °C is a consequence of the large peaks in tissue-specific CF for digestive gland and byssus at this time. However, the biological cause of these peaks is less obvious. As \(^3\text{H}\) rapidly equilibrates with suspended sediment particles (Turner et al. 2009), one possible explanation is that the initial dampening of CF at 25 °C is related to reduced consumption of such material. This idea is supported by the digestive gland peak for 15 °C, suggesting food intake is an important source of \(^3\text{H}\) at this temperature/timepoint. Previous authors have reported that \textit{Mytilus} individuals acclimatised to 15 °C and then exposed to > 20 °C respond by reducing clearance rate (CR) with only partial acclimatisation at 25 °C (e.g. Theede 1963, Bayne 1976). Quantification of CR (as in chapters 3 and 5) during combined heat shock and HTO exposure could potentially address this question. Regardless of the biological cause, the variation in CFs with time and temperature adds further weight to the idea that \(^3\text{H}\) accumulation in marine mussels is a dynamic and complex
6. Hyperthermia and tritiated water

process with many contributing factors (see Chapter 5).

Despite the variation in tissue-specific accumulation and CFs, there were clear effects of temperature on whole organism activity concentration and therefore dose, although these were dependent on exposure time. At 12 h, exposure to HTO at 15 °C gave a dose 1.7 times higher than that at 25 °C. In contrast, at 72 and 168 h total dose was respectively 1.2 and 1.1 times higher at 25 °C. There is a limited amount of literature with which to compare our results, as to our knowledge this is the first study to look at the combined effects of temperature and time on the effects or accumulation of $^3$H in an aquatic invertebrate. However, Cd exposure in the oyster, *Crassostrea gigas*, resulted in linear accumulation increasing with temperature over 45 days (0.10, 0.53 and 0.56 µg Cd g$^{-1}$ dry mass d$^{-1}$ for 12, 20 and 28 °C; Cherkasov et al. 2007). Where temperature effects have been observed for metal accumulation in mussels, they have been attributed to changes in solution chemistry and physical kinetics, thereby increasing uptake with increased heat (Mubiana & Blust 2007). These factors are thought to be less important with tritium exposure, due to the chemical similarities of $^3$H with H. However, isotopic enrichment of $^3$H in biopolymers, as a result of the preference of $^3$H for weak hydrogen bridges, has been described (Baumgartner & Kim 2000, Baumgärtner et al. 2001). Although, theoretically there is the potential for increased energy (i.e. heat) to weaken hydrogen bridges (Khan 2000) and alter this enrichment, it seems unlikely that this would occur at 25 °C.

6.4.2 Effects of elevated temperature on genotoxicity of tritium to mussel haemocytes

As discussed in Chapter 5, there is much published research on seasonal variation in mussel biomarkers (see Table 5.4), for which temperature and gonadal maturity are the main attributed variables. In terms of genotoxicity, haemocytes of a freshwater mussel (*Dreissena polymorpha*) showed increased DNA damage (as measured by the comet assay) after *in vitro* exposure to sodium hypochlorite and increased temperature (peaking at ~27 % tail DNA after 1 h at 28 °C; Buschini et al. 2003). The temperature-dependent effects reported here took longer to appear, becoming evident only at 72 h. This may be due to the different mechanisms of action of the different stressors used (chemical vs. radiological). Differences between freshwater and marine mussel physiology, different thermal histories of
the animals or the thermal tolerances of these two species may also cause variation in this response. Along this line, it would be interesting to assess the same end-points in hyperthermic, tritium-exposed *M. edulis*, a closely related species that is less thermally tolerant (Bayne 1973). Any species-specific differences would be especially interesting as although the current mussels have been verified as *M. galloprovincialis* (the Mediterranean blue mussel) they are from the north Cornish coast, where temperatures are significantly lower than the Mediterranean. Thus, the *Mytilus* species complex potentially represents an excellent opportunity to look at both the mechanistic and evolutionary basis of temperature effects on radiation toxicity, similar to recent studies on chemical contaminants (Cheung et al. 2006, Dallas et al. 2013).

Future research might also examine a biomarker of genotoxic effect (rather than exposure, as in the comet assay), such as induction of micronuclei which we have shown is closely correlated with comet assay data (see Chapters 3 and 5). To this end, it is interesting to note that elevated water temperature (without irradiation) has been shown to increase the incidence of micronuclei in mussels (Brunetti et al. 1992).

This is the first description of a significant predictive relationship between % tail DNA (from the comet assay) and *rad51* expression in mussels, although a similar trend was reported by AlAlmri et al. (2012). Given the role of *rad51* in HRR and previous demonstration of its upregulation in irradiated mussels, this association is unsurprising (Masson & West 2001, AlAmri et al. 2012). It is tempting to use this correlation to suggest that most of the strand breaks caused by HTO in this study were DSBs, however it is important to note that there is considerable variation in the data, which might be explained by single strand breaks (SSBs). Indeed, *p53* is known to stimulate BER (Zhou et al. 2001), so the observed p53 upregulation at 72 h (for 15 °C HTO) may be in response to SSBs. It is particularly necessary to fully characterise the nature of strand breaks caused by HTO exposure in mussels as DSBs are specifically caused by high LET radiation, i.e. α particles (Natarajan et al. 1993), whilst tritium is a β emitter. Having said this, tritium’s β particles are higher energy than other β emitters and have been predicted to cause DSBs (Chen 2012). The data presented here for *rad51* expression support this idea. Nevertheless, investigation of genes associated with SSB repair processes, such as BER and nucleotide excision repair (NER), are highly recommended for future studies on HTO exposure in mussels. Although the relationship between % tail DNA and *p53* is more complicated (a negative correlation at 72 h and a positive
6. Hyperthermia and tritiated water

one at 168 h), it is still significant at both timepoints and is easily explained when looking at the data (at 72 h, \( p53 \) is repressed where DNA damage is highest - i.e. 25 °C HTO). Together, these results add further weight to the idea that DNA damage may provide an indicator of other biological effects.

In terms of future studies on the links between genotoxic/transcriptional effects and ‘higher’ level biomarkers, one area that may be of interest to explore is the close relationship between anaerobic metabolism and HSPs (Anestis et al. 2009). Thermal shock substantially reduced the ability of mussels to withstand anoxia (i.e. SoS) and induced HSP expression (Anestis et al. 2009). Given that HTO in conjunction with hyperthermia causes induction of similar HSP genes, but the SoS assay was not performed, it might be interesting to explore the potential synergistic effects of these combined physical and radiological stressors on this ecologically relevant biomarker.

6.4.3 Alteration of transcriptional expression of key genes

There is minimal literature on the transcriptional responses of marine invertebrates to ionising radiation (Farcy et al. 2007, 2011), and even less data for Mytilus spp. (only AlAmri et al. 2012). However, comparisons with the mammalian literature yield some interesting comparisons and support the general trends we have seen. For example, there is a well-known link between radiation exposure and increased expression of heat shock genes in mammalian \textit{in vitro} systems. Mouse splenocytes exposed to 0.04 Gy showed increased \( hsp70 \) and \( hsp72 \) along with \( hsc70 \) (Nogami et al. 1993). Chinese hamster cells (CH3 10T 1/2) also showed upregulated heat shock genes after exposure to \( \gamma \) radiation (Calini et al. 2003) and inhibition of \( hsp90 \) has been reported to increase radiosensitivity in tumour cells (Dote et al. 2006). Protective effects of metallothionein proteins in \( \gamma \) and UV-irradiated human cell culture systems have also been reported and are attributed to their oxygen radical scavenging ability (Cai et al. 1999, 2000). In aquatic invertebrates, only one transcriptional study has included laboratory exposure to a radionuclide (waterborne \( \gamma \) radiation \([^{60}\text{Co}]; \) Farcy et al. 2011). The current results show similar upregulation of metallothionein genes to that reported by Farcy et al. (2011), which suggests this defence mechanism may be generally important in radiation exposure. It is, however, important to remember that comparisons of these two studies are difficult, as the total doses varied approximately 2.5-fold.
(with concomitant differences in dose rate) and different radionuclides were used. Field studies of transcriptional changes after radiation exposure are also difficult to interpret, with temperature having a significant impact on gene expression in oysters from the French coast (Farcy et al. 2007). Interestingly, these authors report that temperature was inversely correlated with expression of genes such as \textit{hsp70}, \textit{hsp90} and \textit{MT}, a trend only reflected in the current results for HTO exposure (but not for temperature alone). The field-based work of Al-Amri et al. (2012) found significant upregulation of \textit{rad51} in mussels exposed to dose rates as low as 0.61 $\mu$Gy h$^{-1}$. In the current work, significant upregulation of this gene after 72 h exposure to HTO (but not for Cu or the 25 $^\circ$C control) supports the idea that this is radiation-induced change.

Gourgou et al. (2010) report rapid induction of \textit{hsp70} and \textit{mt20} during hyperthermia in \textit{M. galloprovincialis} (30 $^\circ$C for up to 8 h), which is at odds with our hyperthermia only treatment (25 $^\circ$C control). It is, however, important to note that the higher temperature (30 $^\circ$C) caused 95 % mortality by 24 h, suggesting this difference results in a considerably more stressful environment for mussels. Despite the difference in outcome, use of selective inhibitors to potentially link \textit{hsp70} and \textit{mt20} expression during HTO exposure with p38-MAPK or JNKs (as in hyperthermic mussels; Gourgou et al. 2010), might be interesting from a mechanistic point of view.

The data reported here indicate significant upregulation of \textit{hsc70} after only 1 h in two of the treatment groups (HTO at 25 $^\circ$C and Cu at 15 $^\circ$C). This is initially surprising as \textit{hsc70} is usually regarded as a constitutively expressed gene in mussels, encoding a chaperone or cognate protein with ATPase and protein-binding abilities (Hofmann et al. 2002). However, a more thorough examination of the literature reveals that this gene has been found to vary in response to stress in fish (Deane & Woo 2005) and other molluscs (Clegg et al. 1998). Changes in transcriptional expression of \textit{hsc70} have previously also been reported in \textit{M. galloprovincialis} after exposure to 150 $\mu$g L$^{-1}$ Hg (downregulation at 1 d and upregulation after 6 d) and 1 - 50 ng L$^{-1}$ Cr (upregulated after 7 d; Franzellitti & Fabbri 2005). The expression profile of \textit{hsc70} reported here is different from that induced by these metals (particularly the sharp initial rise at 1 h), but considering the temporal variation in our \textit{hsc70} expression profiles, it is possible that previous studies have failed to observe an early peak. Alternatively, there may be stress-specific differences in the response, although in the current study both Cu and HTO exposure resulted in a similar \textit{hsc70} signal at this early timepoint. In
mammalian systems, significantly elevated hsc70 expression has been reported in response to radiation (e.g. mice after 0.04 Gy γ radiation; Nogami et al. 1993) and the importance of the protein interaction between HSC70 and HSF1 (see Fig. 6.1) has been underlined (in HEK-293 cells; Ahn et al. 2005).

There are several splice-variants of p53-like genes, specifically p53 (with a sterile alpha motif [SAM] on the C-terminal end), p63/73 (with a SAM and a transactivation domain [TA] at the N-terminal end) and ΔNp63/73 (which contain a SAM but are truncated at the N-terminus, therefore lacking the TA). The ΔN isoforms have no ability to induce apoptosis and actually suppress functional p53/63/73, meaning they are oncogenic (Muttray et al. 2008). The nomenclature surrounding which of these variants is present in Mytilus sp. is often confusing (Muttray & Baldwin 2007, Rotchell & Ciocan 2007, Štifanić et al. 2009), but as of 2009 all known p53-like sequences in molluscs had been identified as p63 genes or their expression isoforms (Štifanić et al. 2009). For accurate analysis and interpretation of transcriptional alterations in ‘p53’ it is essential to have clarity regarding these issues. The ‘p53’ primers used herein are derived from a M. galloprovincialis sequence of the p63/73 family (see Table 6.2 for details) and were designed to quantify total p63/73 expression (i.e. to target a sequence found in all the splice variants; Dondero et al. 2006b). As a consequence it is possible that the increased p53 expression observed at 72 h is either anti-oncogenic (p63/73) or oncogenic (ΔNp63/73). The observed increase in genotoxicity at this timepoint at 25 °C (where p53 is downregulated in comparison to the cooler temperature) suggests that at 15 °C p53 is either having a protective function or the protective effects of other genes/proteins are compensatory (e.g. HSPs, MTs).

In general, the temperature-dependent difference between expression profiles for HTO-exposed mussel gill at 72 h suggests that downregulation of key protective genes could be one explanation for the earlier genotoxicity of HTO at 25 °C. Downregulation of these genes has been reported in conjunction with DNA damage before (e.g. p53 in mussels exposed to B[a]P [Banni et al. 2009a] and hsp70 in γ-irradiated C3H 10T 1/2 cells [Calini et al. 2003]). There is, of course, the potential that the lack of resolution in our sampling schedule (i.e. a gap of 60 h) has obscured an earlier response by these genes. For example, Tedengren et al. (1999) have reported that mussels pre-exposed to elevated temperature showed increased resilience to Cd toxicity as a result of more rapid synthesis of stress-induced cytoprotective proteins (e.g. HSPs). However, our mussels had concurrent exposure to heat and HTO, with no pre-treatment, so this effect is unlikely. The more rapid
occurrence of DNA strand breakage in the 25 °C HTO exposed mussel haemocytes also suggests a lack of protection, rather than a temporal shift. It is interesting that there was no reduction in expression of rad51 between the 15 and 25 °C HTO treatments, as this gene is involved in double strand break repair (AlAmri et al. 2012, Di 2012). Similarly, rad51 was not upregulated before significant strand breakage occurred (i.e. < 72 h) for HTO at 15 °C, suggesting other DNA repair genes were involved in maintaining genomic integrity at this stage. Yet again temperature altered this effect, with rad51 upregulated at only 1 h for 25 °C HTO exposure. Future studies to elucidate whether or not this difference is due to different repair mechanisms or a temporal shift would greatly enhance our understanding of the combined effect of radiation and heat on DNA.

The current study only analysed transcriptional expression in one tissue - the gills. Though this tissue has been cited as showing the highest levels of HSP70 and HSP72 proteins in *M. edulis* (Chapple et al. 1997) this does not necessarily equal the highest mRNA expression, nor does it apply to other genes/proteins. Expression of heat shock molecules is notably tissue-specific in *Mytilus* spp. according to (Pantzartzi et al. 2010). Similarly, Banni et al. (2009a) reported significant tissue-specific effects on *p53* expression in mussels exposed to B[a]P, with digestive gland showing upregulation, in contrast to downregulation in the haemocytes. Metallothionein genes (*mt10* and *mt20*) also showed tissue-specific variation in *M. galloprovincialis* after exposure to TiO2 nanoparticles, their bulk equivalent and a Cu positive control (D’Agata et al. 2013). Unfortunately it was logistically difficult to include other tissues here, however it is imperative that future studies consider this important variable, in order to fully characterise the response of these key genes to radiation and/or heat stress.

### 6.5 Interaction of other physical factors with radiation

In addition to temperature effects, there are also many other physical factors which may produce interactive effects with radiation exposure in aquatic animals such as mussels. In particular, for euryhaline organisms, salinity can have a pronounced and complicated effect on their ability to tolerate the stress of irradiation. For example, the grass shrimp has has salinity dependent LD50 values for radiation
dose, with individuals at the lowest salinity (15 psu) tolerating the highest dose of radiation (6 Gy; Engel et al. 1974). Both blue crabs and grass shrimp show changes in osmoregulatory capacity after irradiation (Engel et al. 1971, 1974). This indicates that in addition to salinity affecting radiation tolerance, radiation also interferes with the capacity of these organisms to deal with changes in salinity. In addition to salinity there are other known environmental stressors which could be examined in conjunction with radionuclide exposure. One such example is oxygen concentration (e.g. hypoxia and hyperoxia), which has been shown to affect physiological, histopathological and DNA damage in fish (Mustafa et al. 2011, 2012). Given the increasing number of ‘dead zones’ in coastal waters (Diaz & Rosenberg 2008), their interaction with IR could have implications for observed biological responses.

6.6 Conclusions

This study is the first to investigate temperature effects on radiation-induced genotoxicity in an ecologically representative marine invertebrate, *M. galloprovincialis*. This represents an important step forward in radioecology in general, as to date there are temperature-dependent laboratory exposure data for only two other molluscs - *Physa* spp. (a freshwater snail; Ravera 1966, Cooley 1973) and *Crassostrea gigas* embryo-larvae Nelson 1971). From an ecological perspective, this research suggests that mussels (or similar marine species) exposed to increased temperature and HTO may have a compromised ability to defend against genotoxic insult.
Chapter 7

General discussion and future research
7.1 Ecologically relevant species for biomarker studies

The primary aim of ecotoxicology is to protect populations, communities and ecosystems, in contrast to human toxicology where protecting the individual is paramount. There are, however, innumerable different species, occupying various niches and trophic levels, and it is logistically difficult to test contaminants on all of them. As such, it is essential that the organisms used in ecotoxicological tests adequately represent the wider biota. Although the mussel is a commonly used bioindicator organism in ecotoxicology with many beneficial qualities, there is also some debate about the appropriateness of using such a hardy animal in ecotoxicological investigations. It has been suggested that such studies should concentrate on more sensitive species to yield more ecologically relevant results (Wilson 1994). Certainly the HTO results presented here suggest that, at the tissue-organism levels, mussels are relevantly tolerant to this radionuclide (Chapters 5 and 6). Research questions focusing on such effects and on their vertical transmission (see section 7.5) may require animals with shorter life histories, more rapid growth and/or more sensitive responses. Candidate species include mysid shrimp, for which a 7 d toxicity test measuring survival, growth and fecundity has already been developed (Lussier et al. 1999); *Daphnia magna*, the water flea, a very well established model species in ecotoxicology (e.g. Biron et al. 2011, Poynton et al. 2011, Asselman et al. 2012); and the zebrafish (*Danio rerio*) which presents many advantages, especially in molecular ecotoxicology (Hill et al. 2005). Furthermore, the extremely limited amount of information available on the environmental impacts of ionising radiation, make multiple species comparisons essential to produce accurate risk assessment for the environment (Dallas et al. 2012).

7.2 Relative impact of different contaminants

Nickel, zinc pyrithione and tritiated water were selected as the contaminants of interest in these studies as a result of their input into the marine environment via several different sources (e.g. mining, boating and nuclear power). They also represent various classes of contaminants (radionuclides, metals, organometals) with differing modes of action. It is, however, important to remember that the
7. General discussion

sources and distribution of such pollutants vary with time, in response to regulation, changing attitudes or technological advances. It is also pertinent to note that improved analytical techniques now allow the quantification of previously undetectable compounds in the aquatic environment. The environmental effects (i.e. impact on the biota) have not been evaluated for many of these emerging contaminants, but they have the potential to cause toxicity (Hutchinson et al. 2013). As such, an awareness of potential future contaminants should inform ecotoxicological studies on metals and radionuclides. In terms of metals and radionuclides with environmental relevance, important contaminants for the future might include naturally occurring radioactive material (NORM) from the oil and gas industry (Betti et al. 2004), radionuclides from accidental releases (e.g. $^{137}$Cs after the Fukushima accident; Buesseler et al. 2011), interactions with natural biotoxins (e.g. Tiedeken & Ramsdell 2009) and manufactured nanoparticles, which are increasingly reaching the aquatic environment via consumer waste and have shown genotoxic effects in marine species, including mussels (e.g. Vevers & Jha 2008, D’Agata et al. 2013).

As the importance of biological responses to contaminants in maintaining good ecological status is being emphasised (Borja et al. 2004), several authors have proposed mathematical methods by which multiple biomarkers can be combined to give an overall indication of impact or risk. For the purposes of evaluating the contaminants used here, three of these methods were considered. These were (1) the integrated biomarker response (IBR) first proposed by Beliaeff & Burgeot in 2002 and subsequently modified (Serafim et al. 2012, Sanchez et al. 2013), which is derived from the area of star plots for standardised biomarker responses; (2) the health status index (HSI; Dagnino et al. 2007), which ranks significant differences according to threshold limits and then applies an algorithm to categorise samples into one of five health levels (A [healthy] - E [pathological]); and (3) the biomarker response index (BRI; Hagger et al. 2008), itself a modification of the HSI, incorporating aspects of the bioeffects assessment index (BAI; Adams et al. 1993).

After careful consideration, BRI was chosen to evaluate the contaminants of this study, for the following two reasons: its calculation is relatively simple, relying on ranking without the complex algorithms of HSI; and biomarkers are weighted by level of biological organisation within the BRI calculations, thus fitting with the integrated approach of this programme of study. In addition to the three contaminants of interest for this programme of study, an additional contaminant,
benzo(a)pyrene, was included as a representative organic pollutant (56 $\mu$g L$^{-1}$ for 12 days; Di et al. 2011).

To calculate BRI, biomarker responses were first ranked for each contaminant according to the magnitude of their difference from their respective control(s). The ranks used were $4 = \text{no response or slight response}$, $3 = \text{moderate response}$, $2 = \text{major response}$ and $1 = \text{severe response}$ (Hagger et al. 2008). Weightings for biological level of $1 = \text{molecular}$, $2 = \text{cellular}$ and $3 = \text{physiological}$ were also used. BRI values for HTO, Ni and ZnPT were calculated according to equation 7.1 and are displayed in Table 7.1 along with individual biomarker rankings and weightings.

$$BRI = \frac{\Sigma(BR_n \times BW_n)}{\Sigma BW_n}$$

(7.1)

where $BR_n$ and $BW_n$ are the rank and weighting of the $n$th biomarker respectively. Lower BRI values indicate a more toxic set of responses.

Typically, such indices are mostly used to compare biomarkers measured in field-sampled organisms from multiple sites or sampling periods (e.g. Hagger et al. 2008, 2010, Serafim et al. 2012, Tlili et al. 2013). Therefore it is essential to bear in mind some caveats when using these techniques to assess the multiple laboratory-based studies on different classes of contaminants that are presented here. First and foremost, the concentrations of contaminants used are not equivalent to each other (nor is it possible to truly compare dose from radiation with that from metals). Secondly, exposure durations varied and it is entirely possible that Ni would have produced different responses after 14 days exposure instead of only 5, especially given the temporal variation seen in Chapters 4 and 6. This is especially important to bear in mind as many of our biomarkers relate to gene expression for a specific tissue (gill or digestive gland), which could vary considerably depending on exposure duration (in addition to other compounding factors).

Given these important caveats, it is interesting to note that ZnPT received the lowest BRI score of 1.80, followed by B(a)P at 2.14 and Ni at 2.63, all of which correspond to ‘Severe alteration’ according to Hagger et al. (2008). In contrast, HTO scores $> 3.01$ classifying it as ‘No or slight alteration from normal response’ (Hagger et al. 2008). The idea that ZnPT is by far the more harmful of the three agents used here is further emphasised when the environmental relevance of
Table 7.1. Assigned biomarker values and calculated biomarker response indices (BRI) for responses of *Mytilus galloprovincialis* to different contaminants \(^{a}\). These were the three contaminants used in the current work - tritiated water (HTO; a radionuclide), nickel (Ni; a metal) and zinc pyrithione (ZnPT; an organometallic compound) - and a reference organic contaminant, benzo(a)pyrene (B[a]P).

<table>
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<th>Biomarker</th>
<th>Weighting (^{b})</th>
<th>HTO (^{c})</th>
<th>Ni (^{d})</th>
<th>ZnPT (^{e})</th>
<th>B(a)P (^{f})</th>
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</table>

**BRI values** 3.15 2.63 1.80 2.14

\(^{a}\) where 4 = no response or slight response, 3 = moderate response, 2 = major response and 1 = severe response. In all cases `response` was classified as alteration from control specimens in the same experiment; \(^{b}\) where 1 = molecular, 2 = cellular, 3 = physiology; \(^{c}\) values are based on 15 MBq L\(^{-1}\) responses from Chapter 5 or 15 °C HTO treatments in Chapter 6; \(^{d}\) values are based on the 3600 µg L\(^{-1}\) treatment in Chapter 3; \(^{e}\) 0.2 µM; \(^{f}\) data from Di et al. (2011) for 56 µg L\(^{-1}\) benzo(a)pyrene exposure for 12 days; \(^{g}\) gene expression is for either gill or digestive gland, whichever had the larger deviation from the control(s).
our contaminant concentrations is examined. HTO concentrations in the English Channel are estimated to be 10 Bq L$^{-1}$ (Bailly du Bois & Dumas 2005), i.e. $1.5 \times 10^6$ times less than those used here. Similarly, although Ni concentrations in the coastal marine environment are not well defined, the concentrations used in Chapter 3 most likely represent acute contamination of the environment. In contrast, the concentrations of ZnPT used are potentially of the same order of magnitude as those reported for contaminated marinas (Mackie et al. 2004). Examination of Table 7.1 also emphasises the need for consistent experimental design so that true like-for-like comparisons can be made. Having said that, the BRI calculation does remove weightings for unused biomarkers, thereby making comparison fairer. Future studies would benefit from using such an approach, especially in terms of disseminating key results to regulators, stakeholders and the general public.

7.3 Relationships between biomarkers

Significant relationships between a biomarker of exposure at the genetic level (comet assay) and biomarkers of effect at both the genetic and tissue levels (micronucleus assay, gene expression and histopathology) were found in experiments using both chemical (Ni, ZnPT) and radiological toxicants (HTO). Overall, the work carried out during this study programme confirms the potential of genotoxic responses to predict changes in mussels at these levels as suggested by previous authors (e.g. Hagger et al. 2005a, Jha et al. 2005). It is, however, the first to explicitly define these relationships (i.e. with regression/correlation) in marine mussels. The relationships between gene expression and DNA damage are especially interesting as the mechanistic links between these parameters are not well established, especially in mussels, and may be dynamic processes. For example, different genes may be induced either in response to DNA strand breaks or directly by ROS or other causative agents (i.e. simultaneous to strand breakage).

It is especially interesting that significant correlations were observed between biomarkers in different tissues. The experiments reported here exclusively measured DNA strand breakage in mussel haemocytes, whereas gene expression and histopathology were monitored in gill and/or digestive gland. Further research to investigate the mechanisms behind this inter-tissue pattern would inform future studies on mussels, and contribute to the limited amount of information linking effects at multiple levels.
Unfortunately, the methodology of many of the higher level biomarkers used herein prevented their correlation with genotoxic effects at the level of the individual mussel, for example protein samples had to be pooled for 2D gel preparation in Chapter 4. Future studies could address this issue by careful experimental design, potentially utilising smaller more focused experiments to establish robust links between biomarkers, before expanding to the larger, fully integrated approach used in the current project. This methodology would also enable mechanistic investigation of the links between effects at different levels of biological organisation, with resulting benefits for future ecotoxicological studies. Such information could be incorporated into wider environmental management and risk assessment (Moore et al. 2004).

7.4 Oxidative damage to DNA, as measured by the comet assay

Despite the successes of the integrated approach adopted here, the mechanism(s) of action behind many of the observed effects require further exploration. An interesting example of this is the lack of significant oxidative damage in mussel haemocytes, as measured by the modified comet assay. Oxidative damage plays an important role in a range of pathophysiological conditions in humans and other mammals, including ageing and reduced fertility (Evans et al. 2004). In mussels, capacity for oxygen radical scavenging (i.e. protection from oxidative damage) has also been shown to correlate closely with DNA integrity under environmental stresses (eutrophication; Frenzilli et al. 2001). Furthermore, the lack of oxidative damage observed in mussel haemocytes during exposure to the three contaminants used here contradicts the majority of the published literature on mechanisms of genotoxicity for metals and ionising radiation (Riley 1994, Costa et al. 2002, Valko et al. 2006). As such, it certainly requires more investigation. Technical error seems unlikely as successive validation experiments with hydrogen peroxide under \textit{in vitro} conditions yielded a positive response, whilst the same technique has also revealed significant oxidative damage in fish (\textit{Cyprinus carpio}) exposed to hypoxia in our laboratory (Mustafa et al. 2011).

As genotoxicity is a tissue-specific phenomenon (Jha 2008), it seems likely that the lack of effect observed under \textit{in vivo} conditions here is related to the haemo-
cytes themselves. Contrasting the haemocytes of mussels with erythrocytes of fish (which do show significant stress-induced base oxidation; Mustafa et al. 2011) potentially reveals an important difference. The haemocytes of mussels have no oxygen carrying capacity and are primarily involved in digestion and immunity (Bayne 1976), including the release of ROS to kill pathogens (Pipe et al. 1993, García-García et al. 2008). To protect both themselves and surrounding tissues, mussel haemocytes are known to have high anti-oxidant enzyme activity (Pipe et al. 1993), potentially reducing their usefulness as a tissue for the investigation of oxidative DNA damage. In addition, although most authors agree that there are three main types of haemocyte in _Mytilus_ spp. (eosinophils, basophils and hyalinocytes; Carballal et al. 1997, Parisi et al. 2008, Venier et al. 2011) there is still much debate about the subtypes and it is generally agreed that these cells are dynamic and interactive (Le Foll et al. 2010). The differential stress responses of various cell types within a single tissue has been demonstrated in mammalian cell culture models, for example epithelial cells from the liver are more resistant to hypoxia-induced cell death than hepatocytes (Abe et al. 2012). Such cell-specific responses complicate comet assay measurement (which assumes a single, or similar, cell type[s]; Jha 2008) and may be the cause of the variability reported herein, which may result in limited statistical power (especially in Chapters 3 and 6 where Fpg treatment produced elevated % tail DNA but not significantly so).

To further elucidate the potential oxidative DNA damage caused by metals or radionuclides, future studies would benefit from several alternative approaches. Firstly, direct measurement of ROS in haemolymph or mussel tissues would determine a physical basis for any damage. Within ecotoxicological studies, the oxidation of dichlorofluorescein has already been used to monitor ROS production _in vivo_ in _Daphnia magna_ exposed to Ni (Xie et al. 2007). It is also possible to use novel techniques, such as electron spin resonance (ESR), to identify the ROS generated by cells _in vitro_ (as in goldfish skin cells exposed to TiO₂ nanoparticles; Reeves et al. 2008) or in an aqueous solution of the contaminant alone (e.g. for TiO₂ nanoparticles themselves; Dodd & Jha 2009). This latter technique has already been used to characterise radical generation for one contaminant of interest here, revealing that β particles from HTO produced a lower radical yield than ⁶⁰Co under the same conditions, attributed to their lower LET (Kroh et al. 1962).

The second recommended approach is to measure anti-oxidant enzyme activity in the haemolymph or haemocytes during/after contaminant exposure. For exam-
ple, glutathione peroxidase (GPX) activity was significantly increased in digestive gland of mussels with tissues concentrations of 200 $\mu$g g$^{-1}$ Ni, i.e. considerably lower than all tissues measured in Chapter 3 except for byssus. This indicates that significant detoxification of hydrogen and lipid peroxides was occurring (Tsangaris et al. 2008). Other anti-oxidant enzymes of interest commonly measured in mussels include catalase (Bocchetti et al. 2008, Lopez-Galindo et al. 2010) and superoxide dismutase (Liu et al. 2010). Such enzyme studies also have the potential to reveal substances with protective effects, such as the increase in GPX activity (and subsequent decrease in genotoxicity) associated with treatment with Se prior to Hg or Cu exposure (Tran et al. 2007, Trevisan et al. 2011).

Use of the enzyme modified comet assay in tissues other than haemocytes would fully elucidate the tissue-specific nature of oxidative genotoxicity in mussels. Other researchers have performed the comet assay on alternative mussel tissues, including gills (Pruski & Dixon 2002, Emmanouil et al. 2006, 2007, Large et al. 2002) and digestive gland (Shaw et al. 2000, Large et al. 2002). Of these studies, only one used the enzyme modified version of the assay, where significant base oxidation was detected after injection of each mussel with 10.4 $\mu$g sodium dichromate but not after metal exposure (Emmanouil et al. 2007). The majority of ecotoxicological studies examining mussel genotoxicology still use haemocytes as there are technical challenges when using other tissues/organs to detect DNA strand breaks in aquatic organisms, in contrast to mammalian systems, where different organs are routinely used (Jha 2008). Lastly, it is important to remember that all of the above methods are potentially confounded by endogenous production of ROS, so careful use of controls is necessary.

Whilst no oxidative damage to nucleic acid bases was observed in the current work, no oxidation of other subcellular components was measured for comparison. Lipid peroxidation is a commonly used biomarker in mussel studies, and has been reported to increase in response to a wide range of contaminants (Emmanouil et al. 2007, Vlahogianni & Valavanidis 2007, Kadar et al. 2010, Gomes et al. 2011). In particular, 10 d exposure of *M. edulis* to Cd resulted in a significant increase in strand breakage (comet assay) in gill cells but no subsequent increase with Fpg treatment. This indicates no oxidation of DNA and is similar to the results reported here. Despite this, significant levels of lipid peroxidation were observed in the same tissue (Emmanouil et al. 2007). Although the three major biological macromolecules (nucleic acids, proteins and lipids) could all be grouped into the same level of biological organisation (subcellular/molecular), future studies which
consider separate biomarkers for the potential oxidation of each would be beneficial. Multiple biomarker studies can, however, present logistical difficulties. As such, research into the relationships between lipid, protein and DNA oxidation for a range of contaminants (and contaminant mixtures), would potentially enable extrapolation and provide regulators with a useful tool for ERA. The current project provides an example of this approach using biomarkers of genotoxicity (comet assay, induction of micronuclei) and gene expression.

7.5 Future perspectives

This present project assessed a range of biomarkers in *M. galloprovincialis* with the aim of correlating effects at multiple levels of organisation. Although this study spans biomarkers across the genetic, tissue, and individual organism levels, effects at even higher levels (i.e. populations, ecosystems) were not addressed. Current trends in ecotoxicology and radiobiology provide several suggestions by which future studies on similar contaminants could expand their focus into such higher level biomarkers. In particular, bystander effects (Nagasawa & Little 1992, Lorimore et al. 1998, Watson et al. 2000, Xue et al. 2002, Bertucci et al. 2009), transgenerational effects (Parker et al. 1999, Dubrova et al. 2002, Baverstock & Belyakov 2010) and epigenetic effects (Vandegehuchte & Janssen 2011, Head et al. 2012, Vandegehuchte & Janssen 2013) all have the potential to cause alterations in population structure. While there has been extensive work on these areas in the human health arena, there has only been a very limited amount of studies on aquatic animals. Despite this, all three types of effect have been reported in aquatic species (see below).

Bystander effects are changes in non-irradiated cells, tissues or organisms following radiation exposure in their neighbours (Osterreicher et al. 2003). They are an important area of research in radiation biology (Baverstock & Belyakov 2010), and have previously been reported in aquatic species. For example, unirradiated fish of two species (rainbow trout and zebrafish) have been shown to develop a classic bystander response (induced cell death) after exposure to irradiated individuals (Mothersill et al. 2006, 2007). Lorrimore et al. (2001) reported that $p53$ is linked to macrophage activation and neutrophil infiltration (i.e. inflammatory bystander responses) in $\gamma$-irradiated mice (4 Gy, whole body dose). This effect was attributed to $p53$-mediated clearance of radiation damaged cells via apopto-
sis. As closely related genes \((p63/73)\) were upregulated in HTO-exposed mussels, it would be particularly interesting to explore the potential inflammatory impacts on surrounding tissues and/or cells, especially after longer-term and/or lower dose exposures. Although current research on bystander effects in fish have used relatively high doses of irradiation (e.g. 0.5 Gy; Mothersill et al. 2006), these effects typically occur predominantly at low doses of IR (Seymour & Mothersill 2000, CERRIE 2004). An accurate understanding of their effects is therefore essential before it is possible to extrapolate from high to low radiation doses (CERRIE 2004). This is especially important for groups where there is limited data available, such as the aquatic invertebrates. Therefore, as the understanding of bystander effects grows, it is likely that this area will become increasingly important in studies of radiation- and/or metal-exposed aquatic invertebrates.

Genomic instability has been observed in F1 somatic cells of medaka (\(Oryzias latipes\)) after irradiation of paternal spermatids (Shimada & Shima 2004). Until recently there were no studies of radiation-induced transgenerational effects or germ line mutations in aquatic invertebrates; however, recent work on \(Daphnia magna\) exposed to total doses of 100 and 1000 mGy from \(\gamma\)-rays has shown significant decreases in life span in both the exposed F0 and un-exposed F1 generations (Sarapeltseva & Gorski 2013). Furthermore, exposure to non-radioactive compounds has also caused transgerational effects. For example, B(a)P exposed \(D. magna\) passed alterations in the DNA to offspring (Atienzar & Jha 2004). This species has also been used to demonstrate alterations in DNA methylation (i.e. an epigenetic response) after metal exposure (Vandegehuchte et al. 2010a,b). It is interesting to note that, in this case, single generation Zn exposure resulted in a different profile of organism-level effects (i.e. growth and reproduction) in the F1 and F2 generations than for the F0 generation and for all generations with multigenerational Zn exposure (where adaptation developed by the F2 generation). The propensity for organisms to adapt during chronic, long-term exposure to contaminants (as discussed in reference to ZnPT in marinas in Chapter 4) provides another compelling reason for transgenerational studies on aquatic animals exposed to such compounds.

Transgenerational studies can also reveal instances where successive generations are more susceptible to a contaminant than their parents, as was the case for \(D. magna\) exposed to the \(\alpha\) emitter \(^{241}\)Am. This radionuclide causes detrimental effects on \(D. magna\) at doses \(\geq 0.99\) mGy h\(^{-1}\) (Alonzo et al. 2006, 2008) in the parental generation, and at \(\geq 0.3\) mGy h\(^{-1}\) in the F1 and F2 generations (Alonzo...
7. General discussion

In this context it is important to remember that although the current project did not reveal significant effects of HTO on mussels at the physiological or organism levels, transgenerational effects were not examined and there is the potential that subsequent generations might show heightened sensitivity. A detailed understanding of potential transgenerational impacts of contaminant exposure is also especially relevant for aquatic species which reproduce by spawning and have planktonic larvae, such as mussels. The potentially long range of such offspring from their parents' location could result in deleterious effects at unexposed sites. As such ideas are explored these types of studies will become essential for accurate risk assessment in the aquatic environment.

Epigenetic changes are heritable alterations to gene expression, passed on via mitosis or meiosis but without any change to the genetic code (Vandegehuchte & Janssen 2013). Such effects have already been identified in response to metal exposure in mammalian models, where changes in DNA methylation and histone modification (with concomitant impacts on gene expression) have been implicated in mammalian carcinogenesis (Salnikow & Zhitkovich 2007). Nickel, one of the contaminants used here, has been shown to have an inhibitory effect on cytosine 5-methyltransferase activity in Chinese hamster cells (Lee et al. 1998). The highly conserved nature of these enzymatic mechanisms (Posfai et al. 1989) suggests that Ni might be an ideal candidate for the investigation of similar effects in marine mussels, or other ecologically relevant aquatic species. With respect to studies of radiation-induced epigenetic effects, GM10115 cells showed alterations in DNA methylation (primarily hypomethylation with some hypermethylation) after exposure to X-rays or Fe ions (Aypar et al. 2011). Interestingly, comparison of these two radiations caused differential epigenetic responses in GM10115 cells, especially with regard to miRNA which showed greater alterations for the low LET irradiation (X-ray; Aypar et al. 2011). This is especially pertinent to the current work, as tritium has relatively low LET (compared to γ radiation or Fe ions). Despite widespread interest in environmental epigenetics (Vandegehuchte & Janssen 2011, Head et al. 2012, Vandegehuchte & Janssen 2013), there are to date no studies looking at exposure-induced changes to DNA methylation or histone modification in mussels. As such any future studies on contaminant-induced epigenetic effects would need to be underpinned by basic research on the phenotypic impacts of epigenetic changes in this species (Vandegehuchte & Janssen 2011). Future studies might also compare epigenetic responses to high and low LET radiation in ecologically relevant organisms to further elucidate the...
Improvements in molecular biology have resulted in new avenues of investigation for ecotoxicological studies on metals and radionuclides. In addition to the work on epigenetic effects described above, this also includes the development of microarrays for mussel species (e.g. Mussel MytArray [Canesi et al. 2011], Immunochip [Domeneghetti et al. 2011], the ‘Mytox chip’ [Dondero et al. 2006b] and others [Place et al. 2008]). Such arrays consist of many oligonucleotide probes attached to a solid surface or ‘chip’ and enable simultaneous measurement of expression for multiple genes. This approach is particularly useful where little is known about the mechanisms of action or predicted responses to a given contaminant. It is therefore well suited for the study of radiation-induced effects in aquatic invertebrates, on which there is limited information (Dallas et al. 2012). With regard to metals, a transcriptomic assessment has already been carried out on mussels exposed to a binary mixture of nickel and the organophosphate pesticide, Chlorpyrifos (Dondero et al. 2011). This study revealed significantly different expression profiles for the two contaminants, with metal exposure alone resulting in increased expression of nucleic acid and lipid metabolism genes. In contrast, the pesticide exposure caused upregulation of carbohydrate catabolism genes and mixture toxicity resulted in a different, even more complicated profile (Dondero et al. 2011). As such, this study provides an insight into the different mechanisms of these contaminants and their potential interactive effects. Although there are also many challenges with such approaches (Moore 2002, Snape et al. 2004b), mechanistic information from microarray studies could also be used to link genetic effects, such as those in the current project, to effects at higher (more ecologically relevant) levels of biological organisation.

Although the three contaminants used here were selected based on their environmental relevance, it is important to note that, as discussed in Chapter 6, organisms are rarely (if ever) exposed to a single contaminant. The study presented in Chapter 6 investigated the impact of one of the many physical factors which an aquatic organism may be exposed to in addition to a contaminant (elevated temperature), however it is also likely that other contaminants would be present. It is therefore essential that future ecotoxicological studies on metals and radionuclides investigate mixture toxicity. This may initially take the form of binary mixtures (e.g. Dondero et al. 2011), but with the advent of new molecular technologies it is now also possible to investigate the impacts of complex pollutant mixtures (Altenburger et al. 2012). Careful design of these studies is essential to ensure that
7. General discussion

it is possible to tease apart the contributions of each contaminant and accurately interpret antagonistic, synergistic or additive effects.

7.6 Conclusions

The integrated, multiple biomarker approach used here has allowed us to determine that NiCl$_2$ is genotoxic to mussel haemocytes at concentrations $> 1800 \, \mu$g L$^{-1}$ and that the MXR mechanism might have an important role in Ni detoxification at lower (more environmentally relevant) concentrations; that ZnPT has deleterious effects on mussels at levels of biological organisation from genetic to the whole organism; and that in contrast, HTO causes DNA damage without affecting higher level biomarkers, but that its genotoxic effects are modulated by increased temperature. Consequently, we recommend, as have previous studies (e.g. Moore et al. 2004, Hagger et al. 2005a, Jha et al. 2005, Brooks et al. 2009, Canty et al. 2009, Al-Subaii et al. 2011) that a similar approach is adopted for future ecotoxicological work, in order to maximise the ecological relevance and applicability of findings for regulatory bodies.
Appendix A

Publications
Assessing the Impact of Ionizing Radiation on Aquatic Invertebrates: A Critical Review

Lorna J. Dallas, Miranda Keith-Roach, Brett P. Lyons and Awadhesh N. Jha

There is growing scientific, regulatory and public concern over anthropogenic input of radionuclides to the aquatic environment, especially given the issues surrounding existing nuclear waste, future energy demand and past or potential nuclear accidents. A change in the approach to how we protect the environment from ionizing radiation has also underlined the importance of assessing its impact on nonhuman biota. This review presents a thorough and critical examination of the available information on the effects of ionizing radiation on aquatic invertebrates, which constitute approximately 90% of extant life on the planet and play vital roles in ecosystem functioning. The aim of the review was to assess the progress made so far, addressing any concerns and identifying the knowledge gaps in the field. The critical analysis of the available information included determining yearly publications in the field, qualities of radiation used, group(s) of animals studied, and levels of biological organization at which effects were examined. The overwhelming conclusion from analysis of the available information is that more data are needed in almost every area. However, in light of the current priorities in human and environmental health, and concerning regulatory developments, the following are areas of particular interest for future research on the effects of ionizing radiation on nonhuman biota in general and aquatic invertebrates in particular: (1) studies that use end points across multiple levels of biological organization, including an ecosystem level approach where appropriate, (2) multiple species studies that produce comparable data across phylogenetic groups, and (3) determination of the modifying (i.e. antagonistic, additive or synergistic) effects of biotic and abiotic factors on the impact of ionizing radiation. It is essential that all of these issues are examined in the context of well-defined radiation exposure and total doses received and consider the life stages and life span of the species studied. The review also provides future directions for studies in this stimulating area of research to protect human and environmental health.

INTRODUCTION

Anthropogenic impacts on the environment are a major source of concern for governments and regulatory bodies, and they can have significant economic and social consequences (1, 2). Applications of nuclear technologies have made a very significant contribution to modern civilization. However, the effect of man-made radionuclides on the environment is a growing concern (3), especially where nuclear power generation has been identified as a key part of low-carbon energy strategies (4). Although highly regulated (for example, radionuclides with a half-life of more than 10 days are covered by Descriptor 9 of the Marine Strategy Framework Directive of the EU (5)), the discharge of radionuclides is permitted as part of the normal operational procedures of nuclear institutions (6). For example, in 2003, the Flamanville nuclear power plant (NPP) in France discharged $1.21 \times 10^7$ Bq of liquid $\beta$-particle and $\gamma$-ray emitters and $5.99 \times 10^{12}$ Bq of liquid tritium into the sea. In the same year, the Sellafield nuclear fuel reprocessing plant in Cumbria, UK, discharged $3.43 \times 10^9$ Bq of liquid $\beta$-particle and $\gamma$-ray emitters, $1.03 \times 10^9$ Bq of $\alpha$-particle emitters, and a further $3.73 \times 10^{10}$ Bq of liquid tritium into the marine environment (7). Radionuclides are also released into the environment as a result of nuclear accidents, either in modest amounts or on a larger scale, such as that caused by the earthquake-tsunami at Japan’s Fukushima Daiichi NPP in 2011 or at the Chernobyl NPP (Ukraine, formerly USSR) in 1986. Assessment of the risk these radionuclides pose to the environment (whether by authorized or accidental release) has historically been
determined in accordance with dose limits for the protection of humans. The International Commission for Radiological Protection (ICRP) stated that “if humans are protected from the effects of ionizing radiation, then flora and fauna are also adequately protected” (9). As a result, the need to specifically assess the effects of ionizing radiation on the environment and to protect the nonhuman biota from adverse effects has now been recognized (10–13).

Invertebrates make up approximately 90% of life on Earth, and the aquatic environment covers over 70% of the surface of the planet. In addition to the sheer weight of their numbers, aquatic invertebrates are keystone species in many ecosystems (14). They are an important human food source [both commercially and in artisanal fisheries (15, 16)], are a source of food for commercial fish species (17), and are frequently used as model organisms for toxicological tests (18). In general, a greater activity of radionuclides is discharged as liquid waste than into the air (7), and aerial discharges are also ultimately deposited in the aquatic environment. As a result, aquatic invertebrates are likely to be exposed to anthropogenic radionuclides in addition to background radiation.

Detection and quantification of contaminants in the environment is not enough to accurately determine their impact. It is also necessary to identify their effects on biological systems, which are the ultimate recipients of toxicant-induced damage (19, 20). It is possible to use the responses of ecologically relevant species as indicators of ecosystem damage (1, 19). Yet, despite the environmental relevance of aquatic invertebrates and their ongoing exposure to radionuclides, less than 100 papers on the effects of ionizing radiation on aquatic invertebrates have been published in the past 50 years (Fig. 1; see Table 3 for a full list of references). The statistics in the EU-sponsored FREDERICA database (21) suggest that this results from our traditional focus on human radiological protection, since the greatest numbers of hits are for mammals (model species for humans) or plants and fish (sources of human food) (22). While there are numerous studies published on the accumulation of radionuclides by a range of aquatic organisms (e.g. 23–27), studies related to their biological effects are limited. Research in this area has varied over time; however, the number of papers published on the effects of ionizing radiation on aquatic invertebrates has increased since 2000 (Fig. 1). With the adoption of new approaches and methodologies, and to achieve the goal of realistic environmental assessment, it is likely that this trend will continue. In this review we aimed to focus on evaluating the effects of exposure to ionizing radiation in aquatic invertebrates. We also aimed to highlight areas of future research that would most benefit the radiological protection of the environment and to set out conceptual guidelines for future studies while highlighting knowledge gaps and data reporting procedures.

**BIOLOGICAL RESPONSES IN RELATION TO SOURCE OF RADIATION**

**Relative Biological Effectiveness**

It is important to consider the sources and qualities of radiation used in radioecological studies, as even radiation of the same type can vary considerably in its energy level and hence its interaction with biological material. The sources of radiation used in effects-based studies on aquatic invertebrates are illustrated in Fig. 2. To protect the environment, it is necessary to consider the relative biological effectiveness (RBE) of different radionuclides to nonhuman biota (12, 28). RBE is determined empirically by comparing the levels of damage caused by exposure to the radionuclide of interest and by a reference radiation (e.g. typically X or γ radiation) in the same biological system. In human radiobiology, RBE information is collated and used in conjunction with other factors to produce a conservative radiation weighting factor [RWF (29)]. The absorbed dose is then multiplied by the RWF to give an equivalent dose. Currently the RWFs for humans are 20 for α-particle radiation and unity for β-particle radiation (12, 29). However, there is no consensus on the RWFs that should be used for nonhuman animals (30). For α-particle
radiation, RBE values between 40 and 360 have been calculated (30) and RWFs of 5–40 have been recommended (12), depending on the species and end points considered. With respect to β-particle radiation, specifically for tritium and other low-energy β-particle emitters, a RWF of 3 has been recommended for use with wildlife (12, 31).

Only a few studies have exposed aquatic invertebrates to multiple types of radiation. However, any difference in factors that affect the dose–response relationship (e.g. dose, dose rate, LET, end point, gender, life stage) will alter the RBE (12). Consequently the available data for aquatic invertebrates are not appropriate for calculation of RBE values. For example, Knowles and Greenwood (32) compared tritiated water (HTO) exposure with external γ radiation (137Cs) in the polychaete Ophryotrocha diadema and found no significant difference in the decrease in fecundity between radiations. However, the specific end points measured were different for each type of radiation (reduced egg numbers and reduced larval survival), so it is difficult to use these data to determine RBE. Engel and Fluke (33) exposed Artemia eggs to either γ (60Co) or β-particle (electrons from a Van de Graaff accelerator) radiation. Unfortunately, these exposures occurred months apart and under different conditions (air exposed and exposure in a vacuum) and therefore are not directly comparable. Blaylock (34) reported comparable levels of chromosomal aberrations in the midge, Chironomus riparius, after exposure to similar doses from tritiated water (15.25 Gy) and an external γ-ray source (60Co; 16.5 Gy). Thus the available data for aquatic invertebrates appear to indicate that the current (human) RWF of 1 for β-particle radiation might be most appropriate. However, the paucity of data makes this conclusion highly speculative, and as yet potential RBE data from aquatic invertebrates are limited to tritium.

Recently, the first work on exposure of aquatic invertebrates to α-particle emitters—an important group of radionuclides discharged into the marine environment—has been published. Studies in which the α-particle emitters 226Ra (35) and 210Po (36) were used to expose a polychaete (Hediste diversicolor) and a mollusk (Perna perna), respectively, did not find any significant effect of radiation exposure. Massarin et al. (37) also examined the toxicity of an α-particle emitter (mixed isotopes of uranium) in Daphnia magna, a freshwater flea. The doses achieved (≤20 μGy h⁻¹) led the authors to conclude that the radiotoxicity of uranium is negligible in comparison to its chemotoxicity. This view is supported by others who have...
studied uranium toxicity in D. magna (38). The α-particle emitter 241Am causes detrimental effects on D. magna at doses of 0.99 mGy h⁻¹ (39) and above (40) in the parental generation and at ≥0.3 mGy h⁻¹ in the F1 and F2 generations (40). It has been suggested that comparison of this work to γ irradiation of D. magna (41) and D. pulex (42, 43) and exposure of mussels to HTO (44, 45) indicates the differences in RBE between α-particle, β-particle and γ radiation in these aquatic invertebrates (40). However, it is more likely that experimental design, species or end point differences account for this variation, particularly as the mussel studies examined genotoxic effects while the Daphnia work focused on mortality, fecundity and physiology (oxygen consumption). As yet, there is no definitive study on the RBE of α- or β-particle radiation in aquatic invertebrates.

Environmental Relevance of Radiation Sources

In addition to the discussion of RBE and RWFs, it is also important to consider the environmental relevance of the radiation sources used in studies on aquatic invertebrates. In the 1950s and 1960s, studies of the effects of ionizing radiation on aquatic invertebrates were almost exclusively confined to the effects of external exposure to X rays (e.g. 46–50) or γ radiation from 60Co (e.g. 33, 42, 43, 51–53) (Fig. 2). These external sources allow organisms to be exposed to radiation in well-defined conditions and allowed researchers to build on earlier pioneering work (e.g. 54), but they are not representative of environmental exposure.

Waterborne exposure is clearly the most relevant to aquatic invertebrates and can make them particularly vulnerable to internal exposure because their body surface, respiratory apparatus (especially gills), and internal organs (in organisms with open vascular systems) are in close contact with the surrounding water. Yet prior to 1970 only three studies (33, 55, 56) used waterborne radionuclides to examine the effect of ionizing radiation on aquatic invertebrates. More recently, tritiated water has been used as an environmentally realistic waterborne source of ionizing radiation in several experiments on aquatic invertebrates (32, 44, 45, 57, 58). Other environmentally relevant radionuclides, such as 137Cs, which was reported to be at significant waterborne concentrations after the nuclear accidents at Chernobyl (59) and Fukushima (60, 61), have so far been used only in external exposures to date (e.g. 32, 41, 62).

Impact of Dose and Dose Rate

The number of papers published on the effects of ionizing radiation on aquatic invertebrates is roughly evenly divided between acute (typically 24 to 96 h in duration, resulting in severe harm and often mortality) and chronic exposure (longer term, having sublethal effects). There has been a trend toward more environmentally relevant, chronic work in recent years. This is indicated by a decline in the use of mortality as the main end point and the resulting reduction in studies using organism-level end points (which are dominated by mortality; Fig. 3). Consideration of dose and dose rate is absolutely vital when comparing ecotoxicolog-

![FIG. 3. Level of biological organization of endpoints studied in papers on the effects of ionizing radiation on aquatic invertebrates from 1950–2010. Articles examining two or more endpoints were counted multiple times.](image-url)
Polikarpov (3) proposed a conceptual model that classified the dose rates for chronic exposure to ionizing radiation in nature as follows: (1) zone of biological uncertainty (<0.00001–0.00004 Gy year\(^{-1}\)) for areas with reduced levels of background irradiation (i.e. shielded); (2) zone of radiation well-being (0.00004–0.005 Gy year\(^{-1}\)) where radiation is at natural background levels; (3) zone of physiological masking (0.005–0.05 Gy year\(^{-1}\)) where doses from anthropogenic inputs may overlap with natural background radiation and where minor changes (e.g. in function, morphology or incidence of disease) may be detected but induce no significant hazard to populations; (4) zone of ecological masking (0.05–4 Gy year\(^{-1}\)) where effects on individuals and populations are no longer masked and there is the potential for wide-ranging damage. Most of the work on aquatic invertebrates spans the zones of ecological masking and damage to ecosystems (3), as would be expected for ecotoxicological studies. Williams and Murdoch (65) used a dose rate of 7.45 \times 10^{-4} \text{ Gy year}^{-1}, which falls well within the zone of radiological well-being. However, no effect was reported until dose rates several orders of magnitude higher (7.45 \text{ Gy year}^{-1}), within the zone of damage to ecosystems. This illustrates the difficulties faced by researchers aiming to detect effects at environmentally realistic doses of ionizing radiation.

To our knowledge, there is only one study that compared dose rates in aquatic invertebrates. In the midge, Chironomus riparius, exposure to 20 Gy of \(\gamma\) radiation at a rate of 252 Gy \(\text{h}^{-1}\) induced chromosome aberrations in the larval stage at a frequency (aberrations larvae\(^{-1}\)) of 0.366 (34). In the same study, exposure to 16.5 Gy at a rate of 0.03 Gy \(\text{h}^{-1}\) caused larval chromosomal aberrations at a significantly lower frequency of 0.035 (34). These figures are not directly comparable because the total accumulated doses are not equal and because in the acute study only the male parents were irradiated, whereas the entire population received the dose in the chronic study. Despite these, this study does provide an indication that altered dose rates may produce significantly different biological responses in aquatic invertebrates, in common with other species (12, 63, 64).

**VARIETY OF ORGANISMS STUDIED**

**Ecological Relevance**

The 77 papers reviewed here used 50 different species of aquatic invertebrates (Table 1), including groups of organisms ranging from mollusks to arthropods and echinoderms. Considering the enormous ecological and taxonomic variation within the group, it is not possible to assess the impact of ionizing radiation on aquatic invertebrates as a whole. There are whole phyla of ecologically relevant aquatic invertebrates for which we have very few or no data on their relative sensitivities to ionizing radiation (e.g. Platyhelminthes, Porifera) and many ecologically important classes that are also unrepresented (e.g. Cephalopoda, Asteroidea and Holothuroidea). The majority of papers published on the effects of ionizing radiation in aquatic invertebrates use saltwater or marine organisms (56 of the 77 papers listed in Table 3). If we exclude Artemia species (which inhabit highly saline tropical ponds rather than seawater), the number of papers using marine or estuarine organisms decreases to 39. Freshwater organisms are slightly under-represented, with a total of 22 publications. Crustaceae dominate the research (Table 1, Fig. 4), and the two most studied genera in this field are Artemia and Daphnia (with 17 and 6 papers, respectively). Despite their widespread use in ecotoxicological studies, these species are not representative of aquatic invertebrates as a whole. Artemia are adapted to tolerate the extremely high salinity (up to 200\(\text{w}^\circ\)) of the environments in which they live and are known for their ability to withstand environmental stress (55, 67). There are also concerns that laboratory-cultured Daphnia, selected for high reproductive performance in culture conditions, may as a consequence be more tolerant to other stressors (68). The use of sentinel species, such as Artemia and Daphnia, has evolved from the traditional toxicological approach (6) and has practical advantages; however, future work would benefit from the careful choice of more ecologically and/or phylogenetically representative species of aquatic invertebrates.

**Comparisons Across Phylogenetic Groups**

In classical ecotoxicological studies there has been a move toward a multiple species approach in recent years; this approach has many benefits, such as covering multiple trophic levels and feeding behavior (e.g. carnivores, herbivores) and encompassing differences in physiology (e.g. 69–71) to obtain a broader picture of environmental impact. Studying the toxicity of a contaminant in multiple species also allows comparisons between groups and provides alternatives when a classical test species is not present at a contaminated site (19). Although 10 studies on aquatic invertebrates used multiple species, only five used species from across multiple phylogenetic groups. Radiation resistance (as LD\(_{50}\)) is the property that was most often compared in these studies (Table 2). In this context Artemia salina was shown to be more radiosensitive than a variety of other aquatic invertebrates (40), which is unsurprising but ecologically unrepresentative, as discussed above. Comparisons of radiation-induced LD\(_{50}\) values for other aquatic
invertebrates have shown less dramatic differences, however, including comparable radiosensitivity between two gastropod mollusks (*Ilyanassa obsoleta*, *Urosalpinx cinerea*) and an echinoid echinoderm (*Arbacia punctulata*) (72).

The only study to compare an effect other than mortality across multiple phylogenetically distinct species is that of Williams and Murdoch (65). They examined a variety of end points in a selection of marine invertebrates (Table 1) after continuous low-level γ-ray exposure (60Co) over 3–7 months. It was concluded that the sponge (*Hymenacidon heliophila*) was the most radiosensitive, as it showed reduced growth at only 5 mGy h⁻¹. However, there were no consistent radiation effects, and comparisons between species and end points are speculative. Moreover, the study

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also did not relate the total dose received to the life span of the species. The conclusion that a poriferan is more radiosensitive than a chordate (the urochordate sea squirt, *Molgula manhattensis*) is contradictory to general idea that “higher” organisms are more radiosensitive (12, 73). If these results can be reproduced with consistent end points, it might be necessary to make a distinction between lower chordates and the vertebrata with respect to radiosensitivity. However, it is more likely that there is simply extensive variation within and across taxa and that such generalizations are nonrepresentative.

**Effects of Age, Sex and Size**

It is also of crucial importance to consider the life stage, sex and size of aquatic invertebrates used in experiments, as these factors have important implications for absorbed dose. To date, the impact of age and size on irradiation of aquatic invertebrates has been examined only in two species of aquatic snail, *Astralorbis glabratus* (74) and *Physa acuta* (75). Although this data set is not extensive, both studies found that older snail embryos were considerably less radiosensitive (Table 2). Older embryos whose growth had been retarded by exposure to radiation were as sensitive as younger ones of similar size (74), indicating that size is potentially the more important factor in determining radiosensitivity under these conditions. However, without accurately determining adsorbed dose or examining metabolic rate and other physiological changes as a function of development, it is possible that alterations in any of these factors may still be responsible for the observed effect. It is also important to consider that for organisms with significantly different life spans, the same total dose, dose or dose rate might have altered impacts, as a greater or lesser proportion of the organism’s life occurred during exposure. Improved clarity in reporting should ensure that any such differences are readily apparent and allow for more effective comparisons.

In addition to effects of age or size, several studies in aquatic invertebrates have shown generational effects on radiosensitivity, i.e., that the F1 and/or F2 generations are more radiosensitive than the parental generation (40, 42, 43, 62, 76). This indicates that exposure of parental generations should also be taken into account when considering radiosensitivity. Finally, the impact of sex on radiosensitivity has been assessed in one aquatic invertebrate, the shrimp, *Panaeus japonicas*, where males are more susceptible to reproductive effects after exposure to 10–20 Gy of γ radiation from 60Co (77). This finding is in line with some earlier work on fruit fly (*Drosophila*) and mammalian (*Muntiacus*, Indian barking deer) model organisms, where males have been shown to be more radiosensitive with respect to cytogenetic end points after exposure to external radiation sources (78, 79). Such effects need to be investigated further in aquatic invertebrates.

**FIG. 4.** Categories of species used in laboratory studies on the effects of ionizing radiation on aquatic invertebrates from 1950–2010. Articles examining two or more species were counted multiple times.
BIOLOGICAL END POINTS STUDIED

For the purposes of data analysis, biological effects were grouped under the following headings: (1) mortality, (2) morbidity, (3) reproductive effects and (4) genetic and cellular effects. These umbrella end points are considered to be crucially important in assessing the impact of ionizing radiation on the natural biota and are defined in detail elsewhere (e.g. 80).

Mortality

Most of the studies on the effects of ionizing radiation on aquatic invertebrates focus on mortality as the main parameter (Table 3). It is important to remember that mortality is an important parameter for acute, high-dose situations but that it may not be relevant in chronic exposure scenarios. Decreased survival after irradiation has been reported in aquatic invertebrates at acute doses of ≥5 Gy (81) and at chronic dose rates of ≥0.3 mGy h⁻¹ (40). It is possible to use LD₅₀ values to coarsely rank species by their radiosensitivity (Table 2) (12, 82). However, a look at the LD₅₀ data in aquatic invertebrates reveals some of the problems with this approach. Artemia species are the most radioresistant organisms listed in Table 2, but out of 17 studies published on the effects of ionizing radiation on Artemia, the encysted eggs, which are extremely resistant to most stresses, were irradiated in nine of the studies (83). The explanation for the increased radiosensitivity of early life stages is that they are the most metabolically active with fast cellular turnover (84). It is therefore important to note that encysted Artemia eggs are dormant and have reduced metabolic processes (85). Consequently, in this species, older life stages are probably more radiosensitive than the eggs, as is the case in the freshwater calanoid copepod, Diaptomus clavipes (81). Although all the studies that report LD₅₀’s for Artemia used eggs, an adult population exposed to 500 Gy of 137Cs radiation (137Cs) survived for approximately 16 days (76). This suggests that 30-day LD₅₀ calculations for adults might have returned results similar to those for other crustaceans (Table 2). The use of LD₅₀ data to label entire phylogenetic groups as radioresistant, or otherwise, is also flawed. For example, based on LD₅₀ data (Table 2), the Crustacea contains both the most radioresistant (Palaeo-

<table>
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<td>6 days⁺</td>
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Note: ⁺wet; ⁶dry.
*Time at which 50% mortality occurred.
⁺dpf: Days postfertilization.
⁺4-blastomer.
⁺Trocophore.
monetes pugio) and the most radioresistant aquatic invertebrate (Artemia salina). Even if we exclude Artemia, the amount of interspecific variation in radiosensitivity within the Crustacea is staggering. For example, in one of the few studies comparing the effects of ionizing radiation on multiple species, Engel (86) calculated 40-day LD₅₀'s for five species of Crustacea that varied by over 400 Gy.

There are also many technical issues with the use and calculation of LD₅₀ values, which limit the use of these data. Engel and Davis (206) found that the sea snail, Ilyanassa obsoleta, has a biphasic survival curve after irradiation with ⁶⁰Co γ rays. These curves are normally sigmoidal, and deviation prevents the calculation of an LD₅₀. LD₅₀ values are also greatly influenced by the time for which they are calculated. Bonham and Palumbo calculated the same LD₅₀ of 200 Gy for the gastropods Lymnaea japonica and Nerucella lamellosa after 1 week. Subsequently, only half of the L. japonica population survived to 40 days at 80 Gy, in contrast to the N. lamellosa population, which had an LD₅₀ of 130 Gy after 160 days. One way to lessen this problem is to use mean lethal dose–time curves such as those produced by White and Angelovic for multiple marine species (72). However, this still relies on the generation of multiple LD₅₀ values and tends to oversimplify the biological information.

Although not always comparable for the reasons discussed above, LD₅₀ values for aquatic invertebrates range from 2.1 to 2780 Gy (Table 2). These figures are consistent with some aquatic invertebrates being more radioresistant than mammals [LD₅₀'s of 1–15 Gy (12, 87)] and fish [LD₅₀'s of 7–60 Gy (87)] when mortality is considered. Yet, using the same approach, the most radiosensitive crustacean, the grass shrimp, has a 40-day LD₅₀ of approximately 2 Gy, similar to that of the more radioresistant mammals and lower than that of fish. When reviewing the data for this paper, it became apparent that the periods used to generate LD₅₀ data in aquatic vertebrates vary dramatically (Table 2). As this parameter can have a pronounced impact on the data, it is essential that future studies either use standard periods (e.g., OECD guidelines) or are explicitly clear about their methodology (i.e., clearly state radiation exposure conditions, including duration of exposure and life stage of test organisms at exposure).

Morbidity

Morbidity can be defined as any detrimental impact on biological processes that causes a decrease in fitness of an organism, in either the short or long term. Several studies on aquatic invertebrates have found such sublethal effects on individuals. The end points most commonly studied after exposure to ionizing radiation are respiration rate/oxygen consumption (39, 40, 88), body mass (39, 40, 89, 90), moultng patterns (41, 91–93), and behavior (46, 93) (Table 3). The lowest reported dose rate to have produced an effect in an aquatic invertebrate is that which altered moultung in the goose barnacle, Pollicipes polynemus (91). Using calculations for HTO described elsewhere (44, 45, 94), the exposure reported in the study results in a dose rate of 1.59 × 10⁻³ Gy year⁻¹. At first, this appeared to be well within the zone of biological uncertainty; however, the authors stipulated that these doses were in addition to background levels, which were not quantified. For chronically exposed aquatic vertebrates, sublethal effects were reported at dose rates that fall within the zone of obvious action (>4 Gy year⁻¹ (3)). Oxygen consumption is the most sensitive end point, showing decreases in Daphnia magna at 0.99 mGy h⁻¹ after 23 days (39) and at >1.5 mGy h⁻¹ after 6 days (40). Other responses were reported at slightly higher doses; for example, 8.5 mGy h⁻¹ of γ radiation (⁶⁰Co) caused a decrease in growth of new tissue in sponges (65). Acrately irradiated aquatic invertebrates can show dramatic changes in behavior. For example, blue crabs (Callinectes sapidus) exposed to between 40 and 640 Gy showed immediately decreased irritability, and those exposed to the highest dose became catatonic and never fully recovered (92). Similarly, juvenile freshwater snails (Lymnaea sp.) ceased crawling activity when exposed to X-ray doses <10 Gy (46). Apart from these limited studies, there is a paucity of information on the morbidity of aquatic invertebrates exposed to ionizing radiation. Future work in this area would benefit from careful selection of appropriate end points (with real biological relevance) in line with the parallel approach in human and mammalian radiobiology.

Reproductive Effects

The long-term survival of a species depends on its reproductive success (i.e., fertility and fecundity). Consequently, alterations to reproductive processes are classified as among the most significant sublethal effects a contaminant can cause (19, 95–97). Aquatic invertebrates exposed to dose rates corresponding to the zone of damage to ecosystems (4–>3000 Gy year⁻¹) show significant decreases in reproductive output with a corresponding impact on populations (32, 43, 51), in agreement with Polikarpov's model (3). In aquatic invertebrate radiobiology, the most commonly studied reproductive end point is fecundity (i.e., the ability of an organism to produce viable gametes; Table 3). However, the specific effect used to measure fecundity can vary. For example, in aquatic invertebrates exposed to¹³⁷Cs γ rays, it has been reported as reduced brood size in Daphnia magna after exposure to 0.38 mGy h⁻¹ for 23 days (41), reduced production of eggs and egg sacs in the polychaete, Ophryotrocha adilena, at 3.2 mGy h⁻¹ over 7 generations (98), and alteration in polyvitelline egg production in Physa heterostropha, a freshwater snail, exposed to 10 mGy h⁻¹ (99). As a result of the use of these different measures of fecundity, it is essential that authors clearly define the parameter they are using in addition to providing background or control data.

Other reproductive parameters can be considered in addition to fecundity. These can provide valuable infor-
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Table 3 Continued
information about the quality of offspring, in contrast to measures of fecundity, which tend to focus on the number produced. Changes to other endpoints can have repercussions that are just as ecologically relevant as a decrease in fecundity. For example, Hagger et al. (45) found that mussel larvae exposed to $\gamma$-radiation showed a significantly increased incidence of developmental abnormality. If such abnormalities are sublethal, they may reduce the ability of offspring to mature normally and produce a subsequent generation. If, however, they are lethal, they may result in a reduction of the population size. If development is significantly delayed, as in sea urchin embryos exposed to X rays (100), eggs or larvae may spend more time at their more vulnerable stages and may be more likely to be preyed upon (101). Tsytsugina and Polikarpov (102) found an alteration in reproductive mode (i.e., an increase or decrease in the relative frequencies of asexual and sexual reproduction), in three species of oligochaetes ($Dero$ obtusa, $Nais$ pseudobtusa and $Nais$ pardalis) exposed to $14 \mu$Gy h$^{-1}$ of combined $\gamma$ and $\beta$-particle radiation (in the field). It has been reported that $\beta$-particle radiation ($^{32}$Po or $^{65}$Zn) can skew the sex ratio of Artemia offspring, although these results were not consistent, with one experiment favoring males (55) and one producing more females (56). If these effects occur in natural populations, the overall reproductive output, population structure and genetic diversity of future generations could be severely affected. Alternative reproductive effects may also occur at doses lower than those that cause an outright decrease in fecundity. For example, Alonzo et al. (39) reported decreased egg mass in Daphnia magna at dose rates of $20 \mu$Gy h$^{-1}$ ($^{241}$Am $\alpha$ particles).

These studies illustrate that effects that do not reduce the overall recruitment of new individuals to the population (i.e., no reduction in fecundity) may still have profound effects. The available data do not support the conclusion that reproduction in fish is more sensitive to ionizing radiation than in aquatic invertebrates. In fish, reproductive effects have been reported ranging from reduction in testes weight

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<td>Apop</td>
<td>Induction of apoptosis</td>
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<td>Cilia</td>
<td>Changes in epithelial cilia motility</td>
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<td>SCGE</td>
<td>Single-cell gel electrophoresis (comet assay)</td>
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Notes:
- Mort: Mortality of exposed individual (not descendants)
- Hist: Histological changes
- RTS: Resistance to starvation
- Moul: Changes in moulting
- GMS: Changes in growth, mass or size
- Ox: Alteration in oxidative stress response
- ION: Changes in ionic or osmotic regulation
- HBs: Changes in haemoglobin absorption spectrum
- Behav: Behavioural changes
- Resp: Alterations to respiration
- Cyto: Cytotoxicity
- Apop: Induction of apoptosis
- Cilia: Changes in epithelial cilia motility
- SCGE: Single-cell gel electrophoresis (comet assay)
- Gene: Alterations in gene expression
- MN: Induction of micronuclei
- Hist: Histological changes
- NAS: Alterations in nucleic acid synthesis
- SCEs: Induction of sister chromatid exchanges
- Fec: Alterations in fecundity
- T-H: Changes in time to hatch
- ECP: Changes in embryo or larval survival
- M/F (F1): Changes in gender ratio of progeny
- Dev Ab: Induction of developmental abnormalities
- E/L Surv: Alteration in embryo or larval survival
in plaice (*Pleuronectes platessa*) at 240 μGy h⁻¹ (103) to sterility of offspring at 7700 μGy h⁻¹ (104). The NCRP (104) reported reduced fertility in fish at dose rates of 2700 μGy h⁻¹ and stated that significant negative reproductive effects are unlikely to be found below 1000 μGy h⁻¹. A more recent field study of silver carp (*Hypophthalmichthys molitrix*) in the Chernobyl cooling pond reported reproductive effects at lower dose rates (<100 μGy h⁻¹ (105)), however, this may be due to interaction with other contaminants (12). In comparison with the data on aquatic invertebrates, as outlined above, it appears that ionizing radiation can cause reproductive effects in both aquatic invertebrates and fish at similar dose rates. It is important to remember, however, that in most of these cases the specific end points do not correspond, making comparisons invalid and underlining the importance of true comparative studies.

### Genetic and Cellular Effects

Genetic studies into the effects of ionizing radiation on aquatic invertebrates have focused on cytogenetic effects, including induction of chromosomal aberrations (34, 45, 102–105), micronuclei (44, 58) and sister chromatid exchanges [SCEs (45, 108, 109)]. Other biochemical and molecular parameters that have been examined include induction of DNA strand breaks (44, 110), changes to nucleic acid synthesis (100), and gene expression profile (111, 112). The lowest dose rate so far reported to cause genetic changes in an aquatic invertebrate is 12 μGy h⁻¹ from HTO, which induced DNA strand breaks and micronuclei in *Mytilus edulis* (44). However, Hagger *et al.* reported induction of SCEs and chromosome aberrations at doses of 0.03 mGy in embryo-larvae of *M. edulis*. Although dose rates were not explicitly stated in their study, this equates to dose rates of <2 μGy h⁻¹ (45). This is considerably lower than the 400 μGy h⁻¹ dose rate at which the International Atomic Energy Agency (IAEA) predicts no effect would be expected (113). Andersson *et al.* (114) recommended a generic no-effect dose limit of 10 μGy h⁻¹. Based on the data of Hagger *et al.* (45), it seems inappropriate to conclude that this is an appropriate no-effect dose rate limit, at least for *Mytilus edulis*. In contrast, work on fish indicated that plaice (*Pleuronectes platessa*) exposed to much higher dose rates of γ radiation (up to 240 μGy h⁻¹) showed no genotoxic damage in terms of micronucleus induction or flow cytometry measurement of nuclear DNA in erythrocytes (103). This indicates that fish may be less susceptible to genetic damage from ionizing radiation than aquatic invertebrates; however, the radiation sources and exposure scenarios differ, and the sensitivity of micronucleus induction (a cell cycle-dependent phenomenon) in fish erythrocytes has been questioned (19), thereby rendering the comparison invalid. Overall, not enough data are available to establish no-effect dose rates for organism-specific groups (114). An important point when considering studies on the induction of genetic damage is that mutations in the somatic cells may cause cell death or malignancy, but they are relatively unimportant in the context of ecotoxicology in comparison to mutations in the germ cells (97). In acute, high-dose exposures, heritable mutations may not have the predicted deleterious effect at the population level due to selection pressure rapidly eliminating them (96, 115, 116). Yet in chronic, low-level exposure situations, induced heritable mutation could potentially alter the reproductive output of a population (19, 95, 96, 117).

Some of the work on aquatic invertebrates has focused on the direct DNA-damaging effects of irradiating embryos or larvae (45, 100, 108, 109). In addition, work on chromosome aberrations in larvae from irradiated parents has provided an indication of genetic damage to germ cells (34, 106). Given the fact that ionizing radiation is known to be a powerful mutagen/clastogen, it is surprising that there have not been adequate studies on its genotoxic effects in aquatic invertebrates. While terrestrial invertebrates, such as grasshoppers, were studied extensively in the 1950–1970s to elucidate radiation-induced chromosomal damage in germ cells (118), there was a limited amount of work in this area on aquatic invertebrates [e.g., on irradiated *Chironomus riparius* (34, 106)]. By the 1980s, Harrison *et al.* (108) were using the combination of 5-bromodeoxyuridine (BrDU) and fluorescence plus Giemsa (FPG) staining, which was originally developed for mammalian studies, on aquatic invertebrates. It is worth noting that despite ionizing radiation (external radiation) being a poor inducer of SCEs, radionuclides have been found to enhance this cell cycle-dependent cytogenetic damage (45). This indicates that despite technical difficulties [e.g., obtaining metaphase spreads in aquatic species (96, 116)] this technique could be used to evaluate cytogenetic effects of environmentally relevant radionuclides. In addition, there are now rapid, sensitive techniques to measure genetic damage, such as induction of micronuclei, DNA strand breaks and changes in randomly amplified polymorphic DNA (RAPD) profiles (19, 44, 119–121). These have all been used to demonstrate genotoxicity in aquatic invertebrates exposed to ionizing radiation (44, 45, 58, 110). Another emerging area of importance in genetic ecotoxicology that could be readily applied to future work is ecotoxicogenomics, i.e., the transcriptomic, proteomic and metabolomic responses to radiation (122). Gene expression techniques have also been applied successfully to field studies examining the effect of environmentally relevant exposure to ionizing radiation (111, 123). As the technology for such work is no longer limiting, and is easily transferable, it is likely that studies using a molecular approach (e.g. 124) in combination with classical biological responses will be a key area of future development in aquatic invertebrate radioecology.

There are even fewer studies on the cellular or molecular effects of ionizing radiation in aquatic invertebrates than for genetic and cytogenetic impacts. Hagger *et al.* (45)
demonstrated cytotoxicity in Mytilus edulis exposed to β-particle radiation (HTO; 1.2 μGy h⁻¹) using the proliferation rate index (PRI) in the embryo-larval stages. Otherwise, studies on the cellular or molecular effects of exposure to ionizing radiation in aquatic invertebrates have focused mostly on in vitro effects. One such area that has been explored is the induction of apoptosis in irradiated aquatic invertebrate cells. Apoptosis, or programmed cell death, is a highly conserved biological process and is a key immune response in many aquatic invertebrates (125). Lobster cells (126) and Artemia oocytes (127) showed evidence of apoptosis after γ radiation (60Co) by histological examination. There are now biochemical techniques for the detection of apoptosis, such as the TUNEL assay or the measurement of caspase activity (128–131). A combination of histological and biochemical tools (i.e., multiple biomarkers) could be used (both in vitro and in vivo) to quantify the apoptotic response of aquatic invertebrate cells to ionizing radiation, especially at low (environmentally relevant) doses. Patel and Patel (132) reported that in vitro exposure of hemoglobins from marine bivalves to γ radiation (60Co) at a dose rate of 2760 Gy h⁻¹ revealed a surprising difference in radiosensitivity, as indicated by decreased spectroscopic absorbance. The extracellular hemoglobin of the false cockle (Cardita antiquata) was found to be considerably more resistant than the intracellular hemoglobin of the blood cockle, Anadara granosa (132). Other techniques used to assess cytotoxicity such as Trypan blue exclusion and immunological assays (e.g., measurement of phagocytosis) do not appear to have been investigated in aquatic invertebrates after exposure to ionizing radiation. Research in these areas has the potential to advance our knowledge on the cellular, subcellular, and mechanistic effects of radiotoxicity in this group.

In contrast to mammalian studies, there has been a very limited program to develop and maintain invertebrate cell lines (77). Despite this, some recent studies used primary cultures of invertebrate cells to compare the effects of exposure to ionizing radiation on different species (78, 79). In this context, hemolymph from the lobster (Homarus gammarus) and coelomic fluid from the lugworm ( Arenicola marina) showed similar dose–response profiles after in vitro exposure to 0–5 Gy of X rays (78). Lyng et al. (79) examined the induction of apoptosis in response to in vitro exposure of cells to 60Co γ radiation (0.5 and 5 Gy). The hematopoietic cells of the Norwegian lobster, Nephrops norvegicus, showed a higher level of apoptosis (determined by morphological changes) at a total dose of 0.5 Gy compared to granulocytes from a fish (rainbow trout, Oncorhyncus mykiss) at the same dose. Interestingly, the authors concluded that this result supports the conclusion that invertebrates are more radioresistant, because their cells have a protective response (i.e., can undergo programmed cell death before permanent damage occurs). While this may be the case, currently there are no in vivo data comparing the effects of ionizing radiation on these two species, and it has not been verified that the invertebrate is actually more radioresistant at any higher level of biological organisation. This illustrates that although in vitro work has the potential to address many questions on the radiobiology of aquatic invertebrates (particularly on the mechanisms of radioresistance and radiotoxicity) while reducing the number of animals used experimentally, it is essential that it is complemented by more ecologically relevant in vivo work.

One of the main advantages of studies focusing on genetic, molecular, and biochemical aspects is that they enable the detection of effects at much lower doses compared to those at the individual or population level (122, 133). Results in aquatic invertebrates support this idea, as the observations of Hagger et al. (45), discussed above, occurred at very low dose rates (within Polikarpov’s zone of physiological masking). Early detection enables researchers and regulators to see the threshold at which problems start to occur and has the potential to open up new possibilities for management and repair of contaminated ecosystems. In this context, one of the problems that has been identified with studying cytogenetic effects on aquatic organisms is the fact that most tissues of mature organisms have low mitotic activity and slow cell cycles (96, 104, 116). Other biochemical and molecular techniques such as the comet assay do not require actively dividing cells; thus they are ideal candidates to measure genotoxicity in these cases (19).

FIELD STUDIES

Although most of the data on the effects of ionizing radiation in aquatic invertebrates has come from controlled laboratory studies, there is a small body of work that looked directly at natural populations that have been exposed to ionizing radiation. This irradiation has usually taken place over a long period, such as in animals exposed to cooling waters discharged from nuclear sites (111, 134) or in the decades after an acute exposure, such as the Chernobyl accident (135). In accidental discharge situations, it is not always possible to carry out field studies for safety reasons, or gathering biological effects data is not a priority because the focus is on remediation. To the best of our knowledge, the only field study looking at radiation-induced effects in aquatic invertebrates in the short term after the Chernobyl accident was that of Florou et al. (107). They analyzed platyhelminthes samples ( Plagiostomum girardi) collected from the North Aegean Sea in December 1987, 20 months after the Chernobyl disaster, which deposited an estimated 20 kBq m⁻² of 137Cs over the area (136). The authors reported an increase in the number of cells with chromosme aberrations. Other than this, there appear to be no field data on acute exposure of aquatic invertebrates to ionizing radiation in the natural environment. Although the EPIC (Environmental Protection from Ionizing Contaminants in
the Arctic) database (137) may contain such information for Russian-speaking colleagues, translation/publication in English would be a valuable resource.

In contrast, several studies have examined the effects on field populations of aquatic invertebrates subjected to longer-term irradiation (111, 134, 135). All three of these studies report that overall no ecologically detrimental effects were exhibited by the irradiated populations. Cooley et al. (134) reported that a population of the pond snail, Physa heterostropha, that inhabited a lake contaminated by waste from the Oak Ridge National Laboratory showed decreased egg capsule production. Despite this, there was no overall decrease in fecundity because the population had apparently adapted to counteract the effect by producing more eggs per capsule (134). This occurred at the higher end of the ecological masking zone, at an estimated dose rate of 2.37 Gy year⁻¹, and indicates that in this species Pollakarpov’s model accurately predicts the effect of chronic irradiation—individuals are affected but the overall effect is masked by ecological changes. Farcy et al. (111) found no statistically significant difference for expression of selected genes (e.g. heat-shock proteins, superoxide dismutase and metallothionein) between oysters collected from exposed (due to the AREVA reprocessing plant at La Hague) and reference parts of the English Channel/Atlantic Ocean. Similarly, Murphy et al. (135) reported that variation in macroinvertebrate diversity and species abundance (including mollusks) in contaminated lakes around Chernobyl (sampled in 2003) was not associated with the external radiation dose. Despite this apparent consensus, it is worth reiterating that these studies examined different end points in different species.

Additionally, there can be technical problems with field studies. The calculation of dose to the biota can be difficult, particularly when the input of radionuclide(s) has varied with time. The ERICA tool has recently been used to model dose to the biota after the accident at Fukushima (61), and this may prove to be a useful tool in future field studies. The analysis of body tissues for radionuclide content can also provide some information on dose; however, it is important to remember that when the biological half-life is short, this analysis provides only a “snapshot” of the organism’s exposure. Unless the field area has been monitored prior to sampling, it is also possible that other contaminants or stressors may be contributing to the effect of radionuclides on aquatic invertebrates.

In a complex environmental situation, where contaminants could be present in many different combinations, it is important to determine the relative contributions of each contaminant and their potential interactions. For example, the exposed snail (Physa heterostropha) population in the 1969 field study of Cooley (134) had a higher average water temperature than the reference population, which led to increased fecundity. Similarly, Farcy et al. (111) attributed the lack of an effect in their findings for gene expression in oysters to environmental variation, particularly temperature (seasafonality). Transplantation of field-exposed animals (to either an alternative location or the laboratory) is one way to ensure that environmental parameters are consistent across exposure groups (134), and this technique has been used successfully to examine the population genetic structure of mosquitofish (Gambusia affinis) exposed to radionuclides in situ (138). Currently, field studies may generate more questions than they answer, and the difficulty in extrapolation of laboratory data to the field was identified as a key knowledge gap by the ERICA group (139). In future, as laboratory studies provide more information on the potential modifying effects (i.e. synergistic, antagonistic, additive or protective) of environmental factors on radiotoxicity, field studies on aquatic organisms will become less cryptic. They will then present an opportunity to study the ecosystem-level effects that are very difficult to recreate in the laboratory and have the potential to provide the crucial link between laboratory results and ecological application (6).

**INTERACTION OF IONIZING RADIATION WITH OTHER FACTORS**

**Temperature**

Invertebrates inhabit many different niches within the aquatic environment, and many species have geographical ranges that span entire continents or oceans. For example, the greater pond snail, Lymnaea stagnalis, is found in ponds across Europe (140), and Antarctic krill, Euphausia superba, have a circumpolar distribution in the Southern ocean (141). In some cases, a single individual can tolerate an extraordinary range of conditions. For example, the marine mussel, Mytilus edulis, can survive at temperatures as low as −10°C (142) and as high as 27°C (143). It is well known that environmental factors such as temperature, salinity and dissolved organic material can have pronounced effects on the toxicity of pollutants such as metals and pesticides (144–146). When investigating the effect of radiation on the morphology of developing pinfish (Lagodon rhomboides), White (147) noted that radiation, salinity and temperature produced significant interactions. In this context, Woodhead (148) recommended that “as many ecological parameters as possible” be studied to understand the “ecological effects of natural and man-made variations” on the effects of ionizing radiation. Yet there are still relatively few studies that have incorporated this into work on either aquatic invertebrates or other groups of organisms.

In general, it has been agreed that radiation produces damage faster at higher temperatures because the metabolic rate is increased (149, 150). It has been widely reported that an increase in temperature can cause an increase in radiosensitivity in fish (151–153). The available data for mortality in aquatic invertebrates are consistent with this. For snails (Physa heterostropha), a rising temperature of 30°C decreased life span by two-thirds compared to 10°C
Physa also mask the effect of radiation (affected at the same dose). The influence of temperature may show no effect on life span at doses of 10 Gy h at 28°C developed faster but had an increased frequency of abnormalities and decreased survival compared to those reared at 20 and 24°C (157). In aquatic snails of the genus Physa, both Ravera (154) and Cooley (99) reported that higher temperatures caused a more severe effect on fecundity. Engel et al. (158) concluded that the effect of radiation on ionic regulation in Callinectes sapidus was influenced by the crabs’ environment before and after irradiation. It was suggested that temperature had more of an effect than salinity; however, the exact impact of temperature was difficult to determine due to the complex interactions. The hatchability of dried Artemia salina eggs is greater if they are stored at 25°C as opposed to on dry ice (52), so it would appear that—at least for this species and life stage—there is a threshold temperature at which a cooler environment becomes detrimental. This is unsurprising, as at freezing temperatures organisms experience additional stress. Another exception to the general rule was provided by Cooley (99), who noted that, despite the negative effect on fecundity, elevated temperature resulted in a slight protective effect with respect to the adult snail life span. Individuals of Physa heterostropha irradiated at 25°C showed no effect on life span at doses of 10 Gy h (60Co γ rays), whereas those irradiated at 15°C were negatively affected at the same dose. The influence of temperature may also mask the effect of radiation (99), making these studies complex and reinforcing the need for suitable controls.

Thermal discharge from nuclear facilities is considered to be one of the most important environmental issues surrounding these establishments, second only to the release of radionuclides (159). A comprehensive understanding of the effect of temperature on radiotoxicity is therefore essential for protection of the environment. This is especially true for sessile aquatic invertebrates as they are unable to respond behaviorally to temperature increases (i.e., by moving to cooler areas). Furthermore, climate change is one of the biggest issues facing environmental protection today. With sea surface temperatures forecast to rise by 0.5–3.5°C in the next 30–100 years (160), the damaging effects of radiation are likely to vary accordingly. In this context, it is clear that there is a pressing need for more data on the consequences of increasing temperature and ionizing radiation in aquatic invertebrates and aquatic organisms as a whole.

Salinity

The NCRP and IAEA have stated that there is no evidence for disparity between radiation responses of marine and freshwater animals (104, 161). Consequently, aquatic animals are not categorized separately for the purposes of environmental radiological protection. Nonetheless, for euryhaline organisms, salinity can have a pronounced and complicated effect on their ability to tolerate the stress of irradiation. Although changes in salinity and temperature created complex variations in ionic regulation in irradiated crabs (Callinectes sapidus), temperature was thought to be the more influential variable (158). This species is known for its tolerance to both radiation and salinity; it has LD50’s of 420–510 Gy (Table 2), can be found at salinities up to 117‰ (162), and is therefore unlikely to be representative. Similar work by the same group of authors on the less tolerant species of grass shrimp, Palaemonetes pugio, illustrates this point. The grass shrimp has much lower radiation LD50’s, and these are dependent on salinity, with individuals at the lowest salinity (15‰) tolerating the highest dose of radiation (6 Gy) (163). Despite their differential response to irradiation at various salinities, both Callinectes sapidus and Palaemonetes pugio showed changes in osmoregulatory capacity after irradiation (158, 163). This indicates that in addition to salinity affecting radiation tolerance, radiation also interferes with the capacity of these organisms to deal with changes in salinity. Grosch looked at the interaction between salinity and irradiation in Artemia (55, 56). As predicted for a species adapted to hypersaline environments, adults in seawater supplemented with sodium chloride (~90‰) survived longer after irradiation than those in normal seawater (~3‰). Furthermore, individuals kept in diluted seawater (~13‰) had the shortest post irradiation survival time. In contrast, Angelovic and Engel (88) found that both high salinity (200‰) and high radiation dose (60Co γ rays, 800 Gy) had a detrimental effect on Artemia salina. Both these conditions decreased the respiration rate of nauplii, and when combined they acted synergistically to produce the lowest respiration rate observed in the study. Two hundred parts per thousand is considered to be the maximum tolerable salinity for most strains of Artemia salina (66), so the organism is likely to be experiencing osmotic stress at this level. This could explain the negative effects of higher salinity, though as the studies looked at different end points, it is not possible to rule out similar alterations in respiration rate at 13–90‰. This underlines the importance of considering a range of end points when investigating the effects of ionizing radiation on any group of organisms. This is particularly the case for aquatic invertebrates, where the data are scarce and as a result effective comparisons are often not possible. In addition to the above-mentioned abiotic factors, there are other known environmental stressors that could be examined in conjunction with radionuclide exposure. One such example is oxygen.
concentration (e.g., hypoxia and hyperoxia), which has been shown to affect physiological, histopathological and DNA damage in fish (164). Given the increasing number of “dead zones” in coastal waters (165), their interaction with ionizing radiation could have implications for observed biological responses.

**Biotic Factors**

Biotic factors (such as inter-/intraspecific competition, parasitic infection, genetic makeup of individuals and periods of starvation) can also have a pronounced impact on the effect of ionizing radiation on aquatic invertebrates. The work of Marshall (42, 43) reveals the importance of intraspecific competition on the effect of radiation in the freshwater flea, *Daphnia pulex*. The average weight of an individual at any given age increased with radiation dose rate, from 21.3 µg at 0 Gy h⁻¹ to 37.8 µg at 215 mGy h⁻¹ (43). The author attributed this somewhat surprising result to the increased availability of food in populations at higher radiation doses, because of increased mortality (i.e., less competition from other individuals). Unfortunately, as food consumption was not measured directly, no correlation between mortality and food availability was reported. In a similar study, however, where *Daphnia magna* were kept in isolation, no such effect was found (41). Scarcity of food is a relatively common biological stress, and it may occur in situations of radiological contamination if prey is more radiosensitive than its predator, potentially leading to an imbalance in trophic-level structure. Consequently, resistance to starvation is an ecologically relevant end point for consideration in radioecological studies. *Daphnia magna* larvae show decreased resistance to starvation after parental exposure to external γ radiation (41) or waterborne α-particle radiation (39). A significant reduction in the mass rather than the number of offspring suggested that this effect was caused by decreased parental investment as a consequence of chronic irradiation (39). Although no alteration in ingestion rate of adult *Daphnia magna* was reported (39), the increased metabolic cost of coping with irradiation suggests that adults would have had similar problems coping with a reduction in food. Examination of the metabolic cost of radiation exposure and its knock-on effects on population dynamics in a range of aquatic invertebrates (multiple life stages) using computational models should be explored.

**Other Pollutants or Contaminants**

In addition to changes in the physical aspects of their environment, aquatic invertebrates can potentially be exposed to nonradioactive contaminants in conjunction with ionizing radiation. This is especially the case in highly industrialized areas surrounding nuclear facilities, where radionuclides may be discharged in conjunction with stable chemicals, including heavy metals. Additionally, many industrial contaminants are persistent and can bioaccumulate late, meaning that co-exposure with ionizing radiation may result from temporally distinct inputs. To our knowledge, there are no laboratory studies investigating the effects of ionizing radiation on aquatic invertebrates in conjunction with other known pollutants. Similarly, only one field study examined the effects of potential mixtures of radionuclides and other pollutants. Hingston *et al.* (110) compared sites that were suggested to be mainly polluted with either radionuclides or industrial chemicals and looked at comet tail length (DNA strand breaks) in lugworms (*Arenicola marina*) collected from the sites. Although differences in DNA damage were observed between the sites, and mixtures of radioactive and nonradioactive pollutants were expected at each site, this was not quantified. Therefore, it is difficult to use the results to draw conclusions on the possible modifying interactions (e.g. synergistic or antagonistic) of these two types of contaminants. While for human radioprotection, the safety of the individual is paramount, from the perspective of environmental interactions. This information would be vital in situations of industrial accidents or pollutant spills, whereby toxic chemicals are introduced into an environment that is already contaminated with radionuclides or vice versa. The potential for other contaminants, especially metals with antioxidant properties, to exert a protective effect in the presence of radionuclide exposure is also worth exploring. In fish, antioxidants have been shown to dramatically reduce radiation-induced damage in zebrafish embryos if administered 3 h before or up to 15 min after exposure to γ radiation from 137Cs (20–80 Gy) (166). There is currently no evidence of this effect in aquatic invertebrates, although selenium has been shown to reduce DNA damage in mussels exposed to mercury (167). Research on this novel subject has the potential to provide solutions for areas of the environment where contamination with radionuclides is unavoidable and would be a very interesting topic for future work on aquatic invertebrates.

**FUTURE PERSPECTIVES**

It is clear that compared to humans there are many inherent constraints when assessing the impact of ionizing radiation on wildlife and in translating the experimental and field data into meaningful, robust radiological protection guidelines (13, 168). For example, as exposure scenarios can vary enormously, relating exposure to absorbed dose and absorbed dose to effect can be very complex (169). Estimation of absorbed dose from different types of radiation also introduces complexity for nonhuman species, where the data on RBE are often lacking (as in aquatic invertebrates). In addition to these difficulties, the approaches for human and nonhuman biota differ conceptually. While for human radioprotection, the safety of the individual is paramount, from the perspective of environment...
Accurate dosimetric models for reference aquatic invertebrates will be a valuable resource for future studies in this area. The ICRP has developed a set of reference plants and animals for use in environmental protection ([168], [169], [171], [172]). For nonhuman biota, the range of geometries for reference organisms is staggering, and so compromises have had to be made. Nonetheless, the ICRP list is limited (6), containing, for example, only one aquatic invertebrate—the Reference Crab. Copplestone et al. (12) recommended a wider range of reference organisms for different ecosystems, based on the report of Woodhead (173). The use of these organisms, or those of similar size and shape to them, is essential to standardize impact assessment. However, it remains important to collect data on the effects of ionizing radiation on additional species, ideally in comparison to the closest reference animal or plant. This should ensure that data for environmental protection remain consistent while interspecies comparisons of radiosensitivity become more robust.

In recent years many real biological phenomena have been identified in association with radiation exposure that have been suggested to have human health implications for present and future generations. Two key themes emerging are the impact of bystander effects (174–178) and transgenerational effects (179–181). While there has been extensive work on these areas in the human health arena, there have been very few studies on aquatic animals. Despite this, both types of effect have been reported in aquatic species. For example, unirradiated fish develop a classic bystander response (induced cell death) after exposure to irradiated individuals (182, 183). Furthermore, the impact of bystander effects has been observed in F1 somatic cells of medaka (Oryzias latipes) after irradiation of paternal spermatozoids (184). To the best of our knowledge there appear to be no studies of radiation-induced bystander or transgenerational effects or germline mutations in aquatic invertebrates; however, recent work on benzo(a)pyrene (B(a)P)-exposed Daphnia magna indicated that alterations in the DNA are passed to offspring (121). Molecular methods, including the RAPD technique used in that study, could become a more important aspect of aquatic invertebrate radiotoxicological studies as our knowledge of the genetic makeup of these organisms increases (96, 121). Additionally, since bystander effects occur predominantly at low doses of ionizing radiation (185, 186), an accurate understanding of their effects is essential before it will be possible to extrapolate from high to low radiation doses (186). This is especially important for groups for which limited data are available and where irradiation occurs at low environmental levels, such as the aquatic invertebrates. Therefore, as the understanding of bystander effects grows, it is likely that this area will become increasingly important in studies of radiation-exposed aquatic invertebrates.

In human and mammalian studies, the field of toxicogenomics examines the differential expression of genes after toxic exposure. A parallel field in environmental risk assessment, known as ecotoxicogenomics, has been proposed (187) and is now emerging as a key component of some ecotoxicological studies (188–190). The resulting techniques (e.g. metabolic profiling, transcriptomics, DNA microarrays) have been applied to work on aquatic invertebrates exposed to various contaminants, including metals ([191]), polyaromatic hydrocarbons ([192]), PAH-like compounds ([194]). Given the known potential for genotoxicity after exposure to ionizing radiation, and as genes for tumor induction and DNA repair processes are generally highly conserved across phyla ([124, 195]), studies using these techniques have the potential to greatly enhance our knowledge of the impact of ionizing radiation on aquatic invertebrates.

As discussed above, the majority of studies on the effects of ionizing radiation on aquatic invertebrates focus on organism-level or reproductive end points ([Fig. 3, Table 3]). This trend is also found across ecotoxicology as a whole (6). It can be very difficult to extrapolate these data to generate accurate predictions about the unit of interest: populations ([196]). Unfortunately, directly measuring effects at the population level requires extensive knowledge of an ecosystem ([196]) and therefore is not always feasible. This issue is one of many currently being incorporated into new approaches to environmental risk assessment ([96]). Moore et al. (1) proposed an approach that uses multiple diagnostic biomarkers that can be examined in laboratory-based tests and used to link multiple levels of biological organization. Genetic and cellular biomarkers are more responsive and readily detectable, but lack the ecological significance of the more difficult to measure, higher-level indicators (such as population decline and loss of biodiversity (1)). If we can link the biomarkers at lower levels of biological organization with effects at the higher levels or with the Darwinian fitness of the organism, then it may be possible to use them as surrogates for population-level effects with a much greater degree of accuracy ([e.g. 19, 96, 197]). While such an approach has been developed and used in the human health arena, the adoption of this method for nonhuman species has been limited.

There is also a significant amount of interest in developing ecosystem-based approaches to environmental risk management (6). This method uses biomarkers of effect...
CONCLUSIONS

This review has highlighted that the available information on the effects of ionizing radiation on aquatic invertebrates is scarce and fragmented and that there is considerable variation in radiation-induced effects within the group. Lack of transparency in reporting dose, dose rate and length of exposure makes assessment of dose-rate effects in aquatic invertebrates even more difficult. It is therefore essential that authors explicitly state these factors in future publications to move the science forward. In line with studies carried out in the human health arena and in agreement with the recommendation of Tsytsugina and Polikarpov (102), it is suggested that future work should concentrate on dose rates that fall within the physiological and ecological masking zones. This is where the most environmentally relevant doses occur and where the interactions with other factors (physical, biological or anthropogenic) have the greatest effect on the impact of ionizing radiation. Although observing effects at these dose rates can be very challenging, molecular and genetic end points that have already been linked with observable outcomes have great potential in this area. It is crucial that we have a better understanding of effects at these doses and of how to moderate such effects to protect the environment.

Given the technical difficulties outlined above, it is difficult to generate meaningful estimates for the impact of ionizing radiation on the aquatic invertebrates as a whole, and it has not been possible to perform meta-analyses on the available data. Unfortunately, this makes comparisons of the aquatic invertebrates with other groups unrealistic and purely speculative. One hypothesis is that the long-held paradigm of radiation biology—that invertebrates are more radioreistant than higher taxonomic groups—may be challenged as the data set is improved. To address this question and more, it is important that future research fill the knowledge gaps outlined herein, and it is also necessary to consider adopting an integrated approach, incorporating aspects of ecotoxicology, human and mammalian radiation risk assessment (170), and newer technologies, such as “omics”. The establishment of robust links between effects at differing levels of biological organization is essential, as is the incorporation of end points that monitor ecosystem level effects (i.e. the ecosystem approach). As the aquatic invertebrates span such a diverse a range of organisms, it is essential that future work allow comparisons across phylogenetic groups. Finally, ionizing radiation does not affect the environment in isolation; therefore, it is crucial to determine the potential impact of biotic and abiotic factors. Adoption of these ideas will make the field of aquatic invertebrate radioecology challenging but will allow for a greater understanding of the impact of ionizing radiation on this group of organisms and for better protection of the environment as a whole.

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Relative sensitivity of two marine bivalves for detection of genotoxic and cytotoxic effects: a field assessment in the Tamar Estuary, South West England

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Abstract The input of anthropogenic contaminants to the aquatic environment is a major concern for scientists, regulators and the public. This is especially relevant in areas such as the Tamar valley in SW England, which has a legacy of contamination from industrial activity in the nineteenth and twentieth centuries. Following on from previous laboratory validation studies, this study aimed to assess the relationship between genotoxic and cytotoxic responses and heavy metal concentrations in two bivalve species sampled from locations along the Tamar estuary. Adult cockles, Cerastoderma edule, and blue mussels, Mytilus edulis, were sampled from five locations in the Tamar and one reference location on the south Devon coast. Bivalve haemocytes were processed for comet and neutral red retention (NRR) assays to determine potential genotoxic and cytotoxic effects, respectively. Sediment and soft tissue samples were analysed for metal content by inductively coupled plasma mass spectrometry. Sediment concentrations were consistent with the physico-chemical nature of the Tamar estuary. A significant correlation ($P<0.05$) was found between total metal concentration in sediment and *C. edule* soft tissues, but no such correlation was found for *M. edulis* samples. DNA damage was elevated at the site with highest Cr concentrations for *M. edulis* and at the site with highest Ni and Pb concentrations for *C. edule*. Analysis of NRR revealed a slight increase in retention time at one site, in contrast to comet data. We conclude that the comet assay is a reliable indicator of genotoxic damage in the field for both *M. edulis* and *C. edule* and discuss reasons for the apparent discrepancy with NRR.

Keywords Bivalve molluscs · Tamar estuary · Metals · Biomarkers · Comet assay · Neutral red retention

Introduction

Anthropogenic inputs to the environment represent a growing concern for governments, policy makers and the public, particularly in light of increasing global industrialisation and human population growth (Moore et al. 2004; Jha 2004). The marine environment (including estuaries) is often the ultimate recipient of discharged contaminants, and thus identifying and quantifying the potential risks to exposed aquatic biota and human health has become a key challenge for ecotoxicologists, environmental managers and regulatory agencies (Moore et al. 2004). In addition to quantifying levels of contamination with chemical and
water monitoring programmes, sub-lethal biological responses or biomarker studies on key ‘sentinel’ or ‘bioindicator’ species are also essential for environmental risk assessment and protection (Moore et al. 2004; Lyons et al. 2010). In recent years, emphasis on the ecological impact of contaminants has increased. For example, the European Union water framework directive (Directive 2000/60/EC) emphasises the need for ecological quality of the hydrosphere, in particular, focusing on those contaminants which are carcinogenic, mutagenic or show reproductive toxicity (Borja et al. 2004; Fuhracker 2009). In order to assess these effects, it is important to assess the sub-lethal effects in native biota inhabiting potentially different contaminated sites.

It is generally realised that applications of sub-lethal biological responses in native organisms have been implemented in only a few selected organisms (Jha 2008). It is often seen that, at contaminated sites, these species are either absent or, if present, their responses might not be indicative of other species in the community (Cheung et al. 2006; Jha 2008). Marine bivalves, such as the blue mussel (*Mytilus edulis*), are commonly used as bioindicator species in ecotoxicological investigations. Their sessile, filter-feeding mode of life and ease of maintenance in the laboratory make bivalves ideal test species. It is, however, important to bear in mind that sediment is the ultimate depository of contaminants, and as intertidal species, they might not represent the actual biological impact of contaminants on the biota. Many factors have an impact on the biological response to contaminants, including trophic position, feeding methods and habitat; therefore it is imperative to extend the choice of available sentinel species. The fact that bivalves are generally abundant, geographically widespread and display a variety of biological responses when stressed makes them appropriate model organisms in a range of exposure scenarios. In this context, the common cockle, *Cerastoderma edule*, a sediment-dwelling bivalve that is widely distributed from north-east Norway to West Africa is a potentially useful sentinel species. In contrast to *M. edulis*, it is mobile and does not attach to the substrata with byssus threads. Despite its characteristics, there have been a limited number of attempts to explore the usefulness of this species for environmental monitoring purposes (Cheung et al. 2006; Lobo et al. 2010; Jung et al. 2006).

The Tamar estuary is a tidal estuary in the south-west of the UK, extending approximately 22 km landwards from Plymouth Sound, where it meets the sea (Environment Agency 1996). Five rivers (the Tamar, Tavy, Lynher, Plym and Tiddy) run into the Tamar estuary, which then empties into Plymouth Sound (Environment Agency 1996). The Tamar valley is highly mineralised and has been mined (e.g. for Sn, Cu, Pb, Ag and Zn) for the last millennium. In the nineteenth century, mining activities were particularly intensive, and the area was the world’s largest source of arsenic and copper (Shaw and Moore 2011). In addition to mining, industrial influences on the Tamar estuary include various marinas, the city of Plymouth (with ~250,000 inhabitants) and the Devonport Royal Dockyard, where submarine refits are undertaken for the Royal Navy and radioactive waste is discharged. Nutrient enrichment occurs within the upper Tamar estuary mainly from agricultural run-off and sewage discharge (Langston et al. 2003). Chemical data from the Tamar estuary area indicate that, compared with many other estuaries around the British coast, the area contains elevated levels of heavy metals and radio- nuclides in sediments (Lindsay and Bell 1997) and polycyclic aromatic hydrocarbons both in seawater (Law et al. 1997) and in sediments (Woodhead et al. 1999). In addition, as a result of the PREDICT workshop (Shaw and Moore 2011), ecotoxicological effects of contamination on bivalves in the Tamar estuary have been investigated (Bignell et al. 2011; Money et al. 2011; Shaw et al. 2011). The Tamar estuary is designated as ‘An Area of Outstanding Natural Beauty’, and two Tamar Valley estuaries have been designated as Sites of Special Scientific Interest (Langston et al. 2003). Despite the ecological significance of this area and the historical and present input of anthropogenic contaminants, there is currently limited published work on biological responses in native biota. In particular, there is little work that compares sub-lethal toxic responses in different marine species inhabiting the Tamar estuary.

Genotoxic and cytotoxic biomarkers are sensitive sub-lethal indicators of potential environmental toxicity at genetic and cellular levels. In particular, single-cell gel electrophoresis or the Comet assay is a reliable and widely used method for the detection of single- and double-strand DNA breaks at the level of the individual cell. The assay has been implemented in several laboratory and field studies (Jha 2008; Canty et al. 2009). In bivalve molluscs, this assay has been applied to different cell types, including haemocytes.
Furthermore, it is also well established that the genotoxic potency of contaminants or chemicals is closely related to their toxicity at cellular level (i.e. cytotoxicity) for many endpoints (Jha et al. 2000). Cytotoxicity in the haemocytes of bivalve molluscs has also been extensively studied using neutral red retention (NRR) assay developed by Lowe et al. (1995). This assay has been recommended by the International Council for the Exploration of Sea (Moore and Lowe 2004) and has been used in different international monitoring programmes (Moore et al. 2004). Previously, we have compared the relative sensitivity of M. edulis and C. edule haemocytes for induction of DNA damage using the Comet assay under laboratory conditions and found that haemocytes from C. edule are more sensitive (Cheung et al. 2006). In addition, we have also implemented NRR assay in mussels collected from contaminated sites (Cheung et al. 1998). Given our experience with these assays, the goal of this study was to implement them in the field to assess the impact of contaminants on different biota and to evaluate their relative sensitivity.

Against the backdrop of above information, the present study aimed to (1) use the Comet and NRR assays to investigate relative genotoxic and cytotoxic responses in M. edulis and C. edule collected from various sites along the Tamar estuary and (2) determine if any correlation existed between the biomarker responses and levels of metallic contamination present in the organisms, their environment and the ecological niche they inhabit.

Materials and method

Sampling sites

The field sampling for the current investigation was carried out between September and October 2001. Six sampling stations were selected from the Tamar estuary as illustrated in Fig. 1. Site selection was based upon ease of collection for biological and sediment samples in addition to relative location along the estuary. Site 1 was situated at Bantham (Ordnance Survey grid reference: SX 665 438) on the Avon estuary. Relatively little contamination occurs within the vicinity of this site, and an oyster farm is located a few kilometres away—where bivalve molluscs are cultured and farmed for human consumption. This location was therefore considered to be relatively clean and was used as a reference site. Site 2 was located on the shore close to the Torpoint vehicle and passenger ferry terminal (west side of the estuary; SX 442 551). With the exception of the reference site (Bantham), this site was the furthest south and the closest to the sea. Site 3 was located approximately 2 km north-west of Torpoint close to a jetty at Cove Head, Welcombe (SX 434 567). This site was located approximately opposite to the Devonport Royal Dockyard. It was observed that a few small craft were moored at this location. Site 4 was situated at Jupiter Point, at a jetty located on the southern bank of the river Lynher (SX 416 568). It was noted that there were a number of dinghies moored at site 4, which are used for training by the Royal Navy. Site 5 was situated under the Tamar Bridge, close to the marina at Saltash (SX 433 586). Site 6 was situated at Neale Point close to Wearde Quay, where the river Tavy meets the river Tamar (SX 436 612).

Organism sampling

The presence of the species of interest at each potential sampling station was established in advance of the sample collection. In addition, the ease of access to the stations and the distance from the laboratory was taken into account when selecting the sampling stations, to minimise the transportation time and thus the amount of stress placed upon the animals before tissue sampling. Adult M. edulis (4.2–6.2 cm long) were collected from sub-littoral, rocky outcrops at each sampling site at low tide (n=12; 6 for biomarker studies and 6 for metal analyses). The organisms were carefully removed from their substrate by cutting the byssus threads and were then transported back to the laboratory in a cool box to minimise thermal stress. In addition to the sampling of M. edulis, adult C. edule (3.5–4.0 cm from umbo to valve edge; n=12; 6 for biomarker studies and 6 for metal analyses) were collected from the sub-littoral, silty sediments at each sampling site at low tide. The animals were then transported back to the laboratory in a cool box containing damp tissue paper to maintain humidity. On return to the laboratory, the external shells of the mussel and cockle samples were cleaned of epibionts and sediments. Haemolymph samples were extracted from the sampled animals immediately after they had been...
cleaned. The whole animals were then placed into labelled plastic sample bags and stored at −80°C until analysis.

Sediment sampling

Sediment samples were collected at low tide at the same time and close to where the biological samples were collected. Approximately 5 kg of the sediment (wet weight) was collected from each sampling site using a small plastic trowel, transported back to the laboratory in labelled plastic sample bags and then stored at −80°C until analysis.

Water sampling

On the same day that the biological and sediment samples were collected, hydrological parameters (pH, temperature, salinity and dissolved oxygen) were measured in situ at high tide (YSI 550 DO multi-

Fig. 1 a Location of Plymouth within the UK and b location of the sampling stations along the Tamar estuary
incorporated meter; YSI Ltd, Fleet, Hampshire, UK). In addition, approximately 10 L of water was collected from each site for determination of suspended particulate matter (SPM). Sampling of SPM occurred at high tide, avoiding collection of the water surface microlayer. On return to the laboratory, 1 L from each seawater sample was filtered through a pre-weighed 0.45-μm filter assembled in a Buchner funnel filter unit. Filters were then dried in an oven at 40°C for 24 h and re-weighed. The amount of suspended particulate matter was calculated as milligrams per litre of seawater.

Determination of metal content by inductively coupled plasma mass spectrometry

Sediment preparation

Preparation of sediments for metal analysis was carried out as per the methods described by Jha et al. (2000). In brief, the sediment samples were placed in an oven at 60°C and allowed to dry for 7 days. The dried samples were then ground with a mortar and pestle and sieved through a 180-μm nylon mesh. A blank containing only 5 mL of nitric acid and a certified reference material (LGC 6137, Estuarine Sediment; Laboratory of the Government Chemist, Teddington, UK) were prepared simultaneously in an identical manner.

Preparation of bivalve and reference samples

Bivalve samples were prepared for inductively coupled plasma mass spectrometry (ICP-MS) analysis as per the following methods. Whole samples were freeze-dried for 24 h; the soft tissues were then extracted from the shell (and the shells discarded), pooled for each site (n=6) and then pulverised using a pestle and mortar. Blanks were prepared, containing only 5 mL nitric acid and a certified reference material (TORT-2, Lobster hepatopancreas; National Research Council, Canada). Blanks were prepared simultaneously and in an identical manner to samples.

Analysis of sediment and bivalve samples

From each sample, approximately 0.25 g of the sieved sediment was placed into acid-washed beakers and 5 mL of concentrated nitric acid (Fisher Scientific, Loughborough, UK) added. The samples were covered with a sheet of paper to prevent the ingress of extraneous material and left overnight to pre-digest in a fume cupboard. After pre-digestion, the beakers were placed onto a hotplate and boiled until all the biological material had dissolved. The samples were then allowed to cool, after which they were transferred quantitatively into pre-cleaned 25-mL-capacity volumetric flasks. Each sample was spiked with a 0.25-mL aliquot of 10 μg mL⁻¹ indium solution and diluted to volume with Milli-Q water.

Although the nitric acid extraction is insufficient to dissolve the aluminosilicate material, it is capable of extracting 90 % or more of heavy metals, e.g. copper, cadmium, lead, etc. Other analytes would be less efficiently extracted, such as arsenic and chromium, although efficiency is often greater than 50 %. All the dissolved sediment and biological samples were analysed for Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Cd, Hg and Pb using the semi-quantitative analysis software of an ICP-MS instrument (PlasmaQuad PQ+ Turbo, VG Elemental, Winsford, Cheshire, UK). The results of the certified materials (run in parallel) were used to validate the analyses of the biological and sediment samples. Similar calculations were made for most of the elements detected with the exceptions of Al, Ca, Fe, Mg and Na in the sediment samples, since for these analytes, the concentration was so high that the linear calibration range of the ICP-MS instrument did not extend that far.

Determination of biomarker responses

All chemicals were supplied by Sigma-Aldrich Ltd, Gillingham, Dorset, UK, unless otherwise specified.

Sampling of haemolymph

From each individual of both species, 0.20 mL of haemolymph was extracted from the posterior adductor muscle via a 21-gauge hypodermic needle into a 0.50-mL syringe pre-filled with 0.20 mL physiological saline (20 mM HEPES, 435 mM NaCl, 100 mM MgSO₄, 10 mM KCl, 10 mM CaCl₂, pH=7.36). Each sample was transferred into siliconised microcentrifuge tubes (2 mL) and held on ice to minimise cellular stress.
Single cell gel electrophoresis or the Comet assay

Following cell viability assessment using Eosin Y (samples showed viability >90%; data not included), the Comet assay was carried out according to the methods of Cheung et al. (2006). Briefly, 100 μL of haemolymph was transferred to a siliconised Eppendorf tube and centrifuged for 2 min at 200×g. The supernatant was discarded and replaced with 200 μL of 0.5% low-melting-point agarose (LMPA) held at 4°C. After gentle mixing, an 85-μL aliquot was applied to a microscope slide pre-coated with 1.5% normal melting point agarose and was immediately topped with a coverslip. This was repeated with a further 85-μL aliquot to create a replicate microgel on the same slide. Slides were then placed on ice for approximately 10 min, to allow the LMPA to set. The coverslips were gently removed and the slides placed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% sodium sarcosinate, 1% Triton X-100, 10% DMSO; adjusted to pH 10 by dropwise addition of 10 M NaOH) for 1 h at 4°C. After lysis, slides were rinsed with distilled water and transferred to the electrophoresis chamber containing electrophoresis buffer (1 N NaOH) at room temperature for 20 min to allow DNA to unwind. After the unwinding period, electrophoresis was carried out for 30 min (15 V, 320 mA). Slides were then rinsed with distilled water and immersed in neutralisation buffer (0.4 M Tris, adjusted to pH 7 with HCl) for 5 min. Following staining with ethidium bromide (40 μL of 20 μg/mL per microgel), 100 cells per sample (50 per replicate microgel) were scored using an epifluorescence microscope (Leica Microsystems Ltd, Milton Keynes, Buckinghamshire, UK) and Komet 5.0 image-analysis system (Kinetic Imaging, Liverpool, UK). The comet parameters, tail length and tail moment (tail length×% tail DNA/100) were used for data analyses, as these were found to be most sensitive in an earlier study (Cheung et al. 2006).

Neutral red retention (NRR) assay

The NRR assay was used to determine cytotoxicity in the haemocyte samples as described elsewhere in detail (Lowe 1988; Lowe et al. 1995; Moore and Lowe 2004; Cheung et al. 1998; Wedderburn et al. 1998). Briefly, a stock solution of neutral red was prepared by dissolving 20 mg of dye in 1 mL DMSO. The working solution was made by adding 5 μL of stock to 995 μL of physiological saline (Lowe et al. 1995). Of the haemolymph samples collected from each individual, a 40-μL aliquot was used for the NRR assay. Haemolymph was added to a microscope slide pre-coated with 10% poly-L-lysine (to aid cellular adhesion). Slides were incubated in the dark at 20°C for 30 min to allow cells to attach. After removal of the excess cell suspension, 40 μL of neutral red working solution was added, and the slide was left to incubate for a further 15 min (Lowe 1988). Slides were examined under a light microscope (Leica DMR) every 15 min for the first hour and thereafter every 30 min (Lowe and Pipe 1994). The time at which ≥50% of the cells showed leakage of the dye from the lysosomal compartment into the cytoplasm was recorded, and observation of that slide was terminated at that point (Lowe 1988; Lowe et al. 1995; Moore and Lowe 2004; Cheung et al. 1998; Wedderburn et al. 1998).

Statistical analyses

Statistical analyses were carried out using the statistical packages Statgraphics Plus Version 4.0, Minitab 15.1 and Microsoft Excel 2007. All data were tested for normality and the appropriate parametric or non-parametric tests used. Pearson’s correlation coefficient (R) was calculated for the relationship between total metal content in sediment and in bivalve soft tissue. For the comet assay, all data were non-parametric, and consequently, the Kruskal–Wallis test was applied. However, data for comet tail length in C. edule could be normalised by log-transformation, and a one-way ANOVA was performed on transformed data. For the NRR assay, data for M. edulis were normally distributed, whereas data for C. edule were non-normal. C. edule data were normalised by log-transformation, and subsequently, all NRR data were analysed with one-way ANOVAs. Significance was set at P<0.05 for all tests.

Results and discussion

Hydrological parameters

Table 1 presents the hydrological data from each sampling site. The pH of the water was slightly lower at the sites that were located further upstream. This may have been due to a combination of the relatively high levels of contamination and lower amounts of circulation and dilution at these sites. There was minimal
temperature variation between the sites, with the small difference (maximum difference of 1.2 °C) probably attributable to differences in shading from foliage. Salinity ranged from 33.3 to 35.1 and increased towards the open sea, as expected. There was no clear trend in either dissolved oxygen or SPM levels; however, it was noted that the highest level of SPM (35.2 mg L\(^{-1}\)) was measured in water collected from site 2 (Torpoint), which also had the lowest dissolved oxygen levels, suggesting that the aerobic breakdown of organic matter present in the SPM was depressing local oxygen levels.

### Heavy metal analyses

Analysis of certified reference materials indicated that the analytical techniques used were accurate to within 10% of certified values.

### Sediments

Individual concentrations for each heavy metal measured are presented in Table 2. Figure 2a presents the total concentration of heavy metals measured in the sediments collected from the six sampling sites. It should be noted that measurements of the certified sediment material indicated that there was a significant underestimation of the measurements for Fe and Hg. The data collected for these two elements are therefore deemed to be inaccurate and have not been included in total heavy metal calculations. Sediment from site 1 had the lowest total metal concentration and was found to have the lowest concentrations of all the metals measured, except Fe. Total metal concentration was highest at site 5 (Fig. 2a); however, site 6 had the most number of metals at their highest concentrations.

### Table 1 Hydrological measurements at sites along the Tamar estuary

<table>
<thead>
<tr>
<th>Site</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Salinity</th>
<th>Dissolved oxygen (mg L(^{-1}))</th>
<th>SPM (mg L(^{-1}))</th>
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### Table 2 Metal concentration in sediment samples collected from the different sites along the Tamar estuary

<table>
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<tr>
<th>Site</th>
<th>Cr (μg g(^{-1}))</th>
<th>Mn (μg g(^{-1}))</th>
<th>Fe (^{a}) (μg g(^{-1}))</th>
<th>Co (μg g(^{-1}))</th>
<th>Ni (μg g(^{-1}))</th>
<th>Cu (μg g(^{-1}))</th>
<th>Zn (μg g(^{-1}))</th>
<th>As (μg g(^{-1}))</th>
<th>Se (μg g(^{-1}))</th>
<th>Cd (μg g(^{-1}))</th>
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</table>

\(^{a}\) Indicates results that are unreliable due to the ICP-MS instrument underestimating concentration.
Site 4 had the highest concentrations of Cr, Mn and Ni, whereas site 5 had the highest concentrations of Zn and Cd. The results for sites 4, 5 and 6 are consistent with these sites being furthest away from the sea, where water exchange would be the lowest due to the physico-chemical nature of the Tamar estuary.

**Biological material**

The individual metal concentrations for *M. edulis* and *C. edule* are presented in Tables 3 and 4, respectively. Figure 2a illustrates total metal concentrations for both organisms. For consistency with sediment data, soft tissue total metal concentrations do not include Fe or Hg. Both species had the lowest total metal concentration and the lowest concentrations of Mn, Co, Ni, Zn, Se and Pb at the reference site (Fig. 2a; Tables 3 and 4). Cockle samples, however, had the highest concentration of Cd at site 1 (Table 4). Of all the metals measured, Fe concentrations were the highest for both species, with the mean soft tissue concentration across all sites 417±182 μg g⁻¹ for mussels and 1,211±210 μg g⁻¹ for cockles. Hg concentrations were the lowest with 0.11±0.09 and 0.11±0.15 μg g⁻¹ for mussels and cockles, respectively. Highest total metal concentrations in soft tissues were at site 3 for mussels and site 4 for cockles (Fig. 2a). In terms of the number of metals at their highest concentration, site 5 was most contaminated for both species. Mussels from

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**Fig. 2** a Total metal concentration (excluding Fe and Hg) at each site for sediment (dotted bar) and soft tissue of *M. edulis* (black bar) and *C. edule* (grey bar). b Correlation between total metal concentration in sediment and that in soft tissues of *M. edulis* (filled diamonds, solid line) and *C. edule* (open circles, dashed line)

**Table**

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Table 3 Metal concentrations in the soft tissue of adult *M. edulis* collected from different sites along the Tamar estuary

<table>
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<tr>
<th>Site</th>
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<td>3.40 × 10⁰</td>
<td>6.50 × 10⁻¹</td>
<td>1.27</td>
<td>1.37 × 10⁻¹</td>
<td>1.04 × 10⁻¹</td>
<td>1.21 × 10⁻¹</td>
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<td>2.59</td>
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<td>1.49</td>
<td>1.60 × 10⁻¹</td>
<td>1.32 × 10⁻¹</td>
</tr>
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</table>

Table 4 Metal concentrations in the soft tissue of adult *C. edule* collected from different sites along the Tamar estuary

<table>
<thead>
<tr>
<th>Site</th>
<th>Cr (μg g⁻¹)</th>
<th>Mn (μg g⁻¹)</th>
<th>Fe (μg g⁻¹)</th>
<th>Co (μg g⁻¹)</th>
<th>Ni (μg g⁻¹)</th>
<th>Cu (μg g⁻¹)</th>
<th>Zn (μg g⁻¹)</th>
<th>As (μg g⁻¹)</th>
<th>Se (μg g⁻¹)</th>
<th>Cd (μg g⁻¹)</th>
<th>Hg (μg g⁻¹)</th>
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<td>3.70 × 10⁻¹</td>
<td>5.00 × 10⁻²</td>
<td>7.65</td>
</tr>
</tbody>
</table>
site 5 showed the highest levels of Fe, Ni, Zn, Se and Pb. Consistent with the mussel results, cockles from site 5 also had the highest levels of Fe and Se but also had the highest levels of Co and Cu. Mussel soft tissues also showed contamination from numerous heavy metals at site 3, where they exhibited the highest concentrations of Cr, Mn, Co and Cd. As in mussel samples, site 3 also yielded cockle samples with the highest concentrations of several metals, although this was for different elements (Zn, As and Hg). This is inconsistent with the results from sediment analysis, where this site had the highest concentration of Se only. In addition, cockles from this site also contained the highest levels of Cu, As and Pb. This preferential accumulation of metals suggests different rates of uptake, accumulation, metabolism and excretion in these two bivalve species under natural conditions (Philips 1977).

When comparing trends in sediment and soft tissue, only two samples were consistent. Both sediment and *C. edule* samples from site 4 had the highest Mn concentrations across sampling sites. Similarly, both sediment and *M. edulis* samples at site 5 showed the highest levels of Zn. A statistically significant correlation was found between total metal concentration in sediments and total metal concentration in *C. edule* soft tissues ($P<0.05$). Despite a similar trend for *M. edulis* soft tissue samples, the correlation was not significant (Fig. 2b).

### Biological responses or biomarkers

**Comet assay**

Due to the constraints imposed by the quantity of animals that could be collected from each site and the number of samples which could be processed per experimental session, the Comet assay was carried out on six animals per site for each species. There were insufficient *M. edulis* adults within the acceptable size range at Torpoint (site 2) for the Comet assay to be carried out.

Data for DNA damage in haemocytes of *M. edulis* are presented in Fig. 3a and b. The results for tail length (geometric length of the tail) and tail moment (measured in arbitrary units) showed some inconsistencies, hence they both were included for relative comparison (Fig. 3a and b). Normally, data for different Comet assay parameters generated through commercially available software show the same trends (Kumaravel and Jha 2006), but the way measurements of these parameters are made, optical, and programing variables might also influence and potentially alter the observed pattern (Kumaravel et al. 2009).

For tail length, it appears that *M. edulis* sampled from site 3 had significantly less DNA damage than those from the reference site ($P<0.005$). For tail moment, there was a statistically significant increase in DNA damage at the same site ($P<0.0001$). Although tail length has been validated as an appropriate measure of DNA damage in *M. edulis* in previous studies, it is generally not regarded as a robust parameter for Comet assay analysis (Collins 2004; Kumaravel and Jha 2006). In particular, it increases most dramatically at low levels of damage (Collins 2004), so the very small decrease shown here may be statistically significant but not biologically so. As tail moment incorporates a measure of the fraction of DNA that has migrated from the comet head, it is closer to % tail DNA, the recommended measure of DNA damage from the Comet assay (Collins 2004; Kumaravel and Jha 2006). The remainder of our analysis will therefore focus on alterations in tail moment.

Total metal concentration in the soft tissues of *M. edulis* was highest at site 3, which also showed genotoxicity; however, the overall pattern is inconsistent as no increase in DNA damage was observed at site 5. Despite this, an examination of the individual metal concentrations at sites 3 and 6 reveals some possible links between metals and genotoxic effects. Although the highest body burden for a single metal in *M. edulis* at site 3 was Fe (as at all sites; Table 3), Fe concentration was highest at site 5, where there was no significant DNA damage, indicating that perhaps other metals are responsible for this effect. The two metals that were at their highest concentrations in *M. edulis* soft tissues at site 3 were Cr and Mn. Cr has previously been found to cause DNA damage in *M. edulis* at tissue concentrations of $\geq 2.70 \mu g \cdot g^{-1}$ wet weight after laboratory exposure (Emmanouil et al. 2007). Additionally, Rank et al. (2005) found a significant correlation between chromium concentration and DNA damage (tail moment) in field-sampled *M. edulis* off the Danish coast. As Mn is an essential metal for most species, including *M. edulis* (Pipe et al. 1993), and there are currently no reports of Mn causing DNA damage...
damage in mussels, it is more likely that the damage seen here is caused by Cr, although interactive effects of these two and other metals along with other contaminants could not be ruled out. At site 6, the potential cause of the elevated DNA damage is not as clear. The concentration of As was the highest of all the sites (13.21 μg g⁻¹) but was still similar to that at site 5 (13.02 μg g⁻¹) where there was no significant DNA damage. Similarly, Pb, Ni, Zn, Se and Pb were all found at considerably higher body burdens at this site than at the reference site, but all were higher at site 5, where no significant damage occurred.

The data for tail moment in C. edule haemocytes is presented in Fig. 3c. An increase in the mean tail length of the samples collected from C. edule from site 4 compared with the reference site was statistically significant (P<0.0002) in the log-transformed data. However, when the Kruskal–Wallis test was applied to the non-transformed (non-parametric) data, there was not a statistically significant increase when comparing the various sites with the reference site (data not shown). When considering the data for the tail moment, it was found that the level of DNA damage was significantly higher in samples collected from sites 4 and 6 when compared with the reference site (P<0.005).

When the Comet assay data are compared with the concentrations of heavy metals in the sediments and

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Fig. 3 The level of DNA damage, as measured by the Comet assay, in haemocytes of bivalve molluscs sampled from the Tamar estuary; a tail length in M. edulis haemocytes; b tail moment in M. edulis haemocytes; c tail moment in C. edule haemocytes. * denotes a statistically significant difference from the reference site 1 (P<0.005)
the soft tissue of *C. edule* collected from the same sites, there is again no consistent link between total metal concentration in soft tissues and the Comet assay data. Although the highest total metal body burden was found at site 4, which is consistent with increased DNA damage at this site, sites 3 and 5 had higher total metal concentrations than site 6 but did not show correspondingly elevated incidence of genotoxicity. Analysis of individual metal data suggests possible reasons for the genotoxic damage at sites 4 and 6 in particular. Both Ni and Pb were present at their highest concentrations in tissues from cockles sampled at sites 4 and 6, respectively. Nickel is a known mammalian carcinogen (Beyersmann and Hartwig 2008) and has been shown to cause DNA damage in another bivalve, *Mytilus* sp. (Millward et al. 2012). Furthermore, Pb has been correlated with a reduction in delta-aminolevulinic acid dehydratase activity in field-sampled *C. edule* (Company et al. 2011), indicating it has the potential to cause toxic effects in this species. A number of previous reports have reported elevated levels of DNA damage (as measured by the Comet assay) in human lymphocytes (Anderson et al. 1997). Furthermore, Pb has been correlated with a reduction in delta-aminolevulinic acid dehydratase activity in field-sampled *C. edule* (Company et al. 2011), indicating it has the potential to cause toxic effects in this species. A number of previous reports have reported elevated levels of DNA damage (as measured by the Comet assay) in human lymphocytes (Anderson et al. 1997).

Neutral red retention (NRR) assay

The NRR assay was carried out on six animals from each site, with duplicate slides being prepared from each animal. As with the Comet assay, there were insufficient *M. edulis* adults within the acceptable size range at Torpoint (site 2) for the NRR assay to be carried out. The data showing the mean retention time of neutral red dye in *M. edulis* haemocytes are presented in Fig. 4a. There was no statistically significant difference between the means of the samples from each of the sites (*P*=0.7606). Although mussel haemocytes from site 5 appeared to have a lower retention time than the other sites (which would be consistent with the total metal concentration of sediments), this was not found to be statistically significant. With respect to *C. edule*, there was a statistically significant difference between the mean neutral red retention time of the samples collected from the reference site and those collected from site 5 in the log-transformed data (*P*=0.0113; Fig. 4b). However, this difference was actually an increase in relation to the reference site, indicating that the animals were less stressed at site 5 than at the reference site. This results contrasts with the total metal concentration in sediment, the weak trend for *M. edulis* and with the body burden data for *C. edule*. The pattern of NRR across sites was different for both species (Fig. 4a and b), and in general, *M. edulis* haemocytes retained the neutral red for less time than those of *C. edule*. This contradicts the idea that *M. edulis* has higher immunological vigour than *C. edule* (Wootton et al. 2003).

The NRR assay has been used in a number of field studies to assess cell injury (Lowe 1988; Cheung et al. 1998; Wedderburn et al. 1998). Although the acidic nature of the external environment of the lysosomal compartment would not be expected to be suitable for metal accumulation, data demonstrate that lysosomes are nonetheless important sites of metal compartmentalisation in the cell (Viarengo 1985). Both Cu and Cd have been found to cause detrimental effects on the stability of the lysosomal membrane (Moore et al. 1984). However, not all heavy metal ions cause damaging effects on the lysosomal compartment. Some studies have indicated that Zn is able to stabilise the lysosomal membranes (Sternlieb and Goldfischer 1976), and stimulatory effects on the lysosomal enzymes have been observed (Webb 1979). This could potentially explain the surprising increase in retention time for site 5, where body burden data show Zn present at higher levels than the reference site, although still at lower levels than at site 3, where it was highest.

In addition to heavy metals, polycyclic aromatic hydrocarbons (PAHs) have been reported to induce destabilisation of the lysosomal compartment, and the NRR assay has been used to identify cytotoxic effects of such pollutants. In a study of mussels located close to the location of the spillage of oil from the Sea Empress tanker, the stability of the lysosomal compartment was found to be inversely correlated with PAH concentration in the mussel tissues (Fernley et al. 2000). It is therefore likely that, in addition to
metallic contaminants, other contaminants including PAHs or pesticides present in the environment might influence the biological responses. Metal speciation in natural waters is of high importance and relevance since toxicity, bioavailability, environmental mobility and biogeochemical behaviour are all strongly dependent on the chemical species of metals (Fytianos 2001; Money et al. 2011). Furthermore, the kinetics of the rate of adsorption has a direct effect upon the quantities of contaminants taken up by filter feeders (Liu et al. 1998). In addition, biotic factors, such as the feeding rate, size, sex, maturity and disease state of the test organisms can have pronounced effects on the toxicity of a given substance. For example, Hagger et al. (2010) showed that NRR, feeding rate and cardiac output of M. edulis all varied significantly with season. The occurrence of sampling for the current study in September to October, a period when Hagger et al. (2010) report a sharp drop in retention time for the NRR assay, may have masked any toxic effects caused by differential metal concentrations.

The data presented here do indicate potential relationships between metal pollution in the Tamar estuary and genotoxic effects (particularly with respect to Cr for M. edulis, and Ni and Pb for C. edule). Despite this, there are several factors that require further discussion and examination. For example, in addition to seasonal variation for the sampling period (i.e. September–October) influencing biological responses (Hagger et al. 2010), identification of individuals and species with high levels of resistance to contaminant effects would assist in answering questions related to either physiological acclimatisation or evolutionary adaptation (Nevo et al. 1986). The Tamar valley lies in a region previously identified as containing both M. edulis and the closely related species Mytilus gallo-provincialis, in addition to large numbers of hybrids (Hilbish et al. 2002). Although individual mussels...
collected in the present study were preliminarily identified as *M. edulis*, the morphological distinction between these species is unclear. Robust genetic markers to classify species would have provided a useful cross-reference, to ensure that all sampled mussels were correctly identified, or to correlate any discrepancies in the data with species. It is also crucial to note that both the Comet and NRR assays were carried out on bivalve haemocytes, but metal analysis was on soft tissues. This discrepancy may account for the apparent inconsistencies between total metal concentration in the soft tissues and genotoxic/cytotoxic responses.

**Conclusion**

There have been suggestions that ecotoxicological studies are problematic in natural environments due to many inherent limitations and genetic variability within natural populations as well as individuals. This inherent variability makes the selection of suitable “control” or “reference” populations very difficult (Hasspieler et al. 1995). Human and fish cell lines have been suggested as alternatives to the use of aquatic organisms in vivo for the screening and testing of water quality (Papis et al. 2011; Baron et al. 2012). Such studies however do not take into consideration the biotic (e.g. adaptation, inter/intra-species effects) or abiotic (e.g. temperature, salinity) factors that can affect indigenous populations in conjunction with chemical contamination. Additionally, aquatic invertebrates cell-lines are less readily available than the vertebrate equivalents (Dixon et al. 2002), and the use of tissues from vertebrates might overlook effects on this ecologically important group. Furthermore, whilst there have been some studies to determine relative sensitivity of ecologically relevant invertebrates following exposure to contaminants under laboratory conditions (e.g. Canty et al. 2009), there has been limited, concurrent studies pertaining to relative sensitivity of species for sub-lethal toxic effects under natural conditions. In this context, the current study has demonstrated that biomarker responses previously identified and implemented in laboratory studies can be applied to indigenous populations of *M. edulis* and *C. edule* collected from the field. In particular, the results of the Comet assay appeared to correlate with high concentrations of heavy metals within the soft tissues. Measures should however be taken to further reduce confounding physical and biotic factors in order to fully understand the causes of the biological responses observed.

**Acknowledgment** Partial financial support from European Regional Development Fund, INTERREG IVA (grant no. 4059) is acknowledged.

**References**


Oxidative DNA damage may not mediate Ni-induced genotoxicity in marine mussels: Assessment of genotoxic biomarkers and transcriptional responses of key stress genes

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ABSTRACT

Nickel (Ni) is a known carcinogenic and mutagenic compound and an important contaminant of aquatic environments. Ni toxicity and its potential impact on aquatic organisms are, however, not well understood. This study used an integrated approach to evaluate genotoxic effects, tissue-specific accumulation and transcriptional profiles of key genes in mussels, Mytilus galloprovincialis, exposed to a range of concentrations of Ni. The genotoxic effects assessed were total and oxidative DNA damage (DNA strand breaks measured using the enzyme modified comet assay), and induction of micronuclei (MN; clastogenic and/or aneugenic effects) using haemocytes as the target cells. Six genes (gpg, mt20, mt20, sod, hsp70 and gap) were selected for transcriptional analysis in the gills based on their key role in the stress response. Following exposure to sublethal concentrations of Ni (0–3600 µg L−1) for 5 days, mussel haemocytes showed significant genotoxicity at >1800 µg L−1 (4-fold increase for DNA strand breaks and 3-fold increase for MN induction). There was no significant difference between buffer (control) and enzyme treatments which target oxidised DNA bases (formamidopyrimidine glycosylase or endonuclease III). This suggested that, in haemocytes, oxidative DNA damage is not a major mechanism for Ni-induced genotoxicity. The expression of mt20 and gpt genes in gill was up-regulated at genotoxic concentrations, whilst gpg expression was markedly up-regulated, particularly at 18 µg L−1 Ni (19-fold increase). Pearson’s correlation analysis revealed significant associations between TA tail DNA and MN induction in haemocytes (r = 0.88, p < 0.05), and between Ni accumulation in foot (r = 0.47, p < 0.05) and digestive gland (r = 0.41, p < 0.05) and induction of MN in the haemocytes. Our results are the first to suggest that Ni-induced genotoxicity in mussel haemocytes may not be a result of oxidative DNA damage, and that multixenobiotic resistance (MXR) may play an important role in Ni detoxification in this species.

1. Introduction

Nickel (Ni) is a naturally occurring metal of commercial importance, used in a variety of industries including coin production, engineering and the manufacture of stainless steel [1]. It is released into the atmosphere during its mining, smelting and refining, and after disposal of Ni-containing products. As a result, although Ni concentrations are typically low in the open ocean (0.12–0.70 µg L−1; [2]), in rain (<1 µg L−1; [1]) and in rivers and lakes (<10 µg L−1; [1]), it is greatly enriched in freshwater environments close to mining establishments. For example, in lakes near the Sudbury nickel refinery, Canada, dissolved concentrations of 180,000 µg L−1 have been reported [1]. Nickel concentrations in coastal marine waters can also be several orders of magnitude higher than in the open ocean. For example, Ratekevicius et al. [3] report coastal seawater Ni concentrations of 380 µg L−1 in the Cachagua area of Chile, and dissolved Ni concentrations > 70 µg L−1 have been reported in English estuaries (the Tyne, Wear and Tees; [4]). Data for Ni concentrations in coastal, marine areas close to Ni mines are not well reported. For example, it is well accepted that the coral lagoon surrounding the islands of New Caledonia (the third largest producer of Ni globally; [5]) has an extremely high heavy metal input [6], however Ni concentrations are yet to be quantified. Although Ni is an essential trace metal [7,8], it is also a known carcinogen [9,10] and genotoxic agent for mammalian cells under in vitro conditions [11–14,98]. In relation to the aquatic environment there is a growing scientific and regulatory concern about carcinogenic, mutagenic or reproductive (CMR) toxicants [15,48]. In this context, Ni and its compounds have been classified as priority pollutants in the EU Water Framework Directive (WFD) and in the

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Priority Substances Directive amending the WFD [16,17]. Following haemolymph extraction, mussels were dissected into their individual organs for analysis of their Ni content by ICP-MS (n = 6). A small (~5 mm) section of gill was also removed (n = 6), preserved in RNAlater, and stored at −20 °C for transcriptional analysis of target genes.

2.2. ICP-MS measurements to determine tissue-specific acccumulation and water Ni concentrations

For determination of tissue-specific Ni concentration, each individually dissected tissue (gills, mantle, digestive gland, foot, adductor muscle and byssus) was washed with distilled water, blotted dry and transferred to a pre-weighted scintillation vial. Samples were dried overnight at 60 °C and re-weighed. Tissue digestion was achieved by addition of 3 mL concentrated nitric acid (20% v/v analysis grade) and incubation for 2 h at 70 °C. Digested tissue samples were diluted to a final volume of 5 mL with Millipore Milli Q water and stored at room temperature until analysis. For analysis of water Ni beakers were used, water in exposure vessels was mixed thorou- ghly and a 2.5 mL water sample was removed and transferred to a scintillation vial pre-filled with 22.5 mL of 25% HNO3, and stored at room temperature until analy- sis. An internal standard of 15.7 ng was added to both tissue and water samples, to a final concentration of 10 μg/L.1 This verified that instrumental drift was not the cause of sample variation. Indium was selected on its minimal occurrence in marine samples and low polyatomic interference with seawater. Samples were analyzed using an X Series II ICP-MS (Thermo Fisher Scientific Inc., Waltham, MA, USA) with PlasmaLab software (Thermo Fisher Scientific Inc., Waltham, MA, USA). Six replicate analyses of Mili-Q water were used to determine the limit of detection (LOD, three standard deviations) of 0.67 μg/L and limit of quantification (LOQ, ten standard deviations) of 2.20 μg/L for Ni using this apparatus.

2.3. Engine-modified comet assay to determine oxidative DNA damage

DNA strand breaks were detected using the enzyme-modified comet assay. Although assessment of these breaks may be influenced by many factors (e.g. cyto- toxicity, excision repair) and the fact that the tail region could also contain DNA loops [28,29], for the purposes of this study, we will refer to the outcome of the comet assay as DNA damage. The comet assay was performed using the haemocytes of mussels as described in detail by our research group [25], modified to include the use of bacterial enzymes to target oxidised purine and pyrimidine bases [30]. Prior to performance of the comet and microusmus assay, total cell counts were taken and cell viability was checked using Trypan blue and found to be >90% for all treatments (data not shown).

Prior to Ni-exposure, two separate experiments were carried out to validate the enzyme-modified comet assay, using mussel haemocytes after in vitro exposure to various concentrations of hydrogen peroxide. In the first, the bacterial enzyme, formamidopyrimidine DNA glycosylase (Fpg) was used to convert oxidised bases to strand breaks, whilst in the second experiment endonuclease III (Endo III) was used to target oxidised pyrimidines [30]. Briefly, haemolymph from 18 mussels was pooled, and exposed in triplicate to concentrations of f(f) (control), 150, 500 and 500 μM hydrogen peroxide. After 1 h exposure in the dark at 4 °C, cells were collected by centrifugation at 150 g for 10 min at 4 °C, then washed and resuspended. The enzyme-modified comet assay was then performed as detailed below.

Slides were pre-coated with normal melting point agarose (NMPA, 1.5% in Mili- Q water). Haemolymph-saline suspension (200 μL) was centrifuged at 350 g for 3 min at 4 °C. The resulting pellet was resuspended in 150 μL of low melting point agarose (LMPA, 0.75% in PBS) and added to the agarose solution. The agarose was then melted at 95 °C for 1 min and added to 4 °C before allowing it to solidify. Coverslips were gently removed and slides stored at 4 °C overnight, before transfer to lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 mM Na-lauryl-sarcosine, 1% Triton X-100, 10% DMSO, pH adjusted to 10 with NaOH) and incubation for 1 h at 4 °C.

Three slides were prepared per sample, one enzyme buffer control, one with Fpg and one with Endo III. After lysis, slides were washed three times (5 min) in enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.1 mM EDTA, 0.2 M NaCl, 0.1% BSA, pH 8.0), drained and one unit of Fpg or Endo III (diluted in 50 μL buffer) was added to each microgel. Enzyme buffer control slides had 50 μL of buffer only added at this stage. Slides were then transferred to a humidity chamber and incubated at 17 °C for 45 min. All enzymes were used at a final protein concentration of 1500 μg/L.

Following the enzyme incubation step, slides were transferred to an electrophoresis chamber containing chilled electrophoresis buffer (1 mM EDTA, 0.3 M NaCl, pH 3). DNA was allowed to unwind for 20 min at 4 °C. Then electrophoresis was carried out for 20 min (1.25 V/cm). Following electrophoresis, slides were transferred to neutralisation buffer (0.4 M Tris, adjusted to pH 7 with HCl) for 10 min, rinsed (3 ×) with distilled water and allowed to air dry. Slides were usually stained and scored within 24 h, and always within 2 weeks. Each replicate microgel was stained with 20 μL of 20 μg/mL of 4′,6-diamidino-2-phenylindole (100 per slide) and were scored using an epifluorescence microscope (DMR; Leica Microsystems, Milton Keynes, UK) and Konor 5.0 image system (Kinetic Imaging, Inc.).
Liverpool, UK). Slides were coded and randomized to ensure scoring was blind. The software provided a number of different parameters, but tail DNA (T) is considered to be the most reliable [31].

2.4 Induction of microcuple (MN) to determine clastogenic and/or aneugenic effects

A slide centrepiece (Cytospin 4, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to concentrate 200 µL of haemolymph–saline mixture on to a slide precoated with poly-L-lysine solution (by spinning at 800 rpm for 2 min). Slides were prepared in duplicate and incubated at 4°C for 1 h (to allow cells to adhere). Cells were then fixed in ethanol for 2 min and dried. Cells were subsequently exposed to 1% sodium citrate for 5 min at room temperature, before staining with 20 µL of 0.5% ethidium bromide. Slides were coded and randomized and at least 1000 cells per slide were scored. Criteria for classification as micronuclei were those outlined by Czyz and Heddle [31,32] and previously used in mussels [33,34]. In addition, efforts were made to only score cells of similar size, thereby reducing variability in MN counts due to the different haematocyte cell subpopulations, as previously discussed by Biologo and Hayashi [35].

2.5 Determination of transcriptional expression of selected genes using real time PCR (qPCR)

In filter feeding organisms, such as mussels, the gills are the first point of contact and primary uptake site for waterborne contaminants. Consequently, they have been reported to be a sensitive organ for the assessment of genetic [18,33,35] and epithelial tissue damage [18,24,27,35], which could have knock-on effects on the fitness of the whole organism. For these reasons, we selected gill tissue for molecular analysis, to elucidate downstream effects of potential genetic damage. Total RNA was isolated from gill samples (n = 6) using the GenElute Mammalian Total RNA Minoprep Kit (Sigma–Aldrich), according to the manufacturer’s instructions. One µg of total RNA was used for reverse transcription using M–MLV reverse transcriptase and random primers (Promega Corporation, Madison, WI, USA). Following cDNA synthesis, qPCR was performed on samples in duplicate using an Applied Biosystems StepOne Plus PCR System (Life Technologies, Carlsbad, CA, USA) with StepOneTM Software (v2.3.2.1, Applied Biosystems). Each 20 µL PCR reaction contained 10 µL of GoTaq® qPCR Master Mix (Promega Corporation), 0.20 µM of forward and reverse primers, and 4 µL of template cDNA. Thermocycling conditions were initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. A dissociation profile (melt curve) was added to verify the purity of PCR products. PCR efficiency and fluorescence baseline and thresholds were measured using LinRegPCR software [36,37]. The relative expression ratio (RE) of 6 genes associated with xenobiotic defence or oxidative stress (glutathione-s-transferase, two metallothionein isoforms, p-glycoprotein, a heat shock protein and superoxide dismutase) and one housekeeping gene (18s ribosomal RNA, Table 1) was quantified using REST 2009 software [38], from qPCR efficiency and Ct values, relative to the reference gene and compared back to control samples as a calibrator.

As threshold cycle (Ct) values for 18s RNA were consistent across all samples and treatments (10.87 ± 0.09) it was used as a single reference gene [39].

2.6 Statistical analyses

All data were tested for normality using Kolmogorov–Smirnov tests and for homogeneity of variance with Levine’s test, and appropriate parametric or non-parametric tests were used. Where appropriate, correlations were determined using Pearson’s correlation coefficient. Proportional (percentage) data for tissue-specific Ni accumulation was square root transformed to normality, before analysis using a 2-way ANOVA with Tukey’s pairwise comparisons as post hoc tests. To avoid over-estimation of statistical significance for the comet assay data, 5 tail DNA values for individual cells were not used for statistical analysis, as recommended by Lovell et al. [40,41]. Instead, the median was calculated for each mussel (as data were not normally distributed). The same is true for data onewed on mussels on mussels was normal distribution and was analyzed by two-way ANOVA with Tukey’s pairwise comparisons as post hoc tests. Statistics were calculated using PASW Statistics (version 18.00, IBM, Somers, NY, USA) and Mintab (version 15.1.0.1). Mintab Inc., State College, PA, USA). Significance for all tests was set at p < 0.05.

### 3. Results

#### 3.1. Tissue-specific accumulation of Ni

Nickel accumulation occurred in a concentration-dependent manner in all mussel tissues. There were significant overall effects of Ni treatment (p < 0.001) and concentration-dependent increases in Ni accumulation in every tissue for all treatments (p < 0.05). Bysussus accumulated the highest amount of Ni per gram of tissue weight (Fig. 1a) and consequently made up a significantly higher proportion of total tissue Ni, across all treatments (Fig. 1b). Proportion of Ni per tissue varied with Ni treatment (Fig. 1b) but not significantly so (p > 0.05). Nickel was generally accumulated in the order byssus > digestive gland > gills > foot > muscle > mantle, although this trend was reversed for foot and muscle in the 1800 µg L⁻¹ Ni group.

### Figure 1

**Fig. 1.** Tissue-specific accumulation of nickel after 5 days exposure (a) per gram of mussel tissue (dw), and (b) proportion of total nickel in each tissue. Error bars are one SE. Asterisks indicate significant differences from the corresponding control. Concentration-dependent differences (p < 0.05) between similar tissues are indicated by mismatching lower case letters (i.e. gill tissue values for 18, 180 and 1800 µg L⁻¹ Ni are not significantly different [a] but are all significantly different from the value for 3600 µg L⁻¹ Ni [b].

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione-s-transferase</td>
<td>5′-CTCAACTTGCTGGCTGGCCACACT-3′</td>
<td>5′-TGACCTGCATCTGCTGGCCACACT-3′</td>
</tr>
<tr>
<td>Metallothionein MT-10</td>
<td>5′-CAATCAGTGGCCACGTGCGGCT-3′</td>
<td>5′-CACATTACACTTACCAAGGCTCTGAC-3′</td>
</tr>
<tr>
<td>Metallothionein MT-20</td>
<td>5′-ACATCGTGTTGTGCTGGTGGGC-3′</td>
<td>5′-TGACAATCTTGCTGGATGCT-3′</td>
</tr>
<tr>
<td>P-glycoprotein</td>
<td>5′-ACACTGCTGTTGTGCTGGTGGGC-3′</td>
<td>5′-GATAAAGCCTGCTGGATGCT-3′</td>
</tr>
<tr>
<td>Heat shock protein 70</td>
<td>5′-CCTTCTTCCCCATGAGACCCGA-3′</td>
<td>5′-AATGTTGCAATGCTGGCTTCTGTA-3′</td>
</tr>
<tr>
<td>Cu/Zn superoxide dismutase</td>
<td>5′-TGAACGAGATCTTTCGAAAGGCA-3′</td>
<td>5′-TGATCTGTTGCTGCTGGCTGAGCA-3′</td>
</tr>
<tr>
<td>18s ribosomal RNA</td>
<td>5′-CTGCTGCTGCTGGCTGGCTGGCA-3′</td>
<td></td>
</tr>
</tbody>
</table>

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3.2. Total haemocyte counts

Mean total haemocyte counts ranged between $1.17 \times 10^6$ for control mussels and $1.92 \times 10^6$ for those exposed to 3600 $\mu$g L$^{-1}$ Ni for 5 days, although there were no significant differences (1-way ANOVA, $p > 0.2$).

3.3. Induction of DNA strand breaks

Buffer treated haemocytes showed significantly elevated DNA damage after exposure to 50 and 500 $\mu$M H$_2$O$_2$ in the Fpg validation assay, and for 500 $\mu$M in the Endo III validation assay (Fig. 2; $p < 0.005$). Both Fpg and Endo III treated cells had significantly higher % tail DNA than their equivalent buffer treatments (Fig. 2; $p < 0.05$).

The results of the comet assay indicated that DNA damage (% tail DNA) in mussel haemocytes was significantly affected by exposure to Ni ($p < 0.0001$). All enzyme/buffer treatments at the highest Ni concentration (3600 $\mu$g L$^{-1}$) produced a significant increase in DNA damage compared to the equivalent treatment in the control group ($p < 0.005$). At 1800 $\mu$g L$^{-1}$ enzyme treated slides showed significantly more DNA damage than their respective controls. Although the buffer treated slides at this treatment also appeared to show enhanced levels of DNA damage, this was not statistically significant (Fig. 3a). DNA damage in the controls was relatively low, at ~10% tail DNA, and the highest amount of DNA damage (35.31% tail DNA) was seen in haemocytes from mussels in the 3600 $\mu$g L$^{-1}$ Ni group following treatment with Endo III. Fpg and Endo III treatments did not produce consistent results across all Ni treatments, and the effects of enzyme and enzyme/Ni interaction were not significant ($p > 0.05$).

3.4. Induction of MN

Data for MN induction was in good agreement with that of the comet assay, with % tail DNA and incidence of MN significantly correlated for haemocytes (Fig. 4a; $r = 0.882$, $p < 0.05$). Ni concentration had a significant effect on the incidence of MN ($p < 0.05$), although the only concentration-dependent differences were between the highest Ni treatment (3600 $\mu$g L$^{-1}$) and the control and 18 $\mu$g L$^{-1}$ Ni groups (Fig. 3b; $p < 0.05$). Micronuclei induction was highest in the 1800 $\mu$g L$^{-1}$ Ni group at 8.66 MN per 1000 cells, although this was not significantly different from the incidence in the 3600 $\mu$g L$^{-1}$ Ni group (8.49 MN per 1000 cells). Although Ni accumulation could not be determined for the haemocytes themselves, when tissue-specific Ni concentrations were compared with induction of MN in haemocytes there were significant relationships (Fig. 4b and c) for the digestive gland ($r = 0.411$, $p < 0.05$) and foot ($r = 0.474$, $p < 0.05$).
Transcriptional expression in gill tissue samples showed considerable variation for the ppg, gst, and mt20 genes (Table 2). Despite this, qPCR revealed significant overexpression of the gst and mt20 genes after exposure to 3600 µg L⁻¹ Ni, with approximately a 2.8 and 2.2-fold increase, respectively (Fig. 5, Table 2). The p-glycoprotein (ppg) gene was significantly overexpressed in Ni-exposed mussel gills, after exposure to 18 µg L⁻¹ Ni (an approximate 19.3-fold increase compared to the control; Fig. 5).

4. Discussion

4.1. Genotoxicity of Ni to haemocytes

Nickel was only significantly genotoxic to M. galloprovincialis haemocytes at the highest concentration tested (3600 µg L⁻¹). Interestingly, even at this concentration, there was no significant increase in strand breaks after Fpg or Endo III treatment, suggesting that there was no substantial oxidative damage to either purine or pyrimidine DNA bases after Ni exposure. This is in contrast to previous work in mammalian systems, which have shown enhanced concentrations of ROS in CHO cell cultures exposed to >6500 µg L⁻¹ of a variety of nickel compounds [42,43] and oxidised bases in vivo in studies of rats exposed to 50–180 µmol kg⁻¹ of nickel(II) acetate [44,45]. It must, however, be noted that these studies used i.p. injection or aequous Ni at concentrations or doses considerably higher than that reported here. Similarly, exposure to 1.76–17.6 µg L⁻¹ Ni caused an increase in ROS (measured by the dichlorofluorescein assay) in a freshwater aquatic species, Daphnia magna [46], although only the lowest dose was within the range reported here. Interestingly, results similar to our own (i.e. genetic damage with minimal or no oxidative damage) have recently been reported in human lung epithelial cells exposed to Ni in vitro [47]. It is however to be remembered that oxidative DNA damage in the present study was determined only in the haemocytes of the mussels, which might have limited potential for induction of genetic damage [25]. Assessment of other oxidative stress markers in different cells or tissues would confirm if this is a biomolecular (i.e. DNA, protein, lipid), cellular, or generic phenomenon [48].

It is possible that modified comet assay analysis of other mussel tissues may produce contrasting results to that of haemocytes. For example, in goldfish (Carassius auratus) exposed to 10–50 mg L⁻¹ Ni there were tissue-specific differences in the antioxidant response, with liver and spleen having sufficient antioxidant potential to resist Ni-induced oxidative stress. In contrast, the kidney and white muscle showed significant antioxidant enzyme induction (superoxide dismutase, glutathione peroxidase and glutathione reductase) [49,50]. Mussel haemocytes are involved in a variety of essential processes, including wound repair, immune/xenobiotic defence, and transport or digestion of nutrients [51]. Consequently it is likely that they have a high antioxidant potential [52] which may account for the lack of oxidative DNA damage we have observed. To our knowledge, the present study is the first to attempt to quantify oxidative DNA damage in mussels following environmentally relevant Ni exposure; however, future work analysing DNA strand breaks in multiple tissues and using the dichlorofluorescein assay [43] to directly detect ROS might further elucidate the mechanisms at work. This is an area of further development as there

3.5. Transcriptional expression of target genes

The efficiency of qPCR was within normal bounds (18S, 1.861; MT10, 1.911; GST 1.906; HSP 1.844; MT20 1.872; PGP, 1.883; SOD, 1.737).

Table 2

<table>
<thead>
<tr>
<th>Ni (µg L⁻¹)</th>
<th>ppg</th>
<th>gst</th>
<th>hsp</th>
<th>mt10</th>
<th>mt20</th>
<th>md20</th>
<th>nud</th>
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<tbody>
<tr>
<td>18</td>
<td>19.1 (1.2–52.6)</td>
<td>1.2 (0.6–2.6)</td>
<td>0.9 (0.5–1.6)</td>
<td>1.3 (0.3–6.7)</td>
<td>1.0 (0.3–4.2)</td>
<td>0.9 (0.5–1.5)</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>2.9 (0.3–14.6)</td>
<td>1.1 (0.5–2.4)</td>
<td>0.9 (0.5–1.7)</td>
<td>1.8 (0.2–6.9)</td>
<td>1.3 (0.5–4.3)</td>
<td>0.9 (0.6–1.6)</td>
<td></td>
</tr>
<tr>
<td>1800</td>
<td>0.3 (0–4)</td>
<td>1.1 (0.7–2)</td>
<td>0.7 (0.4–1.4)</td>
<td>0.5 (0.1–1.8)</td>
<td>1.0 (0.3–2.1)</td>
<td>0.9 (0.6–1.3)</td>
<td></td>
</tr>
<tr>
<td>5000</td>
<td>1 (0–146.9)</td>
<td>2.8 (1.3–7.1)</td>
<td>1.2 (0.6–2.5)</td>
<td>0.6 (0.1–3.1)</td>
<td>2.2 (0.3–6.6)</td>
<td>1.1 (0.7–1.8)</td>
<td></td>
</tr>
</tbody>
</table>
are considerable technical challenges in using other cell types in aquatic organisms, compared to mammalian systems where different tissues/organs are widely used for the detection of DNA strand breaks [47]. It is possible that technical factors (such as cytotoxicity undetectable by dye exclusion, overnight storage of slides and enzymatic treatment) may have reduced the sensitivity of the comet assay [53]. This modified version of the comet assay has, however, been validated using other species in our laboratory [54,55]. Furthermore, this technique has also been successfully validated for mussel haemocytes after in vitro exposure to hydrogen peroxide (Fig. 2), and thus we consider this effect to be minimal.

Fig. 5. Relative expression ratios (RER) of six genes in gill tissue of mussels after exposure to nickel for 5 days. Mean values for RER were calculated using REST 2009 software. To allow for appropriate scaling, variability in this data is reported in Table 2.
Recently, enzymatic repair of oxidative DNA damage has been demonstrated in mussel (M. edulis) gill tissue [56]. This repair could have masked oxidative DNA damage that occurred at an earlier timepoint (i.e. one not sampled in this study). If that is the case, however, it still suggests that over the longer exposure period used here, mussel haemocytes were able to maintain homeostasis with respect to oxidative DNA damage. Time-course analysis of oxidative DNA damage (sampling earlier and more frequently, perhaps over a longer duration) would confirm if this is the case, although there are limitations in procuring sufficient amount of cells during repeated sampling of the same individual.

It has also been suggested that Ni may interfere with incision or post-incision (e.g. ligase) steps of DNA repair processes (e.g. base excision repair [57,58]). There is, therefore, potential for any residual Ni (released during the lyss stage of comet protocol) to inhibit the activity of endonucleases in vitro. We consider this unlikely, as 4h exposure to 0.17 μM Ni clearly showed oxidative DNA damage, as measured by the Fpg-modified comet assay, in Jurtak cells [98], suggesting that in vitro inhibition of Fpg is not a problem. Future investigations could confirm this by titrating known concentrations of Fpg against a known mutagenic substance (positive control).

We also found significant induction of MN in haemocytes after exposure to 3600 μg·L⁻¹ Ni. This may be as the result of direct genotoxic action of Ni, or as an indirect result of the immune response, in which haemocytes are essential [59]. Proliferating gill cells have been reported to be more sensitive indicators of genotoxicity (in the MN assay) in bivalves [35]. There is, therefore, potential that this tissue may exhibit genotoxic responses at lower concentrations than haemocytes. Unfortunately, there are many practical obstacles to assessing MN induction in gill cells [35,48], and as such we did not attempt to quantify this biomarker in the gills. Our results support those of previous studies that have found a strong link between DNA strand breaks (as measured by the comet assay) and the induction of micronuclei in both mammals [60] and aquatic biota [26,61–63]. This suggests a cause-and-effect relationship between DNA strand breaks and MN formation [48]. Apart from generation of ROS, the known inhibitory action of Ni on DNA repair enzymes [57] may provide a causal mechanism for this relationship.

4.2. Transcriptional expression of mt20 and pgp in response to Ni exposure

The induction of metallothioneins and expression of mt genes in mussels in response to Ni has been subject to some debate [64]. Although we did not determine the induction of metallothionein proteins, our results indicate that after 5 days Ni exposure expression of the mt10 gene is stable in comparison to the control, whereas mt20 is significantly upregulated at 3600 μg·L⁻¹ Ni. This is consistent with previous work indicating that mt10 is expressed constitutively, whereas mt20 expression is highly inducible, particularly in response to heavy metal contamination [65–68]. In contrast, Dondero et al. [69] reported that mt10 expression significantly increased following exposure of M. galloprovincialis to 22–770 μg·L⁻¹ Ni for 4 days, but that mt20 expression remained unchanged. The duration of Ni exposure and time of expression measurement could explain this difference, as it is important to consider temporal variability when examining changes in transcriptional expression. Time-response measurement of expression changes in these genes might elucidate this point [70]. Interestingly, Amiard et al. [71] found no correlation between induction of metallothionein proteins and Ni exposure in field- or lab-exposed mussels (10–100 ng individual⁻¹). Since discrepancies between gene expression and induction of metallothionein proteins have already been reported [72], concurrent measurement of both these parameters in future studies might clarify this apparent variability.

The p-glycoprotein multidrug transporter is induced following exposure to a range of contaminants in aquatic organisms [73], and has been identified as part of the multixenobiotic resistance mechanism (MXR), similar to the multidrug resistance mechanism in mammals [74–76]. Although the transport function of p-glycoprotein is more usually associated with metabolically hydrophobic, amphiphilic molecules (including pesticides and anti-cancer drugs; [77]), it has a wide range of substrates and certain subgroups of p-glycoproteins have been reported to utilise heavy metals [78]. Studies reporting increased transcriptional expression of pgp in response to copper exposure in tropical corals [79], decreased emergence in cadmium-resistant Drosophila treated with a pgp inhibitor [80], and hypersensitivity to heavy metals after deletion of the pgp gene [81] support the suggestion that the MXR mechanism may include heavy metal substrates, at least in invertebrates.

Our results indicate that Ni exposure causes increased expression of the pgp gene in mussels, indicating that MXR may play an important role in Ni detoxification. Although the p-glycoprotein response is often simultaneous with increased heat shock protein or hsp70 expression [73,82], our results did not indicate any over-expression of the hsp70 gene at this timepoint. This suggests that, in Ni-exposed mussels, overexpression of pgp may be part of a general cellular response, distinct from the heat shock response, as in mussels exposed to organic toxins [73]. An increase in MXR protein or transport activity has been reported in mussels exposed to other contaminants (PAHs, PCBs, and heavy metals) in the field [83–86], and increased expression of MXR genes has been shown in response to various stressors (e.g. temperature, salinity) under laboratory conditions [87]. Additionally, increased expression of pgp itself has been reported in various bivalves in response to other metals [Zh, 88; Cd, 84]. However, this is the first report of increased expression of the pgp gene in a Ni-exposed Mytilus sp. in a laboratory study. Recently, Farcy et al. [89] also reported that oysters (Crassostrea gigas), a marine bivalve species, exposed to ionising radiation (60Co; γ rays) showed significant up-regulation of similar genes. After a total dose of 6.20 mGy, delivered over 6 weeks, the multixenobiotic resistance gene, MDRI, increased approximately 3-fold in comparison to control oysters. The metallothionein-encoding gene MT showed an approximate 2-fold increase after only 1 week exposure (total dose 0.50 mGy). Interestingly, chemical toxicity from Co was excluded as a cause of this overexpression, although at much lower metal concentrations than that used here (33.6 ng·L⁻¹ Co). P-glycoprotein has been reported to be induced in response to genotoxicity or xenobiotic stress [77,90], in addition to specific induction by its substrates. It therefore appears that expression of these genes could be influenced by intrinsic or extrinsic stressors other than heavy metals.

4.3. Relative toxicity of Ni

Other metals have shown greater genotoxicity (% tail DNA) in mussels than that reported for Ni here. For example, 56 μg·L⁻¹ copper caused approximately 50% more DNA strand breaks (as measured by the comet assay) than the highest dose of Ni used here [27]. In a previous study, however, Ni concentrations of 180 μg·L⁻¹ resulted in approximately 60% tail DNA [19], in contrast to the present work where DNA damage in the same treatment group was 15.6–18.2% tail DNA. Although the results of the comet assay in mussels can be influenced by seasonality [18], both control groups had approximately 10% tail DNA so there are potentially other factors behind this observed variability. This could include biological factors (e.g. age, size, sex, inter-individual variability, etc.), technical parameters (such as differences in the protocols, cameras and/or microscopes used) and/or physico-chemical factors (e.g. temperature, salinity, etc.) [48,91]. Since the speciation of Ni in our seawater...
A. Publications

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Ni2+

was not determined (see Section 2.1), variation in the free
ion
(i.e. the toxic form) may be one such factor.
The relatively low toxicity of Ni we report is, however, consistent
with examination of metal toxicity in embryos of Mytilus trossolus
[92]. Several other biomarkers have also shown detrimental effects
only at relatively high Ni concentrations. For example, Attig et al.
[64] reported that glutathione-s-transferase and malondialdehyde
increased after exposure of Mytilus galloprovincialis to 770 ␮g L−1
Ni, but showed no effect at 135 ␮g L−1 . Glutathione peroxidase
(GPX) activity did not signiﬁcantly increase in the same study until
the Ni concentration reached 3000 ␮g L−1 . Although toxic concentrations of Ni are several orders of magnitude higher than that
in contaminated estuaries [93] or in open oceans [2,94], they are
considerably lower than in Ni-polluted Canadian lakes [1]. Furthermore, there is considerable uncertainty about Ni concentrations at
high risk coastal locations (e.g. the New Caledonian lagoon).
In conclusion, this study’s integrated multiple biomarker
approach suggested that although NiCl2 is genotoxic to mussel haemocytes (>1800 ␮g L−1 ) under the experimental conditions
reported here, there is no signiﬁcant Ni-induced oxidative DNA
damage. Other genotoxic mechanisms proposed for Ni, including
direct interaction with DNA and inhibition of DNA repair enzymes
require further exploration in this model organism. The study
also identiﬁed a signiﬁcant relationship between a biomarker of
exposure (comet assay) and effect (micronucleus assay), and has
shown signiﬁcant upregulation of pgp transcriptional expression
in response to Ni. This indicates that the multi-xenobiotic resistance mechanism may be involved in Ni detoxiﬁcation in marine
mussels. Consequently, we recommend that a similar, integrated
approach is adopted for future ecotoxicological work, with a particular focus on linking genotoxicity and transcriptional changes with
protein proﬁles and other biomarkers at higher levels of biological
organisation. In line with previous work [e.g. 26,27,85,95–97] we
believe this approach will maximise the ecological relevance and
applicability of ﬁndings for regulatory bodies, whilst also enabling
mechanistic investigation of ecotoxicological effects.

Conﬂict of interest statement
None declared.
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Enhanced toxicity of ‘bulk’ titanium dioxide compared to ‘fresh’ and ‘aged’ nano-TiO$_2$ in marine mussels (Mytilus galloprovincialis)

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Abstract

Marine bivalves (Mytilus galloprovincialis) were exposed to titanium dioxide ($10$ mg L$^{-1}$) either as engineered nanoparticles (nTiO$_2$; fresh, or aged under simulated sunlight for $7$ days) or the bulk equivalent. Inductively coupled plasma-optical emission spectrometry analyses of mussel tissues showed higher Ti accumulation ($\approx$10-fold) in the digestive gland compared to gills. Nano-sized TiO$_2$ showed greater accumulation than bulk, irrespective of ageing, particularly in digestive gland (sixfold higher). Despite this, transcriptional expression of metallothionein genes, histology and histochemical analysis suggested that the bulk material was more toxic. Haemocytes showed significantly enhanced DNA damage, determined by the modified comet assay, for all treatments compared to the control, but no significant differences between the treatments. Our integrated study suggests that for this ecologically relevant organism photocatalytic ageing of nTiO$_2$ does not significantly alter toxicity, and that bulk-TiO$_2$ may be less ecotoxicologically inert than previously assumed.

Keywords: engineered nanoparticles, histopathology, gene expression, oxidative DNA damage, comet assay

Introduction

Despite their economic importance, the recent large-scale production and utilisation of engineered nanoparticles (ENPs) has raised concerns over their environmental impact. Consequently, to meet these concerns, there has been call for proper environmental risk assessment of these anthropogenic particles (European Commission 2005; Moore 2006; Nowack & Bucheli 2007; Handy et al. 2008; Klaine et al. 2008). ENPs have different properties than their bulk counterparts as they present a very large surface area-to-volume ratio. When particle size shrinks, there is potential for enhanced toxicity to biota, even if the material is relatively inert in bulk form (Jarré et al. 2009; Al-Subiai et al. 2012). This enhanced toxicity may be due to a range of mechanisms, including the greater surface reactivity of ENPs and their ability to penetrate into and accumulate within cells and organisms (Xiong et al. 2011). Direct and indirect release of ENPs into aquatic environments via bathing, sewage effluent (Hardy & Shaw 2007) and engineering applications (Chen et al. 2004; Nagaveni et al. 2004) has increased potential exposures to both natural biota and humans (Nowack & Bucheli 2007). Furthermore, the effects of ENPs on aquatic organisms may also impact human health via the food chain. Despite this, relatively few studies have examined the toxic or genotoxic effects of ENPs on aquatic organisms, particularly on invertebrates, which play important role in ecosystem functioning (Baun et al. 2008; Handy et al. 2008; 2012; Jha, 2008; Al-Subiai et al. 2012). Nano-sized titanium dioxide (nTiO$_2$) is a widely used ENP, found in a variety of consumer products (e.g. paper, paint and toothpaste) and used in the decontamination of air, soil and water (Esterkin et al. 2005; Weir et al. 2012). TiO$_2$ nanoparticles have been shown to induce detrimental biological responses in different in vitro and in vivo systems (Reeves et al. 2008; Vevers & Jha 2008). Historically, one of the important applications of TiO$_2$ particles has been in sunscreens. When these particles, with apparently low cutaneous penetration, are washed off the skin they are released into the aquatic environment. Environmental contamination by nTiO$_2$ therefore seems inevitable and raises concerns for both humans and aquatic organisms (Kaegi et al. 2008; Kiser et al. 2009; Weir et al. 2012; Handy et al. 2012). Given the dynamic nature of the marine environment, there is potential for ageing of nTiO$_2$ and generation of new products (Fouqueray et al. 2012), the fate and impact of which are not known (Labille et al. 2010). Consequently, the potential impact of nTiO$_2$ on aquatic ecosystems has attracted special
attention (Oberdörster et al. 2005; Moore 2006; Handy and Shaw 2007; Handy et al. 2008). Despite this, little is known about its toxic potential to marine invertebrates (Moore 2006; Klaine et al. 2008; Ward & Kach 2009; Scown et al. 2012). It is now well accepted that the biological responses of ENPs are dependent on the physico-chemical environment in which they contact cells (Vevers & Jha 2008; Handy et al. 2012). In addition, aggregation of particles in the aquatic environment can influence their interaction and/or uptake and therefore the biological responses. Since TiO$_2$ nanoparticles are photocatalytic (Tsang et al. 2008; Dodd & Jha 2009, 2011), ultraviolet (UV) radiation has been shown to substantially increase nTiO$_2$ toxicity by production of reactive oxygen species (ROS; Nakagawa et al. 1997; Uchino et al. 2002; Reeves et al. 2008) which can induce oxidative stress and cellular damage (Labille et al. 2010; Dodd & Jha 2009, 2011).

Against the backdrop of the above information, this study adopted an integrated approach to evaluate the biological effects of fresh nTiO$_2$, aged nTiO$_2$ and the bulk equivalent in a representative bivalve, Mytilus galloprovincialis. We also compared the biological responses with a reference toxic metal, copper (Cu). Several assays were used simultaneously in different target cell types to obtain a holistic perspective. These techniques have been thoroughly optimised and validated in our laboratories using this organism (Jha 2005; Al-Subiai et al. 2011; Fasulo et al. 2008; 2012; Ciacci et al. 2012, Millward et al. 2012, Dallas et al. 2013). In particular, we determined (a) histomorphological alterations in gill and digestive gland; (b) Alcian blue and Periodic acid-Schiff (AB/PAS) staining of acid mucopolysaccharides in gill; (c) transcriptional expression and localisation in situ of two different isoforms of metallothionein (mt) genes (using RT-PCR products and fluorescent in situ hybridisation); and (d) oxidative DNA damage in the haemocytes, using modified comet assay (Reeves et al. 2008; Vevers & Jha 2008, Mustafa et al. 2012, Dallas et al. 2013). In addition, Ti accumulation in tissues (i.e. gill and digestive gland) and concentrations of different elements in different forms of TiO$_2$ used were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS), respectively.

**Methods**

**Preparation and characterisation of TiO$_2$**

The nano-sized titanium dioxide (DeGussa AG, UK) used had a crystalline composition of 74.7% anatase and 25.3% rutile, average diameter 21 nm and purity >99% (according to manufacturer’s information). The bulk TiO$_2$ used here (titanium(IV)oxide; Acros Organics, UK) was the same as that used by Al-Jubory & Handy (2012), who indicated a crystal structure of 74.7% anatase and 25.3% rutile. The nTiO$_2$ samples have been characterised under our laboratory conditions using a range of techniques (Federici et al. 2007; Ramsden et al. 2009; Windeatt & Handy 2012). Nano-TiO$_2$ was aged by exposing a 200 mg L$^{-1}$ solution of the powder (in seawater) to simulated sunlight (16 h light: 8 h dark) for 7 days. Natural sunlight was simulated using Edison fluorescent lamps, each 230 V, 70 W, 35–535 colour. Light intensity was 4.07 klx, measured using an RS-01 Light Meter (RS components, UK).

Stock solutions were dispersed using a bath sonicator (35 kHz, Fisherbrand FB1010, Germany) as described elsewhere (Federici et al. 2007; Windeatt & Handy 2012). A well-mixed subsample (20 mL) of each stock was also sonicated for a further 1 h immediately prior to each application. Subsamples were then further characterised by observing directly on a plastic film with a copper grid using transmission electron microscopy (TEM; JEM-1200 EX II; JEOL Ltd, USA). The diameter of 100 ENPs was measured for each form using an image analysis program (Image J, v1.44; National Institute of Health, USA).

**Animal collection and maintenance**

Mussels (M. galloprovincialis; 45–50 mm) were collected at low tide from Trebarwith Strand (Cornwall, UK), a relatively clean site, used for reference in previous studies (Al-Subiai et al. 2009, 2011, 2012). After collection mussels were immediately transported to the laboratory and placed in an aerated tank (1 mussel L$^{-1}$) with filtered seawater (<10 μm). Mussels were maintained at 15$^\circ$C, fed daily with micro algae (Isochrysis galbana, Interpet, UK) and water changed daily. At least two weeks were allowed for mussels to acclimatise before exposure.

**Exposure conditions**

Mussels were exposed to a nominal concentration of 10 mg L$^{-1}$ of either bulk, fresh or aged nTiO$_2$ in 2-L glass beakers (4 animals beaker$^{-1}$). Mussels were also exposed to 40 μg L$^{-1}$ CuSO$_4$·5H$_2$O (99% purity) as a positive control (Al-Subiai et al., 2011). Each exposure was carried out in triplicate. The concentration of Cu was based on earlier in vivo studies using bivalve molluscs (Bolognesi et al. 1999; Al Subiai et al. 2009; 2011) while the concentration of TiO$_2$ was based on several studies determining biological responses in various organisms (Canesi et al. 2010; Lapied et al. 2011; Xiong et al. 2011). The 4-day exposure of mussels to Cu and TiO$_2$ was based on earlier studies in our laboratory using a range of toxicants (Jha 2005; Canty et al. 2009; Al-Subiai et al. 2011). Each treatment was renewed daily and the water quality parameters were maintained within acceptable ranges (temperature: 15 ± 1$^\circ$C; dissolved oxygen: 96.1 ± 0.3%; total ammonia: 0.04 ± 0.02 mg L$^{-1}$; pH 7.8 ± 0.02; salinity: 31.5 ± 0.15; Hach HQ40D Multi-meter [Hach-Lange, Germany]). Animals were not fed and did not spawn during the experiment.

**Collection of haemolymph samples for the determination of DNA strand breaks**

Haemolymph was withdrawn via the posterior adductor muscle using a 1-mL syringe and 21-gauge needle (Al-Subiai et al. 2009). Samples were diluted with an equal volume of physiological saline (0.02 M HEPES, 0.4 M NaCl, 0.1 M MgSO$_4$, 0.01 M KCl and 0.01 M CaCl$_2$, pH 7.4) and centrifuged at 350 × g for 2 min at 4$^\circ$C (Al-Subiai et al. 2009).
Samples were then placed on ice until processing for the comet assay.

**Determination of oxidative DNA damage using the modified comet assay**

Induction of DNA strand breaks in the haemocytes was determined as described elsewhere (Jha et al. 2005; Al Subiai et al. 2011, 2012). The comet assay can be modified by addition of bacterial enzymes which specifically target oxidised purine or pyrimidine bases (Collins et al. 1995). Measurement of oxidative DNA damage in mussel haemocytes using this technique has been validated in our laboratory (Dallal et al. 2013). In common with previous studies in our laboratory (Reeves et al. 2008; Veesers & Jha 2008; Mustafa et al. 2012, Dallal et al. 2013), the enzymes used were formamidopyrimidine DNA glycosylase (Fpg) and endonuclease III (Endo III). Briefly, the final haemocyte-agaroose suspension was added to a slide pre-coated with 1% normal melting point agarose as two replicate 75-μl microgels. Three slides were prepared per sample, one enzyme buffer control, one with Fpg and one with Endo III. The slides were covered with chilled lysis solution (pH 10.0) and kept at 4°C for 1 h. After lysis, slides were washed three times (5 min) in enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.29 mg mL⁻¹ BSA, pH 8.0; Sigma-Aldrich, UK), drained and 50 μl of Fpg, Endo III or enzyme buffer added to each microgel. Slides were transferred to a humidity chamber and incubated at 37°C for 45 min. To unwind the DNA, slides were placed in a horizontal electrophoresis box filled with freshly prepared alkaline buffer (0.3 M NaOH, 1 mM EDTA, pH 13) for 30 min at 4°C. After electrophoresis (300 mA, 25 V, 30 min) the slides were washed three times with neutralisation buffer and rinsed with refrigerated distilled water. Each microgel was stained with 50 μl of a 1:1 solution of Sybr Safe (10⁻³; Invitrogen) and anti-fading agent (1:1) and examined with a fluorescence microscope (excitation: 488 nm excitation, emission: 520 nm). The percentage of DNA in the comet tail (% tail DNA) was quantified for 100 cells per slide using Komet 5.0 software (Kinetic Imaging, UK). Comet analysis was carried out on samples of haemocytes from individual mussels. These data were then combined by taking the mean for two mussels from three replicate beakers (n = 6).

There is the potential for cytotoxicity to cause a positive response in the comet assay (i.e. DNA migration); however, there are standard procedures in place in our laboratory to minimise this effect. Cell viability was determined prior to the comet assay (by eosin Y dye exclusion assay) and only samples with >90% viability were used (Henderson et al. 1998). In addition, during scoring, so-called ‘hedgehog cells’ were excluded (Brendler-Schwaab et al. 2005).

**Histology and histochemical analyses**

Following haemolymph sampling, mussels were dissected to obtain gill and digestive gland tissues for histological and histochemical assessments, using standard procedures routinely used in our laboratory (Fasulo et al. 2008; 2010a, Al Subiai et al. 2011, 2012, Cappello et al. 2013). Briefly, dissected tissues were fixed in 10% buffered formal saline, dehydrated in ethanol and embedded in paraffin (Bio-Optica, Italy). Histological sections (4 μm) were cut with a rotary automatic microtome (Leica Microsystems, Germany), mounted on glass slides and stained with haematoxylin/eosin (Bio-Optica, Italy) to visualise typical morphological features. A combined method, using AB/PAS staining (pH 2.5), was used to detect neutral and acid mucocytes in gill epithelium. Micrographs were obtained using an Axio Zoom Z1 microscope (Carl Zeiss AG, Germany) with Axio Vision software (v 4.5; Carl Zeiss AG, Germany) for acid mucocyte quantification. These data were then combined by taking the mean for two mussels from the three replicate beakers (n = 6).

**Determination of transcriptional expression of mt genes**

For the transcriptional expression of mt genes, gill and digestive gland tissues were collected and stored at −80°C until use (Fasulo et al. 2008, 2010a, 2010b). Briefly, total RNA was extracted using TRizol LS reagent (Invitrogen, USA; Chomczynski & Sacchi 1987). The RNA content was quantified using a UV spectrophotometer (Nanodrop™, Thermo Scientific, UK). cDNA was synthesised using 4 μg of total RNA and oligo (dT)20 primers (150 pmol/mL; Invitrogen, UK), with M-MLV reverse transcriptase (Invitrogen, UK) according to the manufacturer’s instructions. One microtitre of the resulting cDNA was amplified in a total reaction volume of 25 μL (2.5 μL of 10× buffer, 0.65 U EuroTag polymerase [Euroclone s.p.a., Italy], 1.5 μL of 50 mM MgCl₂, 2 μL of 10 mM mixed primers and 2 μL of 10 mM dNTPs, in Milli-Q water). The thermocycling program used to amplify fragments was 95°C for 2 min, then 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min and final extension at 72°C for 5 min (Mastercycler EP-Gradient, Eppendorf, Italy).

The sequences of the primers were based on those of mt10-III and mt20-II of *M. galloprovincialis* (GenBank accession ns. AT566248 and AT566247) and were expected to amplify sequences 193 and 161 bp long, respectively. mi10 sense primer: 5' GGTCGTCACCTTGAACTCTGTT-3' mt10 antisense primer: 5'-CTACAGGTAGGGCTCCT GT-3'

mi20 sense primer: 5'-AGGATGCGAGGGGAAATGGTT-3' mt20 antisense primer: 5'-AGGAGCACCAGTTCATC AT-3'

RT-PCR products were characterised by electrophoresis on SYBR-safe-stained agarose gels. Bands of ~193 and ~161 bp for mi10 and mt20 were obtained. The results were normalised to the expression of actin, which was expressed at basal levels in both control and treated animals (data not shown). The actin gene was amplified using sequence primers based on the cDNA sequence of *M. galloprovincialis* (accession number AF157491) to amplify 200 bp.

**Localisation of mt mRNA transcript using fluorescence in situ hybridisation technique**

The mRNA expression and morphological localisation of *mt10* and *mt20* genes in gill and digestive gland were investigated by means of fluorescence *in situ* hybridisation.
controls (way analysis of variance (ANOVA). All treatments showed significantly higher DNA damage than the controls (p < 0.0001).

Statistical analyses

Statistical analyses of data were conducted with GraphPad Instat software (GraphPad Software Inc., USA). All results are presented as mean ± SD. Significant differences were determined using one-way ANOVA, followed by multiple-range tests to differentiate between groups of data. Significance for all tests was set at p < 0.05.

Results

Characterisation of TiO2 ENPs

TEM measurements indicated average diameters (n = 100) of 151.40 ± 6.7 nm for bulk TiO2 and 24 ± 4.6 nm for fresh nTiO2 (Figure S1). In both cases, these measurements were similar to the manufacturer’s specifications and are in line with previous results from our laboratory (e.g. Windeatt and Handy 2012). In contrast, the aged nTiO2 formed aggregates of particles with average diameters ranging from 27.60 ± 6.9 to 108.40 ± 5.2 nm (Figure S1).

Determination of oxidative DNA damage using the modified comet assay

Control animals showed low levels of DNA damage (9.38 ± 3.10 % tail DNA; Figure 1) for buffer controls and enzyme treatments. All treatments showed significantly higher DNA damage than controls (p < 0.0001). Interestingly all TiO2 treatments resulted in approximately 40% tail DNA and there were no significant differences between the treatments. The use of Fpg and Endo III showed no significant differences compared to the buffer controls.

Histological and histochemical observations

In all control organisms, the gills had no pathological tissue alterations (Figure S2). AB/PAS staining indicated that two out of six control specimens had acidophil cells (Figure 2A), but gill filaments in control mussels had proportionally more neutrophil cells than all forms of TiO2 (Figure 2F). Histological and histochemical observations in the gills showed substantial influx of haemocytes along the filaments and a large number of acidophilic mucous cells along the apex of the filament in all Cu-exposed mussels (Figure S2; 2b; Table I). Exposure to both forms of nTiO2 resulted in moderate influx of haemocytes along the gill filaments, but no particular alterations of the tissue (Figure S2; Table I). In all mussels exposed to bulk TiO2, a loss of structural definition of the ciliated epithelium and hypolasia was observed (Figure S2; Table I) and acidophil cells were distributed throughout the branchial filament epithelium (Figure 2C). Mussels treated with nTiO2 and its aged samples were diluted with Milli-Q water. Samples were thoroughly vortex mixed immediately prior to analysis using ICP-OES (Varian 725 ES). To aid in comparisons between nTiO2 and its bulk equivalent, 200 mg L−1 solutions of both forms were sonicated for >1 h, and then analysed directly for a range of additional elements using ICP-MS (Thermo Scientific, X Series 2). The results of these analyses are presented in Table S1.

**Figure 1.** Induction of DNA strand breaks, represented as % tail DNA, in mussel haemocytes following 4 days in vivo exposure to copper, nTiO2, aged nTiO2 and bulk TiO2. Values are mean ± SD. All treatments showed significantly higher DNA damage than the controls (p < 0.0001).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Tail DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Cu</td>
<td>20.5 ± 1.2</td>
</tr>
<tr>
<td>Bulk TiO2</td>
<td>35.7 ± 2.4</td>
</tr>
<tr>
<td>nTiO2</td>
<td>40.3 ± 3.2</td>
</tr>
<tr>
<td>nTiO2 aged</td>
<td>41.2 ± 3.5</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Tail DNA</th>
</tr>
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<td>Cu</td>
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</tr>
<tr>
<td>Bulk TiO2</td>
<td>35.7 ± 2.4</td>
</tr>
<tr>
<td>nTiO2</td>
<td>40.3 ± 3.2</td>
</tr>
<tr>
<td>nTiO2 aged</td>
<td>41.2 ± 3.5</td>
</tr>
</tbody>
</table>

**Figure 2.** Histological and histochemical observations in the gills of mussels exposed to TiO2. (A) Control specimen with no particular histological alterations. (B) Specimen exposed to nTiO2 showing moderate acidophil cell infiltration along the branchial filaments. (C) Specimen exposed to bulk TiO2 showing significant acidophil cell infiltration along the branchial filaments. (D) Specimen exposed to aged nTiO2 showing severe acidophil cell infiltration along the branchial filaments.
form showed intermediate numbers of acidophilic cells around the apex of filaments (Figure 2D and 2E; Table I). Numbers of acidophilic cells varied between the forms of nTiO_2 in the order bulk > Cu > nTiO_2 > aged nTiO_2 (Figure 2F, p < 0.0001). There was a statistically significant difference from the control for all treatments except for aged nTiO_2.

In common with gills, none of the digestive glands from control mussels showed tissue alterations (Figure S2f). All organisms treated with Cu showed damaged tissues with vacuolation of the digestive tubules (Figure S2g). The fresh and aged nTiO_2 caused vacuolation of digestive tubules (Figure S2i and l), in the same percentage of specimens seen previously for gill alterations (i.e. 83.3% for nTiO_2; 66.6% for its aged form; Table I). In all samples treated with the bulk form there was increased vacuolisation of the digestive tubules and substantial haemocyte infiltration (Figure S2h; Table I).

Expression of mt-specific complementary DNA

In gills the expression of the mt10 gene was significantly higher for bulk and nTiO_2 than for controls, in contrast to aged nTiO_2 which had lower band intensity (Figure 3A; p < 0.05). In the digestive gland, expression of the mt10 gene was similar for all treatments including the control. The mt20 gene was over-expressed in mussels treated with Cu and with bulk TiO_2 in both gills and digestive gland (Figure 3A and 3B, p < 0.0001). In digestive glands, this induced mt20 transcriptional expression was significantly higher (for Cu and bulk) than for both nTiO_2 forms (p < 0.001).

FISH analyses

The band intensity data were confirmed by the localisation of the respective mRNAs. In gill samples of control mussels, numerous mt10-positive cells were present along the gill filaments (Figure S3a), but with only a few mt20-positive
cells (Figure S3f). The mussels exposed to Cu (Figure S3b and g) and bulk nTiO2 (Figure S3c and h) showed numerous positive cells for both probes, along the branchial filament and in particular in the apices of the gill. The gills from mussels treated with Cu and bulk TiO2 (Figure S3) showed numerous haemocytes positive to both probes, but less compared to Cu- and bulk TiO2-exposed animals. In the digestive gland, all treated and control mussels showed numerous mt10-positive cells (Figure S5). For mt20, control, nTiO2 and aged nTiO2 showed fewer positive cells than those treated with Cu and bulk TiO2 (Figure S4). Figure 4 summarises this information graphically.

Determination of titanium concentrations in water and tissue samples

Water titanium concentrations did not show any clear trends. Before renewal, mean TiO2 concentrations were 82.32 ± 8.73 μg L⁻¹ for fresh nTiO2 73.92 ± 3.96 μg L⁻¹ for aged and 6.72 ± 0.67 μg L⁻¹ for bulk. After changing water, the mean concentrations were approximately 77.28 ± 8.73 μg L⁻¹ for fresh, 99.12 ± 11.76 μg L⁻¹ for aged and 26.88 ± 1.84 μg L⁻¹ for bulk. This decreased Ti concentration (from nominal values) was sample-specific (i.e. not due to instrumental drift) as all calibration check standards measured during the ICP-OES run were within 10% of their initial value.

Comparison of nTiO2 concentrations in tissue samples revealed that the metal accumulated approximately 10 times lower in the gills compared with digestive gland (Figure 5). The form of TiO2 also had a major impact on its tissue-specific accumulation. Interestingly, nano-sized TiO2, whether fresh or aged, was present at significantly greater concentrations than the bulk form in the digestive gland (p < 0.05; Figure 5B).

Discussion

Whilst there are several studies pertaining to size, aggregation and properties of nTiO2 (e.g. Lin et al. 2006; Labille et al. 2010; Jassby et al. 2012), to our knowledge this study is the first to compare the biological effects of different forms of TiO2 (including fresh or aged) on a representative marine organism. With respect to the Ti concentrations measured in the water samples, these were very low probably due to the fact that ENPs tend to form aggregates (Nowack & Bucheli 2007) and thus sink to the bottom quickly despite vigorous stirring before sampling. Previous studies have observed such precipitation of nTiO2 onto the bottom of tanks during

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Table 1. Summary of histomorphological and histochemical alterations in mussels exposed to Cu or TiO2.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>Histological test/Reaction</th>
<th>Animals showing abnormality (% ± SD)</th>
<th>Main observed effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gills</td>
<td>Control</td>
<td>H/E</td>
<td>0</td>
<td>All normal</td>
</tr>
<tr>
<td></td>
<td>Cu</td>
<td></td>
<td>100</td>
<td>Influx of haemocytes along the filament</td>
</tr>
<tr>
<td></td>
<td>Bulk</td>
<td></td>
<td>100</td>
<td>Hypoplasia, loss of cilia</td>
</tr>
<tr>
<td></td>
<td>nTiO2</td>
<td></td>
<td>83.3</td>
<td>Influx of haemocytes along the filament</td>
</tr>
<tr>
<td></td>
<td>nTiO2 aged</td>
<td></td>
<td>66.6</td>
<td>Moderate influx of haemocytes along the filament</td>
</tr>
<tr>
<td>Digestive gland</td>
<td>Control</td>
<td>H/E</td>
<td>0</td>
<td>All normal</td>
</tr>
<tr>
<td></td>
<td>Cu</td>
<td></td>
<td>100</td>
<td>Vacuolation of the digestive tubules</td>
</tr>
<tr>
<td></td>
<td>Bulk</td>
<td></td>
<td>100</td>
<td>Increased vacuolation of the digestive tubules with substantial influx of haemocytes</td>
</tr>
<tr>
<td></td>
<td>nTiO2</td>
<td></td>
<td>83.3</td>
<td>Vacuolation of the digestive tubules</td>
</tr>
<tr>
<td></td>
<td>nTiO2 aged</td>
<td></td>
<td>66.6</td>
<td>Vacuolation of the digestive tubules</td>
</tr>
<tr>
<td>Gills</td>
<td>Control</td>
<td>AB/PAS</td>
<td>33.2</td>
<td>Acidophilic mucous cells in apex of the filament</td>
</tr>
<tr>
<td></td>
<td>Cu</td>
<td></td>
<td>100</td>
<td>Acidophilic mucous cells in the whole filament</td>
</tr>
<tr>
<td></td>
<td>Bulk</td>
<td></td>
<td>100</td>
<td>Acidophilic mucous cells in apex of the filament</td>
</tr>
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</tr>
<tr>
<td></td>
<td>nTiO2 aged</td>
<td></td>
<td>83.3</td>
<td>Acidophilic mucous cells in apex of the filament</td>
</tr>
</tbody>
</table>

*Percentages were calculated from an n of 6.

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Figure 3. Relative band intensity of mt10 and mt20 (compared to actin) RT-PCR products in control and treated mussels. Values are mean ± SD (n = 6). Significant differences are indicated by * (p < 0.001).

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Figure 4. Comparison of nTiO2 concentrations in tissue samples.
mussel exposures (e.g. Canesi et al. 2010). Also, the presence of organic matter in the natural seawater used in our study could also cause complexation and influence the stability and/or bioavailability of these materials (as suggested by other workers, e.g. Labille et al. 2010).

The ICP-OES measurements confirmed that mussels accumulated TiO$_2$ in a tissue-specific manner, with a significant difference between the behaviour of bulk and nano forms (aged or fresh). In common with other studies, our results suggest differential accumulation of metallic contaminants in mussel tissues, in particular the digestive gland accumulating more compared with gills (e.g. Millward et al. 2012). It is possible to envisage a scenario where a high proportion of larger (bulk) particles are rejected and excreted as pseudofaeces at the gills, but that smaller (i.e. nano) particles are transported to the digestive gland. Our results support this idea and an earlier study on NP agglomerates taken up by the digestive gland (Canesi et al. 2012). However, as the TEM measurements of aged TiO$_2$ revealed larger aggregates, it is interesting that there appears to be no ‘trapping’ effect in the gills for this form. It is, however, possible that – as a result of preparation – particle sizes measured using electron microscopy are different from the real aggregation pattern in seawater. Furthermore, there is little information on the effects of ageing on the bioavailability and uptake of ENPs (e.g. Labille et al. 2010) with which to compare our data. Accumulation of aged nTiO$_2$ in gills and digestive glands of mussels in our study is in contrast to observations made in earthworms (Lumbricus terrestris) exposed to an aqueous solution of aged TiO$_2$ nanocomposite (Lapied et al. 2011). It appeared that nTiO$_2$ was unable to cross the intestinal epithelium/ chloragogenous matrix barrier to enter the coelomic liquid, or the cuticle barrier to reach the muscular layers. Since different nanoparticles (T-lite) and an alternative ageing technique (48 h under white light) were used, these may have resulted in altered physico-chemical properties of the nanoparticles. Apart from the surface chemistry of the nTiO$_2$, routes of exposure (mussels being filter feeders with an open vascular system) and complexation with organic matter (especially for earthworms) could also account for these apparently contrasting results (van Herwijnen et al. 2007).

Histopathologically, the tissues treated with bulk TiO$_2$ showed enhanced damage compared to other treatments. In both the gills and digestive gland more vacuolation and a significant reduction of haemocytes was observed in the bulk-treated group despite the concentration of titanium being much lower following exposure to bulk compared to nTiO$_2$ (aged or fresh). This indicates that, for these forms of damage, bulk TiO$_2$ is considerably more toxic than its nano equivalents. Further support for this hypothesis comes from the histochemical analysis, where the gills of mussels exposed to bulk TiO$_2$ had the highest levels of acidophilic mucous cells, although this was not significantly higher than for nTiO$_2$ forms. Similar histochemical responses have also been observed for several marine organisms following exposure to different environmental stresses (Bettercourt et al. 2008, Fasulo et al. 2008, Brunelli et al. 2011).

Figure 4. Quantification of FISH-positive cells in mussel gill (A) and digestive gland (B) following 4 days in vivo exposure to copper, nTiO$_2$ aged and bulk TiO$_2$. Values are mean ± SD (n = 6). Significant differences are indicated by * (p < 0.001).

Figure 5. Accumulation of titanium in gills and digestive glands. Values are mean ± SD (n = 6). Significant differences from the controls are indicated by * (p < 0.05).
Results for transcriptional expression of mt10 and mt20 genes and for localisation of the respective mRNAs suggest that in digestive gland, mt10 is the basally expressed form, while mt20 is an inducible mt gene. This observation is in line with earlier studies in bivalve molluscs (Lemoine et al. 2000, Dondoro et al. 2005, Fasullo et al. 2008, Dallas et al. 2013). Significant overexpression of the mt20 gene in the digestive gland of mussels exposed to bulk TiO2, compared to those of nTiO2 (fresh and aged), provides further evidence that the bulk analogue induces stress in mussels. Our results indicate a tissue- or concentration-specific difference in mt gene induction as – in contrast to the digestive gland – both mt genes were induced in the gills despite much lower titanium accumulation. Although nTiO2 showed higher mt10 induction in gills, this result was not supported by FISH or histochemical data. However, analysis of gene expression at the transcriptional level does not necessarily reflect the true stress scenario. Concurrent measurement of mt proteins could potentially elucidate the mechanisms involved.

The relative histological and histochemical responses following exposure to different forms of TiO2 (i.e. bulk, aged and fresh nano) are in contrast to acute toxicity (96-h LC50) observations in zebra fish. Where nano forms showed higher toxicity compared to bulk form, which was essentially non-toxic. This differential response was attributed to enhanced production of hydroxyl radicals by nTiO2 (Xiong et al. 2011). Similar acute toxicities for nano compared to bulk form have been reported for soil nematode (Caenorhabditis elegans) and water flea (Daphnia magna) (Zhu et al. 2008; Wang et al. 2009). In our study, we did not evaluate the acute toxicity in terms of lethality/survival of the mussels and used only sublethal concentrations, based on previous studies. Phylogenetic differences may explain differential biological responses in marine mussels compared to zebra fish, nematodes or water fleas.

As a naturally occurring mineral, TiO2 is present in two main forms, rutile and anatase. The relative content of these forms in the bulk and nano materials used in these experiments was very similar. In the environment both forms are bound to impurities and the raw material is chemically processed to remove these, leaving the commercial products. The treatments to produce the bulk and nano materials differ and probably the associated impurities are removed to a different extent. This could potentially explain the enhanced toxicity of the bulk material. Our initial ICP-MS results (Table S1) have identified some differences in the elemental composition of each form of TiO2 (e.g. with regard to Al, K and As) but more research is needed to investigate this issue.

Analysis of induction of oxidative DNA damage in the haemocytes of mussels, as determined by modified comet assay, did not highlight significant differences among the three forms of TiO2 used here. This is in contrast to in vitro studies carried out in our laboratory conditions using fish cells which have shown enhanced oxidative damage to DNA following exposure to nTiO2 (Reeves et al. 2008; Veveres & Jha 2008). Using electron spin resonance techniques, it has been further suggested that nTiO2-induced oxidative damage results from hydroxyl radicals (•OH), carbonyl radical anions (CO2•−) and superoxide radical anions (O2•−; Dodd & Jha 2009, 2011). It has been suggested that aggregation of metal-lic nanoparticles (such as that caused by electrolytes [e.g. NaCl in seawater; Jassby et al. 2012]) can decrease their photocatalytic properties (Lin et al. 2006; Tseng et al. 2006). Roles of aggregate size in decreased production of ROS have, however, not been demonstrated as yet (Jassby et al. 2012).

Apart from generation of ROS, it is known that metallic contaminants exert their genotoxic effects by directly interacting with cellular macromolecules (e.g. formation of metal-DNA adducts (Singh et al. 1998) or inhibition of DNA repair processes (Hartwig et al. 2002)). Therefore, induction of DNA damage in haemocytes could be independent of ROS, as treatment with bacterial enzymes did not show significantly enhanced levels of DNA damage. It is also important to remember that DNA damage in the present study was determined only in the haemocytes of the mussels. These cells play a varied role in the organism and could have the capacity to repair induced-DNA damage (Jha et al. 2005).

Furthermore, in common with mammalian blood cells, haemocytes have a well-known capacity to undergo apoptotic processes (Jha et al. 2005). It has been demonstrated that nTiO2 induces tissue-specific apoptosis in earthworms following exposure to concentrations which would normally decrease survival, growth and fecundity (Lapedi et al. 2011). Assessment of other markers in different tissues could be used to elucidate the role of oxidative stress in the observed biological responses in mussels exposed to nano- and bulk TiO2.

Conclusions
Overall, our results show that the exposures of mussels to three different forms of TiO2 induce responses at the cellular and subcellular level both in gills and digestive gland and DNA damage in haemocytes. The response of these organisms confirms the potential risk of ENPs to aquatic life. It appears that, for these organisms, photocatalytic ageing of the nTiO2 does not cause any appreciable difference in cellular/subcellular toxicity, and the bulk form may be more toxic with respect to histopathological and histochemical changes. The use of Cu as a positive control has validated these findings, which are in contrast to many earlier authors who have reported that bulk is more ecotoxicologically inert than nanoparticulate forms. This study has clear implications for the field of nano-ecotoxicology and environmental protection. Further research is required to investigate the cause of elevated bulk toxicity and to investigate this effect in other species and with other ENPs and their bulk equivalents.

Acknowledgements
Partial financial support from the European Regional Development Fund, INTERREG IVA (Grant No. 4059), is gratefully acknowledged. We thank Richard Handy (Plymouth) and Andrew Collins (Oslo) for providing the samples of nTiO2 and bacterial enzymes, respectively.
Declarative interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


Luxembourg, European Commission: 1-79.


NANOTOXICOLOGY

Nanotoxicology characteristics of aged TiO2 nanoparticles: implications for risk assessment.

A. Publications

Supplementary materials available online

Figures S1, S2, S3, S4 and S5

Table S1

Cyotoxicity and oxidative DNA damage in fish cells. Mutat Res 640:113-122.


Appendix B

Radiation Protection
Documentation
## Control of Substances Hazardous to Health (COSHH) Assessment

### Control of Substances Hazardous to Health Regulations 2002

<table>
<thead>
<tr>
<th>Section A – Administration</th>
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<tbody>
<tr>
<td>Faculty</td>
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<td>Department/Location</td>
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<tr>
<td>Assessor</td>
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<tr>
<th>Section B – Substances</th>
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<td><strong>Process</strong> (ref. Guide Section B (2.4))</td>
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<tr>
<td>Flammable</td>
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<tr>
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Control of Substances Hazardous to Health (COSHH) Assessment

Control of Substances Hazardous to Health Regulations 2002

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Section F – Controls (List those in place / note deficiencies)

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<th>Engineering i.e. local exhaust ventilation</th>
<th>Ensure area well ventilated</th>
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<tbody>
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<td>Respiratory i.e. respiratory protective equipment</td>
<td>Labcoat, Goggles, Gloves</td>
</tr>
<tr>
<td>Personal Protection i.e. gloves, boots, overalls</td>
<td>Store as radioactive material</td>
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<tr>
<td>Monitoring Arrangements i.e. health surveillance</td>
<td>Dispose of as radioactive material</td>
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</table>

Section G – Emergency Procedures (List those in place / note deficiencies)

| Spillage Arrangements | Evacuate and ventilate area |
| First Aid Facilities  | Flush wounds/eyes/mouth with fresh water |

Section H – Line Managers Actions

Could the work continue if any hazardous substances were eliminated?  
Yes  No  ✓

Can any hazardous substances be substituted by a less hazardous substance?  
Yes  No  ✓

Further training necessary?  
Yes  No  ✓

Comments: .................................................................

Departmental Manager

Signature

Assessor’s Signature: L. J. Dallas

Review Date: 16.06.2011
Local rules for the calibration of the liquid scintillation counter, for use in the analysis of the tissue-specific accumulation of tritium in *Mytilus edulis*.

**Laboratory Davy 110**

**General**
These Local Rules are provided in accordance with Regulation 17(1) of the Ionising Radiations Regulations 1999 (IRR99). The aim of the experiment is to ensure that the liquid scintillation counter is calibrated for tritium, prior to the investigation of the bioaccumulation of the tritium ($^3$H or T) in the mussel *Mytilus edulis*. This will involve analysis of mussel tissue samples using scintillation techniques and the spiking of these tissue samples with known concentrations of tritium.

The supervisor will give training in the dispensing of the radioisotopes with the aid of the Radiation Protection Supervisor (RPS). The record keeping and monitoring of the work area will form part of the training and will be assisted, as appropriate, by the RPS and assistant RPS. The student has been trained in appropriate lab technique when handling radioactive materials, e.g. making sure that spills are dealt with effectively, clear labelling of samples so that co-workers are fully aware of the presence of any radioactive substances and the use of a dedicated laboratory coat, gloves and safety glasses. The student has also been instructed as to the proper method for disposal of waste.

**Area Description**
Dilutions of tritium from the stock solution will be done in a fume cupboard in Room 314a of the Davy Building by the assistant RPS, Nick Crocker. Laboratory work will be conducted in Davy 110, a supervised area, with analysis in Davy 110A. For disposal, radioactive substances will be disposed of in Room 110 where a special sink without seals is present, designed for the disposal of such substances.

**Dose Investigation Levels**
Tritium is stored and controlled by the assistant RPS, Nick Crocker, (Room 314a, Davy Building). The paperwork will be completed when isotopes are used or taken away from storage. Record keeping; (i) Pink copy, completed and kept by Nick Crocker; (ii) White copy, completed and kept with the compound. At most 185 MBq will be used, when the stock standard is dispensed by Nick Crocker. A solution containing 1 MBq of tritium will be taken into Davy 110; this is << 0.1 of the ALI (480 MBq).

**Working Instructions**
**Methods.** The tray, "Benchcote", pipettes, pipette tips and plastic gloves used for spiking samples will be kept in Room 110. Scintillation counting will be conducted using the scintillation counter in Room 110a. Weighing will be conducted using the balances in Rooms 110.
The mussels will be dissected, removing the byssus thread, foot, adductor muscle, gut, gills, and mantle. If not used immediately mussel tissue will be stored frozen at -80°C in the freezers in Room 422. The sample is then freeze-dried and weighed. Samples are then transferred to scintillation vials and 100 µl milli-Q water is added, samples are left to rehydrate for 30 min. 1 ml of Soluene-350 is added to each vial. Samples are then placed in an oven at 55°C for 48 h or until the tissue is solubilised. Once solubilisation is achieved, 10 ml Optiphase Trisafe liquid scintillation cocktail and 100 µl glacial acetic acid are added. Samples are transferred to the liquid scintillation counter and left in the dark for 90 min before counting. Beta emissions for each sample are determined using a Beckman 65,000 liquid scintillation counter (Room 110a), for 1 hour. Once counts have been achieved in unspiked samples as described, the samples will be transferred to Room 110. They will then be spiked with known activities of tritiated water (0-1000 Bq), transferred back to the liquid scintillation counter and left in the dark for 90 min before counting, as before. The data for both (unspiked and spiked samples) will then be converted to MBq kg⁻¹ using the dry weight of the tissue samples.

Disposals
The low radioactivity involved allows safe disposal of liquid down the drain whilst a tap is running in a designated sink. Small amounts if solid radioactive waste will also be disposed of. The sealed scintillation vials and other contaminated apparatus (i.e. pipette tips) will be disposed of as solid waste. The remaining isotopes can be removed by rinsing and washed down the sink with plenty of water. All disposals will be recorded.

Restricting exposure
The potential exposure from this experiment is low. The proposed safety practices should be sufficient in maintaining a safe working environment for the researcher. It is necessary to exercise extreme care when handling the radioactive material, especially when in the concentrated state prior to spiking of samples. Spiking samples will take place in a tray, marked with radioactive hazard tape and covered with “Benchcote”. The safety record will be signed by the Radiation Protection Supervisor (RPS). The student will wear a designated lab coat, safety glasses and gloves at all times when working in Rooms 110 and 110a.

Contingency arrangements
During and after spiking vials will be kept in plastic trays large enough to contain all contents if a leak should occur. In case of any spillages a vermiculite spill kit will be kept in the lab, this will be used when any spill of liquid thought to contain radioactive material is encountered. Any spillage onto skin will treated by washing with copious amounts of water. The emergency protocols detailed in the handbook will be applied, including minor accidents and fire risk.

RADIOLOGICAL SAFETY CONTACTS
The Radiation Protection Advisor (RPA), available 24/7 for emergencies, is:
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The Radiation Protection Supervisor (RPS) is:

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Fax: 01752 233048  
E-mail: Miranda.keith-roach@plymouth.ac.uk

The Assistant RPS is:

Mr N Crocker  
School of Biological Sciences  
University of Plymouth,  
Drake Circus, PLYMOUTH,  
Devon PL4 8AA  
Tel: 01752 232928

SUPPORTING INFORMATION

Appropriate COSHH forms are also attached.

REVIEW STATUS AND FREQUENCY

The Local Rules will be reviewed annually by the study team, in consultation with RPS, as appropriate.

Signature of Researcher: ..................................  Date: .................................

Signature of Supervisor: .................................  Date: .................................

Approved by the RPS: .................................  Date: .................................
Local rules for the assessment of genotoxic, histopathological and physiological effects and accumulation of radioactivity in mussels (*Mytilus* sp.) chronically exposed to tritiated water.

Lorn Dallas Local Rules HTO Exp1 June 2011

CORIF Local Rules CLR1108

Researcher: Miss L. J. Dallas  
Supervisor: Dr. A. Jha  
Dept: Biological & Biomedical Sciences  
Tel: 07793 033610  
Email: lorna.dallas@plymouth.ac.uk

Date: June 2011

General

These Local Rules are provided in accordance with Regulation 17(1) of the Ionising Radiations Regulations 1999 (IRR99). The aim of the experiment is to determine the genotoxic, histopathological and physiological effects and accumulation of tritium (³H or T) in mussels (*Mytilus* sp.) after chronic exposure. This will involve *in vivo* exposure of mussels to tritiated water (HTO) for 14d, measurement of mussel feeding rate in live animals after exposure, analysis of mussel haemocytes by the comet and micronucleus assays, dissection of mussel tissues for histopathological examination and analysis of mussel tissue samples using scintillation techniques.

The supervisor will give training in the dispensing of the radioisotopes with the aid of the Radiation Protection Supervisor (RPS). The record keeping and monitoring of the work area will form part of the training and will be assisted, as appropriate, by the RPS and assistant RPS. The student has been trained in appropriate lab technique when handling radioactive materials, e.g. making sure that spills are dealt with effectively, clear labelling of samples so that co-workers are fully aware of the presence of any radioactive substances and the use of a dedicated laboratory coat, gloves and safety glasses. The student has also been instructed as to the proper method for disposal of waste.

Area Description

Dilutions of tritium from the stock solution will be done in a fume cupboard, designated for use with radionuclides, in Room 314a of the Davy Building by the assistant RPS, Nick Crocker. Exposure of mussels will be carried out in a controlled temperature room (CR2) adjacent to Davy 420. This area is designated an ‘other area’ in the UoP Radiation Safety Handbook, therefore maximum activity must be <1/10th ALI. The room will be cordoned off and clearly labelled with trefoil as containing radiological material for the duration of the experiment. It is impractical to carry out this work in Davy 110, due to the need for a controlled temperature room with seawater on tap and a set photoperiod. Extraction of haemolymph and mussel dissection will take place in Davy 110, a controlled area. Preparation for scintillation counting will be conducted in Davy 110, with analysis in Davy 110A. For disposal, tritiated water will be transported in sealed containers to Room 110, where a special sink without seals is present, designated for the disposal of radioactive liquids.
Dose Investigation Levels

Tritiated water will be purchased from Perkin Elmer in batches of 185 MBq. We intend to purchase 4 batches, with a total activity of 740 MBq. Tritium is stored and controlled by the assistant RPS, Nick Crocker, (Room 314a, Davy Building). The paperwork will be completed when isotopes are used or taken away from storage. Record keeping: (i) Pink copy, completed and kept by Nick Crocker; (ii) White copy, completed and kept with the compound. The highest activity used will be 185 MBq when the stock standard is dispensed by Nick Crocker. A solution containing 1.85 MBq/ml of tritium will be taken into Davy 420 CR2.

Working Instructions

Methods. All reagents will be kept in Room 422. Beakers, Perspex, airstones, air pumps, siphon hoses, water containers, stirrers and volumetric flasks will be kept in Room 420 CR2. The tray, “Benchcote”, pipettes, pipette tips and plastic gloves used for haemolymph extraction and dissection will be kept in Room 110. Scintillation counting will be conducted using the scintillation counter in Room 110a. Weighing will be conducted using the balances in Rooms 110 and 422.

Exposure scenario. Nine mussels per beaker (2 L) will be exposed to the following activity concentrations of HTO in triplicate, 0, 1, 5, 15 MBq L⁻¹, and a non-radioactive positive control (32 mg L⁻¹ EMS). All dilutions will be made with seawater obtained from the tap in Davy 420 CR2. The exposure will be for 14 d, with 5 complete water changes (days 3, 6, 9, 12 and 14). At each water change the contaminated seawater will be drained by siphoning the water into a large (>10 l), sealable container and taken to Room 110 for disposal. Fresh HTO concentrations will then be made up and added to the appropriate beakers until the next water change. On day 14, the exposure will finish so there will be no renewal of HTO. At each water change, water samples will be taken before removal of contaminated seawater and after renewal with fresh HTO, this will allow calculation of the activity concentration so that disposed activities can be recorded accurately. This exposure scenario results in a maximum activity of 126 MBq in Davy 420 CR2 at any one time, which is considerably lower than 1/10th total ALI (300 MBq).

Sampling. At 2 sampling points (7 and 14 d) mussels will be removed from their beakers, transported to the coulter counter in Davy 420 and processed through the clearance rate assay as follows. Adult mussels (n=9) will be individually transferred to beakers containing 350 ml of filtered seawater (15 °C) with constant vortex mixing. After a 10 min acclimation period, 500 µl of Isochrysis algal solution is added to produce a final concentration of approximately 2 x10⁴ cells ml⁻¹. Water samples (20ml) are taken on addition of the algae (T₀), and at the end of the measurement period (T₁). Enumeration of algal cells was achieved using a Beckman Z2 Coulter Particle Size and Count Analyser (Beckman Coulter, Brea, CA, USA). All water used during the clearance rate assay will be treated as contaminated and disposed of down the designated sink in Davy 110. Following clearance rate assessment, mussels will be transported to Davy 110 for haemolymph extraction and dissection. Mussels will be contained within trays clearly labelled as radiological material at all times during transportation, haemolymph extraction and dissection. Aliquots of haemolymph will be transferred to pre-prepared slides for the comet and micronucleus assays. Three mussels per beaker will be dissected into their individual organs for determination of radioactivity.
(see below) and three mussels per beaker will be dissected into cross-sections for histology. Histological samples will be transferred to Davidson’s seawater fixative for 24h for fixation, then transferred to 70% IMS for storage until processing (< 1 week). Processing takes place in a Leica TP 1020 Automatic Tissue Processor. After processing, tissues are embedded in paraffin wax, sectioned (3-6 µm) using a rotary microtome, transferred to slides and stained with haematoxylin and eosin. After dissection, any waste mussel tissue will be placed in a plastic bag, sealed in a box (this is more than adequate shielding for $^3$H, which requires <0.1mm plastic for total absorption) and frozen before disposal as solid waste in Davy 110.

**Determination of radioactivity within mussel tissues.** The mussels will be dissected, removing the byssus thread, foot, adductor muscle, gut, gills, and mantle. Each sample transferred to scintillation vials, freeze-dried and weighed. To rehydrate samples, 100 µl milli-Q water is added, and incubated for 30 min at room temperature. After rehydration, 1 ml of Soluene-350 is added to each vial. Samples are then placed in an oven at 55°C for 48 h or until the tissue is solubilised. The oven is located in Davy 422 and will be clearly marked with a trefoil for the duration of the incubation. Once solubilisation is achieved, 10 ml Optiphase Trisafe liquid scintillation cocktail and 100 µl glacial acetic acid are added. Samples are transferred to the liquid scintillation counter and left in the dark for 90 min before counting for 1 hour. Beta emissions for each sample are determined using a Beckman 65,000 liquid scintillation counter (Room 110a), with a chemiluminescence quench correction curve. The data will then be converted to MBq kg$^{-1}$ using the dry weight of the tissue samples.

**Disposals**
The exposure scenario described above will result in a total disposal activity of 630 MBq HTO. This is within the university’s monthly aqueous disposal limit for $^3$H of 4 GBq. The low radioactivity involved allows disposal of liquid down the drain whilst a tap is running in a designated sink (Davy 110). Small amounts of solid radioactive waste will also be disposed of using the designated solid waste disposal bin in Davy 110. The levels of activity associated with the solid waste will be below the monthly disposal limit. The sealed scintillation vials and other contaminated apparatus (i.e. pipette tips) will be disposed of as solid waste. The remaining isotopes can be removed by rinsing and washed down the sink with plenty of water. All disposals will be recorded.

**Restricting exposure**
The potential exposure from this experiment is low. All siphoning will performed manually so there will be nil by mouth. Therefore, the amount of HTO ingested should be negligible and considerably below the 20 mSv ALI$^{\text{ingestion}}$ of 480 MBq. Given maximum evaporative loss from each beaker as 10 ml per water change period, the maximum inhalation activity across the experimental period would be 750 kBq. This is considerably less than the 20 mSv ALI$^{\text{inhalation}}$ of 490 MBq. However, as an extra precaution beakers will be covered with cling film to prevent evaporative loss or contamination of the controlled temperature room. After use, the cling film will be disposed of as solid waste in Davy 110. The proposed safety practices should be sufficient in maintaining a safe working environment for the researcher. It is necessary to exercise extreme care when handling the radioactive material, especially when in the concentrated state prior to dilution. The safety record will be signed by the Radiation Protection Supervisor (RPS). The student will wear a designated lab coat, safety
glasses and gloves at all times when working in Rooms 110, 110a and 420 CR2 (and a mask in the constant temperature room). The surfaces of the constant temperature room will be routinely monitored by the Assistant RPS who will take swabs to assess any increases in activity above background.

**Contingency arrangements**

During the exposure period and at all water changes the beakers will be kept in plastic trays large enough to contain all contents if a leak should occur. In case of any spillages a vermiculite spill kit will be kept in the lab, this will be used when any spill of liquid thought to contain radioactive material is encountered. Any spillage onto skin will be treated by washing with copious amounts of water. The emergency protocols detailed in the handbook will be applied, including minor accidents and fire risk.

**RADIOLOGICAL SAFETY CONTACTS**

The Radiation Protection Advisor (RPA), available 24/7 for emergencies, is:
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The Radiation Protection Supervisor (RPS) is:

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**SUPPORTING INFORMATION**

Appropriate COSHH forms are also attached.

**REVIEW STATUS AND FREQUENCY**

The Local Rules will be reviewed annually by the study team, in consultation with RPS, as appropriate.
B. Radiation Protection Documentation

Signature of Researcher:…………………………..             Date:…………………………..

Signature of Supervisor:.…………………………..             Date:…………………………..

Approved by the RPS:………………………………           Date:…………………………..
Local rules for the assessment of genotoxic and physiological effects and accumulation of radioactivity in mussels (*Mytilus* sp.) and oysters (*Crassostrea gigas*) chronically exposed to tritiated water.

**Laboratories:** Davy 110, 420 & 422

**General**
These Local Rules are provided in accordance with Regulation 17(1) of the Ionising Radiations Regulations 1999 (IRR99). The aim of the experiment is to determine and compare the genotoxic and physiological effects and accumulation of tritium (³H or T) in mussels (*Mytilus* sp) and oysters (*Crassostrea gigas*) after chronic exposure. This will involve *in vivo* exposure of mussels and oysters to tritiated water (HTO) for 14d, analysis of haemocytes by the comet assay, assessment of anoxia tolerance, and dissection and analysis of tissue samples using scintillation techniques.

Training in the dispensing of the radioisotopes and in the use of peripheral equipment has been given by the Technical Director of CORiF. The record keeping and monitoring of the work area was included in the training and will be assisted, as appropriate, by the RPS and assistant RPS. The researchers will be competent in appropriate lab technique when handling radioactive materials, e.g. making sure that spills are dealt with effectively, clear labelling of samples so that co-workers are fully aware of the presence of any radioactive substances and the mandatory use of a dedicated laboratory coat, gloves and safety glasses at all times. The researchers have received instruction as to the proper method for disposal of solid and liquid wastes.

**Area Description**
Dilutions of tritium from the stock solution will be done in a fume cupboard, designated for use with radionuclides, in Room 314a of the Davy Building by the assistant RPS, Nick Crocker. Exposure of mussels/oysters will be carried out in a controlled temperature room (CR2) adjacent to Davy 420. This area is designated an ‘other area’ in the UoP Radiation Safety Handbook, therefore maximum activity must be <1/10⁰ of the annual limit of intake (<300 MBq). The room will be cordoned off, locked when not in use, and clearly labelled
with trefoil as containing radiological material for the duration of the experiment. It is impractical to carry out this work in Davy 110, due to the need for a controlled temperature room with seawater on tap and a set photoperiod. Extraction of haemolymph and dissection will take place in Davy 110, a controlled area. A spill kit will be provided in Davy 420. Safe carriage of radioactive water and/or animals from Davy 420 CR2 to Davy 110 will be ensured by removing excess tritiated water (either by draining beakers or drying individual animals), covering beakers with film to prevent evaporation, and keeping beakers and/or animals within trays clearly labelled as radioactive material at all times. Preparation for scintillation counting will be conducted in Davy 110, with analysis in Davy 110A. For disposal, tritiated water will be transported in sealed containers to Room 110, where a special sink without seals is present, designated for the disposal of radioactive liquids. The effective dilution and disposal of the tritiated water will be achieved by a constant supply of tap water. Any solids (gloves, tissues etc) will be disposed of in the specified solid waste disposal bin in Davy 110. The liquid disposal record sheets will be completed immediately afterwards, see also the section on Disposals.

**Dose Investigation Levels**

Tritiated water will be purchased from Perkin Elmer in batches of 185 MBq. We intend to purchase 6 batches, with a total activity of 1,110 MBq. This is considerably less than the University's storage limit for tritium of 10 GBq. Tritium is stored and controlled by the assistant RPS, Nick Crocker, (Room 314a, Davy Building). The paperwork will be completed when isotopes are used or taken away from storage. Record keeping; (i) Pink copy, completed and kept by Nick Crocker; (ii) White copy, completed and kept with the compound. The highest activity concentration used will be 37 MBq/ml when the stock standard is dispensed by Nick Crocker. A solution containing 1 MBq/ml of tritium will be taken into Davy 420 CR2.

**Working Instructions**

**Methods.** All reagents will be kept in Room 422. Beakers, Perspex, airstones, air pumps, siphon hoses, water containers, stirrers and volumetric flasks will be kept in Room 420 CR2. The tray, “Benchcote”, pipettes, pipette tips and plastic gloves used for haemolymph extraction and dissection will be kept in Room 110. Scintillation counting will be conducted using the scintillation counter in Room 110a. Weighing will be conducted using the balances in Rooms 110 and 422.

**Exposure scenario.** Fifteen mussels per beaker (2 L) will be exposed to the following activity concentrations of HTO in triplicate, 0, 1, or 15 MBq L⁻¹. The same experiment will be run in parallel with oysters (i.e. 18 beakers in total). All dilutions will be made with seawater obtained from the tap in Davy 420 CR2. The exposure will be for 14 d, with 5 complete water changes (days 0, 3, 6, 9, and 12). At each water change the contaminated seawater will be drained by siphoning the water into a large (50 L), sealable container and taken to Room 110 for disposal. Fresh HTO concentrations will then be made up and added to the appropriate beakers until the next water change. On day 14, the exposure will finish so there will be no renewal of HTO. At each water change, water samples will be taken before removal of contaminated seawater and after renewal with fresh HTO, this will allow calculation of the activity concentration so that disposed activities can be recorded accurately. This exposure scenario results in a maximum activity of 192 MBq in Davy 420.
CR2 at any one time, which is considerably lower than 1/10th total ALI (300 MBq). The aqueous waste is also far below the monthly waste limit for tritium on the Plymouth campus (4 GBq).

**Sampling.** On day 0 control organisms (those with no exposure to HTO) will be sampled. These will be treated as if radioactively contaminated for the purpose of ‘road-testing’ day 14 sampling procedures. One animal per beaker will be removed, dried (to minimise contamination from drops of tritiated water) and transported to Davy 110 contained within trays and labelled as radioactive material. Haemolymph will be extracted and transferred to pre-prepared slides for the comet assay. Potentially radiolabelled tissue (haemolymph) will be taken into Davy 422 for the comet assay, and will be clearly labelled. Previous work indicates haemolymph has an activity below the limit of detection for liquid scintillation counting. Animals will then be dissected into their individual organs for determination of tritium activity, as detailed below. After dissection, any waste mussel tissue (shells) will be placed in a plastic bag, sealed in a box (this is more than adequate shielding for $^3$H, which requires <0.1mm plastic for total absorption) and frozen before disposal as solid waste in Davy 110. On day 14 sampling will occur as above, except that five animals per beaker will be removed from their beakers and maintained in Davy 420 CR2 for stress on stress assessment. Three animals per beakers will be transported to Davy 110 *in situ* (i.e. in beakers with HTO). It is necessary to keep the animals exposed until the point of dissection, as HTO is depurated quickly. Radioactive water in beakers will be drained to a minimum level (i.e. just above the animals) during transportation. Beakers will be covered with film to prevent evaporative contamination of corridors and will be contained within trays clearly labelled as radiological material at all times. Those organisms used to assess the stress on stress response will be kept in Davy 420 CR2 on petri dishes and monitored for lethality for ~14 days after the exposure ends. These dishes will be labelled as containing radioactive material and the cold room will remain labelled and restricted access whilst this occurs. After death, these animals will be disposed of as solid waste in the bin in Davy 110.

**Determination of radioactivity within mussel tissues.** Mussels will be dissected into seven tissues; the byssus thread, foot, adductor muscle, digestive gland, mantle, gills and ‘other’. Oysters will be dissected into 5 tissues; adductor muscle, digestive gland, mantle, gills and ‘other’. Shells will be disposed of as above. Each tissue sample will be transferred to pre-weighed, pre-labelled scintillation vials, weighed (to obtain wet weight for dose calculations), freeze-dried and re-weighed (to obtain dry weight for accumulation calculations). Samples will be homogenised in a fume hood, and then rehydrated by addition of 100 µl milli-Q water and incubation for 30 min at room temperature. After rehydration, 1 ml of Soluene-350 is added to each vial. Samples are then placed in an oven at 55°C for 48 h or until the tissue is solubilised. The oven is located in Davy 422 and will be clearly marked with a trefoil for the duration of the incubation. Once solubilisation is achieved, 10 ml Optiphase Trisafe liquid scintillation cocktail and 100 µl glacial acetic acid are added. Samples are transferred to the liquid scintillation counter and left in the dark for 90 min before counting for 2 hours. Beta emissions for each sample are determined using a Beckman 65,000 liquid scintillation counter (Room 110a), with a chemiluminescence quench correction curve. The data will then be converted to MBq kg$^{-1}$ using the dry weight of the tissue samples and to a dose rate in μGy h$^{-1}$ using wet weights and the ERICA tool.
Disposals

The exposure scenario described above will result in a total disposal activity of 960 MBq HTO. This will be spread out over 2 weeks (as a result of 5 individual water changes at 192 MBq each, totalling 576 MBq in the first week of exposure, and 384 MBq in the second week) is within the university’s monthly aqueous disposal limit for $^3$H of 4 GBq and weekly limit of 1 GBq. The low radioactivity involved allows disposal of liquid down the drain whilst a tap is running in a designated sink (Davy 110). Small amounts of solid radioactive waste (animal tissues) will also be disposed of using the designated solid waste disposal bin in Davy 110. Any broken glass from beakers containing radioactive material will be poured into a sieve, washed several times to remove any contamination, and disposed of as solid waste. Water from such washing will be disposed of in the designated sink. The solid waste limit is 400 kBq in 0.1 m$^3$ of waste, with any one item having a maximum activity of 40 kBq. The levels of activity associated with the solid waste will be below the disposal limit of solid Very Low Level Waste (VLLW). The sealed scintillation vials and other contaminated apparatus (i.e. pipette tips) will be disposed of as solid waste. The remaining isotopes can be removed by rinsing and washed down the sink with plenty of water. All disposals will be recorded.

Restricting exposure

The potential exposure from this experiment is low. All siphoning will performed manually so there will be nil by mouth. Therefore, the amount of HTO ingested should be negligible and considerably below the 20 mSv AL_{ingestion} of 480 MBq. Given maximum evaporative loss from each beaker as 10 ml per water change period, the maximum inhalation activity across the experimental period would be 4.8 MBq. This is considerably less than the 20 mSv AL_{inhalation} of 490 MBq. However, as an extra precaution beakers will be covered with cling film to prevent evaporative loss or contamination of the controlled temperature room. After use, the cling film will rinsed in the designated sink (to remove any droplets of tritiated water) and will be disposed of as solid waste in Davy 110. The proposed safety practices should be sufficient in maintaining a safe working environment for the researchers. It is necessary to exercise extreme care when handling the radioactive material, especially when in the concentrated state prior to dilution. The safety record will be signed by the Radiation Protection Supervisor (RPS). The researchers will wear a designated lab coat, safety glasses and gloves at all times when working in Rooms 108, 110, 110a and 420 CR2 (and a mask in the constant temperature room). The surfaces of the constant temperature room will be routinely monitored by the Assistant RPS who will take swabs to assess any increases in activity above background.

Contingency arrangements

During the exposure period and at all water changes the beakers will be kept in plastic trays large enough to contain all contents if a leak should occur. In case of any spillages a vermiculite spill kit will be kept in the lab, this will be used when any spill of liquid thought to contain radioactive material is encountered. Any spillage onto skin will treated by washing with copious amounts of water, and the RPS will be contacted as soon as possible. The researchers will have mobile phones at all times when handling radioactive material and will contact the RPS for assistance without leaving the affected area in the event of a spill. After clean up the Assistant RPS will take swabs to measure $^3$H levels, these will be counted in Davy 110a. In the event of minor emergency or accident the RPS or RPA will be contacted immediately. In the case of fire, the alarm will be activated, labcoats and gloves removed...
and the researchers will evacuate the building and call the RPA via the emergency number. In the event of a major flood, labcoat and gloves will be removed and researchers will evacuate the area and call the RPS.

RADIOLOGICAL SAFETY CONTACTS

The Radiation Protection Advisor (RPA), available 24/7 for emergencies, is:
Mr C Ellis, HP Squared Ltd
Tel: 01235 820049 Mobile: 07786 405769
E-mail: cliff.ellis@hpsquared.co.uk

The Radiation Protection Supervisor (RPS) is:

Professor Richard Handy
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E-mail: r handy@plymouth.ac.uk

The Assistant RPS is:

Mr N Crocker
School of Biological Sciences
University of Plymouth,
Drake Circus, PLYMOUTH,
Devon PL4 8AA
Tel: 01752 584593

SUPPORTING INFORMATION

Appropriate COSHH forms are also attached.

REVIEW STATUS AND FREQUENCY

The Local Rules will be reviewed annually by the study team, in consultation with RPS, as appropriate

Signature of Researcher:…………………………..             Date:…31/10/12…..

Signature of Supervisor:…………………………..             Date:…1/11/12…..

Approved by the RPS:……RHandy………………………           Date:………2nd November 2012……..
Local rules for the assessment of genotoxic and physiological effects and accumulation of radioactivity in oysters (*Crassostrea gigas*) chronically exposed to tritiated water.

**Laboratories:** Davy 110, 420 & 422

**General**

These Local Rules are provided in accordance with Regulation 17(1) of the Ionising Radiations Regulations 1999 (IRR99). The aim of the experiment is to determine and compare the genotoxic and physiological effects and accumulation of tritium ($^3$H or T) in oysters (*Crassostrea gigas*) after chronic exposure. This will involve *in vivo* exposure of oysters to tritiated water (HTO) for 14d, analysis of haemocytes by the comet assay, assessment of anoxia tolerance, and dissection and analysis of tissue samples using scintillation techniques.

The supervisor will give training in the dispensing of the radioisotopes with the aid of the Radiation Protection Supervisor (RPS). The record keeping and monitoring of the work area will form part of the training and will be assisted, as appropriate, by the RPS and assistant RPS. The researchers have been trained in appropriate lab technique when handling radioactive materials, e.g. making sure that spills are dealt with effectively, clear labelling of samples so that co-workers are fully aware of the presence of any radioactive substances and the use of a dedicated laboratory coat, gloves and safety glasses. The researchers have also been instructed as to the proper method for disposal of waste.

**Area Description**

Dilutions of tritium from the stock solution will be done in a fume cupboard, designated for use with radionuclides, in Room 314a of the Davy Building by the assistant RPS, Nick Crocker. Exposure of oysters will be carried out in a controlled temperature room (CR2) adjacent to Davy 420. This area is designated an ‘other area’ in the UoP Radiation Safety Handbook, therefore maximum activity must be <1/10th ALI. The room will be cordoned off, locked when not in use, and clearly labelled with trefoil as containing radiological material for the duration of the experiment. It is impractical to carry out this work in Davy 110, due to the need for a controlled temperature room with seawater on tap and a set photoperiod.
Extraction of haemolymph and dissection will take place in Davy 110, a controlled area. A spill kit will be provided in Davy 420. Safe carriage of radioactive water and/or animals from Davy 420 CR2 to Davy 110 will be ensured by removing excess tritiated water (either by draining beakers or drying individual animals), covering beakers with film to prevent evaporation, and keeping beakers and/or animals within trays clearly labelled as radioactive material at all times. Preparation for scintillation counting will be conducted in Davy 110, with analysis in Davy 110A. For disposal, tritiated water will be transported in sealed containers to Room 110, where a special sink without seals is present, designated for the disposal of radioactive liquids.

**Dose Investigation Levels**

Tritiated water will be purchased from ARC in batches of 185 MBq. There is 125 MBq of tritiated water remaining from our previous experiment, whereas a total activity of 480 MBq is needed. Therefore, we intend to purchase 2 more batches. This is considerably less than the University’s storage limit for tritium of 10 GBq. Tritium is stored and controlled by the assistant RPS, Nick Crocker, (Room 314a, Davy Building). The paperwork will be completed when isotopes are used or taken away from storage. Record keeping; (i) Pink copy, completed and kept by Nick Crocker; (ii) White copy, completed and kept with the compound. The highest activity concentration used will be 37 MBq/ml when the stock standard is dispensed by Nick Crocker. A solution containing 1 MBq/ml of tritium will be taken into Davy 420 CR2.

**Working Instructions**

*Methods.* All reagents will be kept in Room 422. Beakers, Perspex, airstones, air pumps, siphon hoses, water containers, stirrers and volumetric flasks will be kept in Room 420 CR2. The tray, “Benchcote”, pipettes, pipette tips and plastic gloves used for haemolymph extraction and dissection will be kept in Room 110. Scintillation counting will be conducted using the scintillation counter in Room 110A. Weighing will be conducted using the balances in Rooms 110 and 422.

*Exposure scenario.* Nine oysters per beaker (2 L) will be exposed to the following activity concentrations of HTO in triplicate, 0, 1, or 15 MBq L\(^{-1}\). All dilutions will be made with seawater obtained from the tap in Davy 420 CR2. The exposure will be for 14 d, with 5 complete water changes (days 0, 3, 6, 9, and 12). At each water change the contaminated seawater will be drained by siphoning the water into a large (50 L), sealable container and taken to Room 110 for disposal. Fresh HTO concentrations will then be made up and added to the appropriate beakers until the next water change. On day 14, the exposure will finish so there will be no renewal of HTO. At each water change, water samples will be taken before removal of contaminated seawater and after renewal with fresh HTO, this will allow calculation of the activity concentration so that disposed activities can be recorded accurately. This exposure scenario results in a maximum activity of 96 MBq in Davy 420 CR2 at any one time, which is considerably lower than 1/10\(^{th}\) total ALI (300 MBq).

*Sampling.* On day 0 control organisms (those with no exposure to HTO) will be sampled. These will be treated as if radioactively contaminated for the purpose of ‘road-testing’ day 14 sampling procedures. One oyster per beaker will be transported to Davy 110 contained within trays and labelled as radioactive material. Haemolymph will be extracted and
transferred to pre-prepared slides for the comet assay. Oysters will then be dissected into their individual organs for determination of tritium activity, as detailed below. After dissection, any waste oyster tissue (shells) will be placed in a plastic bag, sealed in a box (this is more than adequate shielding for $^{3}$H, which requires $<0.1$mm plastic for total absorption) and frozen before disposal as solid waste in Davy 110. On day 14 sampling will occur as above, except that five oysters per beaker will be removed from their beakers and maintained in Davy 420 CR2 for stress on stress assessment. Three oysters per beakers will be transported to Davy 110 in situ (i.e. in beakers with HTO). It is necessary to keep the oysters exposed until the point of dissection, as HTO is depurated quickly. Radioactive water in beakers will be drained to a minimum level (i.e. just above the oysters) during transportation. Beakers will be covered with film to prevent evaporative contamination of corridors and will be contained within trays clearly labelled as radiological material at all times. Those organisms used to assess the stress on stress response will be kept in Davy 420 CR2 on petri dishes and monitored for lethality for $\sim$14 days after the exposure ends. These dishes will be labelled as containing radioactive material and the cold room will remain labelled and restricted access whilst this occurs. After death, these oysters will be disposed of as solid waste in the bin in Davy 110.

**Determination of radioactivity within oyster tissues.** Oysters will be dissected into 5 tissues; adductor muscle, digestive gland, mantle, gills and ‘other’. Shells will be disposed of as above. Each tissue sample will be transferred to pre-weighed, pre-labelled scintillation vials, weighed (to obtain wet weight for dose calculations), freeze-dried and re-weighed (to obtain dry weight for accumulation calculations). Samples will be homogenised in a fume hood, and then rehydrated by addition of 100 µl milli-Q water and incubation for 30 min at room temperature. After rehydration, 1 ml of Soluene-350 is added to each vial. Samples are then placed in an oven at 55°C for 48 h or until the tissue is solubilised. The oven is located in Davy 422 and will be clearly marked with a trefoil for the duration of the incubation. Once solubilisation is achieved, 10 ml Optiphase Trisafe liquid scintillation cocktail and 100 µl glacial acetic acid are added. Samples are transferred to the liquid scintillation counter and left in the dark for 90 min before counting for 2 hours. Beta emissions for each sample are determined using a Beckman 65,000 liquid scintillation counter (Room 110a), with a chemiluminescence quench correction curve. The data will then be converted to MBq kg$^{-1}$ using the dry weight of the tissue samples and to a dose rate in µGy h$^{-1}$ using wet weights and the ERICA tool.

**Disposals**
The exposure scenario described above will result in a total disposal activity of 480 MBq HTO. This will be spread out over 2 weeks (as a result of 5 individual water changes at 96 MBq each, totalling 288 MBq in the first week of exposure, and 192 MBq in the second week) is within the university’s monthly aqueous disposal limit for $^{3}$H of 4 GBq and weekly limit of 1 GBq. The low radioactivity involved allows disposal of liquid down the drain whilst a tap is running in a designated sink (Davy 110). Small amounts of solid radioactive waste (animal tissues) will also be disposed of using the designated solid waste disposal bin in Davy 110. Any broken glass from beakers containing radioactive material will be poured into a sieve, washed several times to remove any contamination, and disposed of as solid waste. Water from such washing will be disposed of in the designated sink. The solid waste limit is 400 kBq in 0.1 m$^{3}$ of waste, with any one item having a maximum activity of 40 kBq. The
levels of activity associated with the solid waste will be below the disposal limit of solid Very Low Level Waste (VLLW). The sealed scintillation vials and other contaminated apparatus (i.e. pipette tips) will be disposed of as solid waste. The remaining isotopes can be removed by rinsing and washed down the sink with plenty of water. All disposals will be recorded.

Restricting exposure
The potential exposure from this experiment is low. All siphoning will performed manually so there will be nil by mouth. Therefore, the amount of HTO ingested should be negligible and considerably below the 20 mSv AL\textsubscript{ingestion} of 480 MBq. Given maximum evaporative loss from each beaker as 10 ml per water change period, the maximum inhalation activity across the experimental period would be 2.4 MBq. This is considerably less than the 20 mSv AL\textsubscript{inhalaion} of 490 MBq. However, as an extra precaution beakers will be covered with cling film to prevent evaporative loss or contamination of the controlled temperature room. After use, the cling film will be disposed of as solid waste in Davy 110. The proposed safety practices should be sufficient in maintaining a safe working environment for the researchers. It is necessary to exercise extreme care when handling the radioactive material, especially when in the concentrated state prior to dilution. The safety record will be signed by the Radiation Protection Supervisor (RPS). The researchers will wear a designated lab coat, safety glasses and gloves at all times when working in Rooms 110, 110a and 420 CR2 (and a mask in the constant temperature room). The surfaces of the constant temperature room will be routinely monitored by the Assistant RPS who will take swabs to assess any increases in activity above background.

Contingency arrangements

\textbf{In Davy 110}
In case of any spillage a response kit for hazardous liquids will be kept under the sink in Davy 110. The spill will be controlled using a chemical sorbent sheet which will be double bagged and disposed of in the designated bin in Davy 110. Any spillage onto skin will be treated by washing with copious amounts of water. Contaminated clothing, paper tissues, towelling and other absorbents will be placed in sealed plastic bags and disposed of as a solid radioactive waste. Swabbed test samples and liquid scintillation counting will be used to check for tritium contamination.

\textbf{In Davy 420 CR2}
During the exposure period and at all water changes the beakers will be kept in plastic trays large enough to contain all contents if a leak should occur. In case of any spillages a vermiculite spill kit will be kept in the lab, this will be used when any spill of liquid thought to contain radioactive material is encountered. Any spillage onto skin will treated by washing with copious amounts of water. The emergency protocols detailed in the handbook will be applied, including minor accidents and fire risk. Swabs will be taken of the far end of the equipment and any background radioactivity measured.

\textbf{In both areas}
In the event of an accident causing radioactive contamination of personnel or of the work area, immediate assistance from the RPS and/or the Assistant RPS will be sought. The area
containing the spill will be identified with a marker pen and a notice of contamination will be displayed. The spill will be clean up immediately and the area will be re-monitored to confirm that the spill has been effectively removed. If contamination cannot be removed, the affected area must be carefully monitored, clearly demarcated e.g. with radioactive hazard warning tape and a risk assessment carried out by the RPS and RPA to determine what future actions may be required to render the area safe. Work will not be resumed until the room/area and personnel concerned have been pronounced fit by the RPS/RPA.

In the case of fire, the alarm will be activated, labcoats and gloves removed and the researchers will evacuate the building (via the evacuation procedure detailed in the radiation safety handbook). The RPS or RPA will be contacted immediately, in order that they can attend and deal with the fire services. In the event of a major flood, labcoat and gloves will be removed and researchers will evacuate the area and call the RPS.

In the event of suspected theft of radioactive material, the RPS will be contacted immediately and a thorough search will be conducted. If the material is not located within one hour, the RPS will contact the police.

RADIOLOGICAL SAFETY CONTACTS

The Radiation Protection Advisor (RPA), available 24/7 for emergencies, is:
Mr C Ellis, HP Squared Ltd
Tel: 01235 820049 Mobile: 07786 405769
E-mail: cliff.ellis@hpsquared.co.uk

The Radiation Protection Supervisor (RPS) is:

Professor Richard Handy
School of Biomedical and Biological Sciences
University of Plymouth,
Drake Circus, PLYMOUTH,
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The Assistant RPS is:

Mr N Crocker
School of Biological Sciences
University of Plymouth,
Drake Circus, PLYMOUTH,
Devon PL4 8AA
Tel: 01752 584593

SUPPORTING INFORMATION
Appropriate COSHH forms are also attached.

**REVIEW STATUS AND FREQUENCY**

The Local Rules will be reviewed annually by the study team, in consultation with RPS, as appropriate.

Signature of Researcher: ……………………… Date: …13-3-13………………………..

Signature of Supervisor: ……………………… Date: …13-3-13………………………..

Approved by the RPS: ……………………… Date: …13-3-13…………..

**B. Radiation Protection**

**Documentation**
Local rules for the assessment of genotoxic and physiological effects and accumulation of radioactivity in mussels (*Mytilus galloprovincialis*) chronically exposed to tritiated water and elevated temperature.

**Laboratories:** Davy 110, 420 & 422

**General**

These Local Rules are provided in accordance with Regulation 17(1) of the Ionising Radiations Regulations 1999 (IRR99). The aim of the experiment is to determine and compare the genotoxic and physiological effects and accumulation of tritium (³H or T) in mussels (*Mytilus galloprovincialis*) after chronic exposure at an elevated temperature. This will involve *in vivo* exposure of mussels to tritiated water (HTO) for 7d, analysis of haemocytes by the comet assay, dissection and analysis of tissue samples using molecular and scintillation techniques.

The supervisor will give training in the dispensing of the radioisotopes with the aid of the Radiation Protection Supervisor (RPS). The record keeping and monitoring of the work area will form part of the training and will be assisted, as appropriate, by the RPS and assistant RPS. The researcher has been trained in appropriate lab technique when handling radioactive materials, e.g. making sure that spills are dealt with effectively, clear labelling of samples so that co-workers are fully aware of the presence of any radioactive substances and the use of a dedicated laboratory coat, gloves and safety glasses. The researcher has also been instructed as to the proper method for disposal of waste.

**Area Description**

Dilutions of tritium from the stock solution will be done in a fume cupboard, designated for use with radionuclides, in Room 314a of the Davy Building by the assistant RPS, Nick Crocker. Exposure of mussels will be carried out in a controlled temperature room (CR2) adjacent to Davy 420. This area is designated an ‘other area’ in the UoP Radiation Safety Handbook, therefore maximum activity must be <1/10⁰ ALI. The room will be cordoned off, locked when not in use, and clearly labelled with trefoil as containing radiological material for the duration of the experiment. It is impractical to carry out this work in Davy 110, due to the need for a controlled temperature room with seawater on tap and a set photoperiod. Extraction of haemolymph and dissection will take place in Davy 420 CR2. A spill kit will be provided in Davy 420. Safe carriage of radioactive water and/or samples from Davy 420 CR2 to Davy 110 will be ensured by use of a carboy to transport tritiated water (closed tightly to prevent evaporation), and keeping samples within closed scintillation vials within trays clearly labelled as radioactive material at all times. Preparation for scintillation counting will
be conducted in Davy 110, with analysis in Davy 110A. For disposal, tritiated water will be transported in sealed containers to Room 110, where a special sink without seals is present, designated for the disposal of radioactive liquids.

**Dose Investigation Levels**

Tritiated water will be purchased from ARC in batches of 185 MBq. A total activity of 300 MBq is needed. Therefore, it is intended to purchase 2 batches of 185 MBq, totalling 370 MBq. This is considerably less than the University’s storage limit for tritium of 10 GBq.

Tritium is stored and controlled by the assistant RPS, Nick Crocker, (Room 314a, Davy Building). The paperwork will be completed when isotopes are used or taken away from storage. Record keeping; (i) Pink copy, completed and kept by Nick Crocker; (ii) White copy, completed and kept with the compound. The highest activity concentration used will be 37 MBq/ml when the stock standard is dispensed by Nick Crocker. A solution containing 1 MBq/ml of tritium will be taken into Davy 420 CR2.

**Working Instructions**

**Methods.** All reagents will be kept in Room 422. Beakers, Perspex, airstones, air pumps, siphon hoses, water containers, stirrers and volumetric flasks will be kept in Room 420 CR2.

The tray, “Benchcote”, pipettes, pipette tips and plastic gloves used for haemolymph extraction and dissection will be kept in Room 420 CR2. Scintillation counting will be conducted using the scintillation counter in Room 110a. Weighing will be conducted using the balances in Rooms 110 and 422.

**Exposure scenario.** Nine mussels per beaker (2 L) will be exposed to 15 MBq L\(^{-1}\) at either 15 or 26 °C. All dilutions will be made with seawater obtained from the tap in Davy 420 CR2. The exposure will be for 7 d, with 2 complete water changes (days 0 and 3). At each water change the contaminated seawater will be drained by siphoning the water into a large (50 L), sealable container and taken to Room 110 for disposal. Fresh HTO concentrations will then be made up and added to the appropriate beakers until the next water change. On day 7, the exposure will finish so there will be no renewal of HTO. This exposure scenario results in a maximum activity of 240 MBq in Davy 420 CR2 at any one time, which is below 1/10\(^{th}\) total ALI (300 MBq).

**Sampling.** At each sampling point (0, 1 h, 12 h, 3 d, 7 d) nine mussels (i.e. one beaker) will be sampled within Davy 420 CR2. At 0 and 1 h this sampling will consist only of removal of a small section of gill, to be flash frozen in liquid nitrogen and stored for gene expression analysis. At all other timepoints, haemolymph will be extracted and transferred to pre-prepared slides for the comet assay. Mussels will then be dissected into their individual organs for determination of tritium activity, as detailed below. Small sections of gill tissue will be taken from each mussel and flash frozen in liquid nitrogen for gene expression analysis. After dissection, any waste mussel tissue (shells) will be placed in a plastic bag, sealed in a box (this is more than adequate shielding for \(^3\)H, which requires <0.1mm plastic for total absorption) and frozen before disposal as solid waste in Davy 110.

**Determination of radioactivity within mussel tissues.** Mussels will be dissected into 7 tissues; adductor muscle, digestive gland, mantle, gills, foot, byssus and ‘other’. Shells will be disposed of as above. Each tissue sample will be transferred to pre-weighed, pre-labelled
scintillation vials, weighed (to obtain wet weight for dose calculations), freeze-dried and re-weighed (to obtain dry weight for accumulation calculations). Samples will be homogenised in a fume hood, and then rehydrated by addition of 100 µl milli-Q water and incubation for 30 min at room temperature. After rehydration, 1 ml of Soluene-350 is added to each vial. Samples are then placed in an oven at 55°C for 48 h or until the tissue is solubilised. The oven is located in Davy 422 and will be clearly marked with a trefoil for the duration of the incubation. Once solubilisation is achieved, 10 ml Optiphase Trisafe liquid scintillation cocktail and 100 µl glacial acetic acid are added. Samples are transferred to the liquid scintillation counter and left in the dark for 90 min before counting for 2 hours. Beta emissions for each sample are determined using a Beckman 65,000 liquid scintillation counter (Room 110a), with a chemiluminescence quench correction curve. The data will then be converted to MBq kg\(^{-1}\) using the dry weight of the tissue samples and to a dose rate in µGy h\(^{-1}\) using wet weights and the ERICA tool.

Disposals
The exposure scenario described above will result in a total disposal activity of 300 MBq HTO. This will consist of two water changes over a week (the initial fill with 240 MBq - disposed of on day 3 - and the 3rd water change of only 60 MBq as several beakers will have already been emptied). This activity is well within the university’s monthly aqueous disposal limit for 3H of 4 GBq and weekly limit of 1 GBq. The low radioactivity involved allows disposal of liquid down the drain whilst a tap is running in a designated sink (Davy 110). Small amounts of solid radioactive waste (animal tissues) will also be disposed of using the designated solid waste disposal bin in Davy 110. Any broken glass from beakers containing radioactive material will be poured into a sieve, washed several times to remove any contamination, and disposed of as solid waste. Water from such washing will be disposed of in the designated sink. The solid waste limit is 400 kBq in 0.1 m\(^3\) of waste, with any one item having a maximum activity of 40 kBq. The levels of activity associated with the solid waste will be below the disposal limit of solid Very Low Level Waste (VLLW). The sealed scintillation vials and other contaminated apparatus (i.e. pipette tips) will be disposed of as solid waste. The remaining isotopes can be removed by rinsing and washed down the sink with plenty of water. All disposals will be recorded.

Restricting exposure
The potential exposure from this experiment is low. All siphoning will performed manually so there will be nil by mouth. Therefore, the amount of HTO ingested should be negligible and considerably below the 20 mSv ALI\textsubscript{ingestion} of 480 MBq. Given maximum evaporative loss from each beaker as 10 ml per water change period, the maximum inhalation activity across the experimental period would be 1.5 MBq. This is considerably less than the 20 mSv ALI\textsubscript{inhalation} of 490 MBq. However, as an extra precaution beakers will be covered with cling film to prevent evaporative loss or contamination of the controlled temperature room. After use, the cling film will be disposed of as solid waste in Davy 110. The proposed safety practices should be sufficient in maintaining a safe working environment for the researchers. It is necessary to exercise extreme care when handling the radioactive material, especially when in the concentrated state prior to dilution. The safety record will be signed by the Radiation Protection Supervisor (RPS). The researchers will wear a designated lab coat, safety glasses and gloves at all times when working in Rooms 110, 110a and 420 CR2 (and a mask in the constant temperature room). The surfaces of the constant temperature
room will be routinely monitored by the Assistant RPS who will take swabs to assess any increases in activity above background.

Contingency arrangements

In Davy 110
In case of any spillage a response kit for hazardous liquids will be kept under the sink in Davy 110. The spill will be controlled using a chemical sorbent sheet which will be double bagged and disposed of in the designated bin in Davy 110. Any spillage onto skin will be treated by washing with copious amounts of water. Contaminated clothing, paper tissues, towelling and other absorbents will be placed in sealed plastic bags and disposed of as a solid radioactive waste. Swabbed test samples and liquid scintillation counting will be used to check for tritium contamination.

In Davy 420 CR2
During the exposure period and at all water changes the beakers will be kept in plastic trays large enough to contain all contents if a leak should occur. In case of any spillages a vermiculite spill kit will be kept in the lab, this will be used when any spill of liquid thought to contain radioactive material is encountered. Any spillage onto skin will be treated by washing with copious amounts of water. The emergency protocols detailed in the handbook will be applied, including minor accidents and fire risk. Swabs will be taken of the far end of the equipment and any background radioactivity measured.

In both areas
In the event of an accident causing radioactive contamination of personnel or of the work area, immediate assistance from the RPS and/or the Assistant RPS will be sought. The area containing the spill will be identified with a marker pen and a notice of contamination will be displayed. The spill will be clean up immediately and the area will be re-monitored to confirm that the spill has been effectively removed. If contamination cannot be removed, the affected area must be carefully monitored, clearly demarcated e.g. with radioactive hazard warning tape and a risk assessment carried out by the RPS and RPA to determine what future actions may be required to render the area safe. Work will not be resumed until the room/area and personnel concerned have been pronounced fit by the RPS/RPA.

In the case of fire, the alarm will be activated, labcoats and gloves removed and the researchers will evacuate the building (via the evacuation procedure detailed in the radiation safety handbook). The RPS or RPA will be contacted immediately, in order that they can attend and deal with the fire services. In the event of a major flood, labcoat and gloves will be removed and researchers will evacuate the area and call the RPS.

In the event of suspected theft of radioactive material, the RPS will be contacted immediately and a thorough search will be conducted. If the material is not located within one hour, the RPS will contact the police.

RADIOLOGICAL SAFETY CONTACTS
B. Radiation Protection

The Radiation Protection Advisor (RPA), available 24/7 for emergencies, is:
Mr C Ellis, HP Squared Ltd
Tel: 01235 820049  Mobile: 07786 405769
E-mail: cliff.ellis@hpsquared.co.uk

The Radiation Protection Supervisor (RPS) is:
Professor Richard Handy
School of Biomedical and Biological Sciences
University of Plymouth,
Drake Circus, PLYMOUTH,
Devon PL4 8AA
Tel: 01752 584630
E-mail: r.handy@plymouth.ac.uk

The Assistant RPS is:
Mr N Crocker
School of Biological Sciences
University of Plymouth,
Drake Circus, PLYMOUTH,
Devon PL4 8AA
Tel: 01752 584593

SUPPORTING INFORMATION
Appropriate COSHH forms are also attached.

REVIEW STATUS AND FREQUENCY
The Local Rules will be reviewed annually by the study team, in consultation with RPS, as appropriate

Signature of Researcher:…………………………..             Date:…8-5-13………………………..
Signature of Supervisor:…………………………..             Date:…8-5-13………………………..
Approved by the RPS:…………
RHandy………           Date:…22nd May 2013………………..


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