

Investigation of waterborne cadmium toxicity in the green-lipped mussel, *Perna canaliculus* using biomarkers – a potential bioindicator of coastal metal pollution in New Zealand



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Dedicated to my mum

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	xi
ABSTRACT.....	xiv
LIST OF FIGURES	xvi
LIST OF TABLES	xx
LIST OF ABBREVIATIONS AND ACRONYMS	xxii
1 General Introduction	1
1.1 Coastal ecosystems and pollution	2
1.1.1 Types of pollutants.....	2
1.2 Metal contamination in coastal ecosystems.....	3
1.3 Cadmium.....	5
1.3.1 Sources of cadmium.....	6
1.3.2 Cadmium concentrations in the coastal environment	6
1.3.3 Regulatory guidelines for cadmium.....	7
1.3.4 Sensitivity to cadmium exposure	8
1.3.5 Cadmium toxicity and its effects	10
1.3.6 Cadmium food safety levels and effects on humans.....	15
1.4 Environmental monitoring	16
1.5 Bioindicators	17
1.5.1 Bivalves as bioindicators	19
1.6 Biomarkers.....	19

1.6.1	Classification of biomarkers	19
1.6.2	Physiological biomarkers	20
1.6.3	Biochemical biomarkers	20
1.6.4	Immunotoxic biomarkers	22
1.6.5	Genotoxic biomarkers	23
1.7	The New Zealand perspective – metals and bioindicators.....	24
1.8	The green-lipped mussel, <i>Perna canaliculus</i>	25
1.9	Objectives	26
1.10	Synopsis of the thesis.....	27
2	General Methodology	29
2.1	Collection and maintenance of mussels.....	30
2.2	Algal culture maintenance	30
2.3	Waterborne exposure to cadmium: experimental set-up	31
2.3.1	Acute and subchronic cadmium exposures.....	31
2.4	Physiological biomarkers.....	32
2.4.1	Clearance rate.....	33
2.4.2	Excretion rate	33
2.4.3	Respiration rate	34
2.4.4	Absorption efficiency.....	34
2.4.5	Oxygen to nitrogen ratio	35
2.4.6	Scope for growth.....	35
2.5	Biochemical biomarkers	36

2.5.1	Collection of haemolymph and tissues	36
2.5.2	Tissue homogenisation for metallothionein-like protein, catalase, and alkaline phosphatase	37
2.5.3	Metallothionein-like protein estimation.....	38
2.5.4	Catalase	38
2.5.5	Alkaline phosphatase	39
2.5.6	Lipid peroxidation.....	39
2.5.7	Na ⁺ , K ⁺ -ATPase	40
2.5.8	Glycogen	40
2.5.9	Haemolymph protein	41
2.6	Immunocytotoxic biomarkers	41
2.6.1	Differential cell count	41
2.7	Cytogenotoxic biomarkers	42
2.7.1	Micronucleus test	42
2.7.2	Comet assay	42
2.8	Metal analysis	43
2.9	Statistical analysis	44
3	Impairment of green-lipped mussel (<i>Perna canaliculus</i>) physiology by waterborne cadmium: relationship to tissue bioaccumulation and effect of exposure duration[†]	46
3.1	Introduction.....	47
3.2	Materials and methods	48
3.2.1	Collection and maintenance of mussels	48

3.2.2	Acute cadmium exposure.....	49
3.2.3	Subchronic cadmium exposure	49
3.2.4	Physiological biomarkers and cadmium analysis	50
3.2.5	Statistical analysis.....	51
3.3	Results.....	51
3.3.1	Cadmium exposure levels and mortality.....	51
3.3.2	Cadmium accumulation	52
3.3.3	Clearance rate.....	57
3.3.4	Absorption efficiency.....	58
3.3.5	Respiration rate	60
3.3.6	Excretion rate	62
3.3.7	Oxygen:nitrogen ratio	63
3.3.8	Scope for growth.....	65
3.4	Discussion.....	66
3.4.1	Acute toxicity.....	66
3.4.2	Cadmium accumulation	67
3.4.3	Physiological biomarkers – does tolerance to cadmium come at a cost?	68
3.4.4	Importance of duration of exposure.....	71
3.5	Conclusions.....	72
4	Biochemical biomarker responses of green-lipped mussel, <i>Perna canaliculus</i>, to acute and subchronic waterborne cadmium toxicity[†]	74
4.1	Introduction.....	75
4.2	Materials and methods	76

4.2.1	Biochemical biomarkers	76
4.2.2	Statistical analysis	77
4.3	Results.....	77
4.3.1	Metallothionein-like protein	77
4.3.2	Catalase	80
4.3.3	Lipid peroxidation.....	82
4.3.4	Alkaline phosphatase	85
4.3.5	Na ⁺ , K ⁺ -ATPase	87
4.3.6	Glycogen.....	89
4.3.7	Haemolymph protein	90
4.4	Discussion.....	92
4.4.1	Cadmium detoxification.....	92
4.4.2	Oxidative stress.....	94
4.4.3	Effects of cadmium on cellular homeostasis enzymes	97
4.4.4	Effects of cadmium on energy substrates	98
4.5	Conclusions.....	99
5	Waterborne cadmium impacts immunocytotoxic and cytogenotoxic endpoints in green-lipped mussel, <i>Perna canaliculus</i>[†]	101
5.1	Introduction.....	102
5.2	Material and methods.....	103
5.2.1	Immunocytotoxic and cytogenotoxic biomarker measurements	103
5.2.2	Statistical analysis	105

5.3	Results.....	106
5.3.1	Differential cell count	106
5.3.2	Micronucleus test	111
5.3.3	Comet assay	117
5.4	Discussion	118
5.4.1	Immunocytotoxic effects of cadmium	118
5.4.2	Cytogenotoxic effects of cadmium	121
5.5	Conclusions.....	123
6	Trace metal concentrations in sediment and tissues of green-lipped mussels from coastal regions of the South Island, New Zealand	125
6.1	Introduction.....	126
6.2	Materials and methods	129
6.2.1	Description of sampling sites.....	129
6.2.2	Mussel collection and maintenance	130
6.2.3	Sediment collection.....	131
6.2.4	Trace metal analysis in mussel tissue and sediment samples	134
6.3	Results.....	136
6.3.1	Metal concentrations in the gill.....	136
6.3.2	Metal concentrations in the digestive gland.....	140
6.3.3	Metal concentrations in the mantle	143
6.3.4	Metal concentrations in the foot	146
6.3.5	Metal concentrations in the sediment	149

6.4	Discussion.....	152
6.4.1	Metal concentrations in mussel tissues.....	153
6.4.2	Metal concentrations in the sediments.....	161
6.4.3	Possible effects of environmental factors.....	165
6.5	Conclusions.....	169
7	Field-to-laboratory transport protocol impacts subsequent physiological biomarker response in the marine mussel, <i>Perna canaliculus</i>[†].....	170
7.1	Introduction.....	171
7.2	Materials and methods.....	172
7.2.1	Collection and maintenance.....	172
7.2.2	Experimental design.....	173
7.2.3	Physiological biomarkers.....	174
7.2.4	Statistical analysis.....	175
7.3	Results.....	175
7.4	Discussion.....	180
7.4.1	Physiological biomarkers.....	181
7.4.2	Transportation treatments.....	184
7.4.3	Aquaculture and ecological implications.....	185
7.5	Conclusions.....	186
8	General Discussion.....	187
8.1	Summary of findings.....	188
8.2	Implications of the findings.....	191

8.3	Methodological considerations	193
8.4	Future studies	197
8.5	Conclusions.....	200
9	References.....	202
10	Appendices.....	254

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ABSTRACT

Coastal metal pollution is a major concern to the health and well-being of aquatic organisms. Bioindicator organisms such as mussels have the potential to monitor coastal metal pollution. In New Zealand, the feasibility of employing green-lipped mussels, *Perna canaliculus* as a bioindicator species is yet to be investigated. This thesis focuses on applying a suite of biomarkers on green-lipped mussels exposed to cadmium (Cd) to evaluate the utility of the biomarkers and investigate the utility of the mussels in assessment of metal pollution.

Cd is a non-essential metal and is known to be highly toxic to many aquatic organisms. This research consisted of a laboratory study to understand the mechanistic effects of Cd toxicity in green-lipped mussels. Physiological, biochemical, immunocytotoxic and cytogenotoxic biomarker responses were measured in mussels exposed to acute (96 h; 2000 $\mu\text{g L}^{-1}$ and 4000 $\mu\text{g L}^{-1}$) and subchronic (28 d; 200 $\mu\text{g L}^{-1}$ and 2000 $\mu\text{g L}^{-1}$) Cd treatments. The 96 h LC₅₀ value for *P. canaliculus* was 8160 $\mu\text{g L}^{-1}$, indicating that the green-lipped mussels were relatively tolerant to Cd exposure.

Results from the Cd exposures, indicated that Cd had a negative impact on physiological processes such as feeding and oxygen consumption. Cd-induced physiological impairments caused an imbalance between energy gain and energy loss in the mussels that led to negative scope for growth. Detoxification (metallothionein-like protein) and defence mechanisms (catalase) were induced in the mussels to provide protection against the toxic effects of Cd. However, the defence mechanisms were not sufficient to protect the mussels from damage due to lipid peroxidation. DNA damage was also observed in the haemocytes of mussels as a result of Cd exposure. Cellular homeostasis (alkaline phosphatase) mechanisms were also perturbed. The immunocytotoxic endpoints reflected differences in haemocyte proportions in

the haemolymph of Cd-exposed mussels. Exposure to Cd also led to the formation of several nuclear aberrations in the gill cells of mussels. Overall the laboratory study highlighted toxic effects of Cd on green-lipped mussels that were dependent on the dose and/or the duration of exposure to Cd. Among the biomarkers tested, clearance rate, metallothionein-like protein induction and the formation of nuclear aberrations in mussel gill cells correlated strongly to Cd accumulation levels and reflected Cd exposure effects.

The feasibility of employing green-lipped mussels as bioindicators was tested during the field study. Green-lipped mussels were collected from different coastal sites along the South Island in NZ. Metal concentrations in the sediment and in four different mussel tissues were analysed. The findings indicated a significant geographical difference in metal concentration in the environment and in the metal accumulation levels in the mussels. Overall, the field study indicated that the green-lipped mussel, *Perna canaliculus* has the potential to be used as a bioindicator species for assessment of coastal metal pollution levels in NZ.

LIST OF FIGURES

Figure 1.1 Green-lipped mussel, <i>Perna canaliculus</i>	25
Figure 1.2 List of biomarkers and assays used in this study.....	26
Figure 2.1 Internal structure of the green-lipped mussel, <i>Perna canaliculus</i>	37
Figure 3.1 Flow chart illustration of cadmium exposure and physiological biomarkers used in this study.	50
Figure 3.2 Cd accumulation in <i>Perna canaliculus</i> gill and digestive gland following acute and subchronic exposure.....	54
Figure 3.3 Clearance rate measurements of green-lipped mussels (<i>Perna canaliculus</i>) during acute and subchronic exposures to Cd.....	58
Figure 3.4 Absorption efficiency measurements of green-lipped mussels (<i>Perna canaliculus</i>) during acute and subchronic exposures to Cd.....	60
Figure 3.5 Respiration rate measurements of green-lipped mussels (<i>Perna canaliculus</i>) during acute and subchronic exposures to Cd.....	61
Figure 3.6 Excretion rate measurements of green-lipped mussels (<i>Perna canaliculus</i>) during acute and subchronic exposures to Cd.....	63
Figure 3.7 Oxygen:nitrogen ratio of green-lipped mussels (<i>Perna canaliculus</i>) during acute and subchronic exposures to Cd.	64
Figure 3.8 Scope for growth measurements of green-lipped mussels (<i>Perna canaliculus</i>) during acute and subchronic exposures to Cd.....	66
Figure 4.1 Flow chart illustration of cadmium exposure and biochemical biomarkers used in this study.....	77
Figure 4.2 Metallothionein-like protein levels in green-lipped mussel (<i>Perna canaliculus</i>) gill and digestive gland during acute and subchronic exposures to Cd.....	79

Figure 4.3 Catalase activity in green-lipped mussel (<i>Perna canaliculus</i>) gill and digestive gland during acute and subchronic exposures to Cd.....	82
Figure 4.4 Lipid peroxidation in green-lipped mussel (<i>Perna canaliculus</i>) gill and digestive gland during acute and subchronic exposures to Cd.....	84
Figure 4.5 Alkaline phosphatase activity in green-lipped mussel (<i>Perna canaliculus</i>) gill and digestive gland during acute and subchronic exposures to Cd.	87
Figure 4.6 Na ⁺ , K ⁺ -ATPase activity in green-lipped mussel (<i>Perna canaliculus</i>) gill during acute and subchronic exposures to Cd.	89
Figure 4.7 Glycogen levels in green-lipped mussel (<i>Perna canaliculus</i>) digestive gland during acute and subchronic exposures to Cd.....	90
Figure 4.8 Haemolymph protein levels in green-lipped mussel (<i>Perna canaliculus</i>) haemolymph during acute and subchronic exposures to Cd.....	91
Figure 5.1 Flow chart illustration of cadmium exposure and immunocytotoxic and cytogenotoxic biomarker assays used in this study.	103
Figure 5.2 Haemocytes observed in the haemolymph of green-lipped mussels.....	104
Figure 5.3 Nuclear abnormalities observed in the gill cells of green-lipped mussels	105
Figure 5.4 Haemocyte count in haemolymph of green-lipped mussels (<i>Perna canaliculus</i>) following acute exposure to Cd	108
Figure 5.5 Haemocyte count in haemolymph of green-lipped mussels (<i>Perna canaliculus</i>) during subchronic exposure to Cd.	110
Figure 5.6 Number of nuclear aberrations observed in the gill cells of green-lipped mussels (<i>Perna canaliculus</i>) following acute exposure to Cd.	113
Figure 5.7 Number of nuclear aberrations observed in the gill cells of green-lipped mussels (<i>Perna canaliculus</i>) during subchronic exposure to Cd	116

Figure 5.8 Percentage of DNA tail intensity of haemocytes in green-lipped mussel (<i>Perna canaliculus</i>) during subchronic exposure to Cd.....	118
Figure 6.1 Location of sampling sites within each region in the South Island, New Zealand.	132
Figure 6.2 Flow chart illustration of field sampling and trace metal analysis	134
Figure 6.3 Concentration of trace metals in the gill of green-lipped mussels collected from different sites in New Zealand.	139
Figure 6.4 Concentration of trace metals in the digestive gland of green-lipped mussels collected from different sites in New Zealand.	143
Figure 6.5 Concentration of trace metals in the mantle of green-lipped mussels collected from different sites in New Zealand	145
Figure 6.6 Concentration of trace metals in the foot of green-lipped mussels collected from different sites in New Zealand.	148
Figure 6.7 Concentration of trace metals in the sediments collected from different sites in New Zealand.	151
Figure 7.1 Flow chart illustration of the experimental design of the field-to-laboratory transportation protocols used in this study.....	174
Figure 7.2 Clearance rate of <i>Perna canaliculus</i> after initial 24 h transport treatment and a subsequent 48 h recovery in natural seawater.....	176
Figure 7.3 Absorption efficiency of <i>Perna canaliculus</i> after initial 24 h transport treatment and a subsequent 48 h recovery in natural seawater.....	177
Figure 7.4 Excretion rate of <i>Perna canaliculus</i> after initial 24 h transport treatment and a subsequent 48 h recovery in natural seawater.....	178

Figure 7.5 Respiration rate of *Perna canaliculus* after initial 24 h transport treatment and a subsequent 48 h recovery in natural seawater.....179

Figure 7.6 Scope for growth of *Perna canaliculus* after initial 24 h transport treatment and a subsequent 48 h recovery in natural seawater.....180

LIST OF TABLES

Table 1.1 Cadmium concentrations in seawater and sediment from different regions	7
Table 1.2 Australian and New Zealand Water Quality Guidelines for cadmium	8
Table 1.3 Cadmium LC ₅₀ values for different freshwater and marine organisms	9
Table 1.4 Characteristics of a bioindicator species	17
Table 1.5 Mussel species used in different regions for trace metal contamination and biomonitoring purposes.	18
Table 3.1 Nominal and measured concentrations of Cd analysed in seawater from acute and subchronic exposures.....	52
Table 3.2 Relationship between Cd exposure level and Cd accumulation in gill and digestive gland of mussels after acute and subchronic exposure to waterborne Cd.	55
Table 3.3 Relationship between Cd accumulation in gill and digestive gland of mussels and physiological biomarkers after subchronic exposure to waterborne Cd.....	56
Table 4.1 Relationship between Cd accumulation in gill and digestive gland of mussels and biochemical biomarkers after subchronic exposure to waterborne Cd.....	92
Table 5.1 Relationship between Cd accumulation in gill of mussels and cytogenotoxic biomarker response in gill cells after subchronic exposure to waterborne Cd.	117
Table 6.1 Description of sampling sites and characteristics of seawater at the different sampling sites.....	133
Table 6.2 Detection limits and recovery percentage of metals in standard reference material used for mussel tissue and sediment analysis	135
Table 6.3 Correlations between metals in different mussel tissues and sediment across all collection sites.....	152

Table 6.4 Metal concentrations in soft tissue of different mussel species. 160

Table 6.5 Australian New Zealand interim sediment quality guideline values for metals... 162

Table 6.6 US EPA sediment quality guidelines and average sediment metal concentrations measured in three regions in this study. 162

Table 6.7 Sediment metal concentrations reported in New Zealand and in other countries. 167

LIST OF ABBREVIATIONS AND ACRONYMS

ADP	Adenosine diphosphate
ANOVA	Analysis of Variance
ANZECC	Australian and New Zealand Environment Conservation Council
ARC	Auckland Regional Council
ARMCANZ	Agriculture and Resources Management Council of Australia and New Zealand
As	Arsenic
ASW	Artificial seawater
ATP	Adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
Ca	Calcium
Cd	Cadmium
Cu	Copper
D	Days
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOM	Dissolved Organic Matter
DTNB	5,5-dithiobis-2-nitrobenzoic acid
DDT	Dichlorodiphenyltrichloroethane
EC	European Commission
ECAN	Environment Canterbury
EDTA	Ethylenediaminetetraacetic acid
EMS 5°C	Emersed at 5°C
EMS 15°C	Emersed at 15°C
Fe	Iron

FSANZ	Food Standards Australia New Zealand
g	grams
h	hours
H ₂ O ₂	Hydrogen peroxide
Hg	Mercury
IARC	International Agency for Research on Cancer
ICP-MS	Inductively Coupled Plasma Mass Spectrophotometry
ISQG	Interim Sediment Quality Guideline
J	Joules
K ⁺	Potassium
KCl	Potassium chloride
kDa	kilodalton
KOH	Potassium hydroxide
L	Litres
LC ₅₀	Median Lethal Concentration
LMPA	Low Melting Point Agarose
LSD	Least Significant Differences
MDA	Malondialdehyde
MED POL	Programme for the Assessment and Control of Pollution in the Mediterranean Region
MfE	Ministry of Environment, New Zealand
Mg ²⁺	Magnesium
MgCl ₂	Magnesium chloride
min	Minutes
Mn	Manganese
MT	Metallothionein
MTLP	Metallothionein-like protein
Na ⁺	Sodium

NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
Na ₂ EDTA	Ethylenediaminetetraacetic Acid, Disodium Salt
NaOH	Sodium hydroxide
NKA	Na ⁺ , K ⁺ -ATPase
NMPA	Normal Melting Point Agarose
NOM	Natural Organic Matter
NRC	National Research Council
NZ	New Zealand
NZTDS	New Zealand Total Diet Survey
O:N ratio	Oxygen:Nitrogen ratio
PAH	Poly Aromatic Hydrocarbon
Pb	Lead
PCB	Poly Chlorinated Biphenyl
pO ₂	partial pressure of oxygen
POM	Particulate Organic Matter
ROS	Reactive Oxygen Species
s	Seconds
SEM	Standard Error of Mean
SFG	Scope for growth
SQG	Sediment Quality Guideline
SRM	Standard Reference Material
SSW	Site seawater
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TDC	Tasman District Council
UNEP	United Nations Environmental Programme
US EPA	United States Environmental Protection Agency

WCRC	West Coast Regional Council
WHO	World Health Organisation
Zn	Zinc
x g	times gravity
‰	Parts per thousand

1 General Introduction

1.1 Coastal ecosystems and pollution

The coastal ecosystem is multifunctional, biologically diverse and highly productive. The functioning of coastal ecosystem is influenced by several natural processes and anthropogenic factors (Aubrey and Elliott, 2006). For example, estuaries act as a transition zone between marine and freshwater environments characterised by fluctuations in physical and chemical conditions such as salinity and temperature (Hodgkin, 1994). They are ecologically important acting as nursery grounds, migration routes and refuge areas for several fish and bird species (McLusky and Elliott, 2004).

However, the increasing pressure from human activities and overexploitation of resources has led to the substantial degradation and loss of coastal habitats (UNEP, 2006). This is primarily due to heavy human settlement along coastal regions, with almost 60% of the world's human population living within 100 km to the coast (Martínez et al., 2007). Coastal environments provide several goods and services to mankind including food resources, recreation, navigation, social and economic development (Costanza, 1997). Thus, disruption of the coastal ecosystems not only affects the aquatic inhabitants but also impacts humans.

1.1.1 Types of pollutants

Some of the primary man-made activities leading to degradation of the coastal ecosystems are discharge of pollutants, conversion of coastal habitats for aquaculture, mining activities, exploration for oil and natural gas, building dams, construction of roads and sedimentation (UNEP, 2002). Apart from directly affecting the well-being of resident organisms, the presence of pollutants also causes several undesirable environmental conditions. This includes depletion of oxygen, production of algal blooms, bacterial or pathogenic infections

and increase in ammonia that are toxic to aquatic organisms (e.g. Moncheva et al., 2001; Zhu et al., 2012).

Industrial, agricultural and domestic effluents are major sources of pollutants that enter into the coastal environment (e.g. Sanchez-Avila et al., 2009). These include organic pollutants such as organochlorinated pesticides, organophosphates, carbamates, herbicides, phenols, polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB) and organotin compounds that are priority pollutants found in effluents (e.g. Martí et al., 2011). Substantial amounts of trace metals also find their way into coastal areas as a result of urbanisation and industrialisation. Additionally, metals are also released into the aquatic ecosystem by natural processes such as weathering of rocks, dust transport and sea upwelling.

1.2 Metal contamination in coastal ecosystems

The presence of trace metals is a major concern in coastal ecosystems. This is due to the fact that metals are persistent, toxic, tend to bioaccumulate, and pose a risk to both the ecosystem and to humans (Philips and Rainbow, 1993). These characteristics of metals have led to the introduction of regular metal monitoring programs in many countries (e.g. Mussel Watch; O'Connor and Lauenstein, 2005).

Trace metals are known to cause several harmful effects in aquatic organisms (Rainbow, 2002). Essential metals such as copper (Cu), zinc (Zn), manganese (Mn) and iron (Fe) play a major role in biochemical pathways of aquatic organisms. For example, Cu is important for the function of the protein haemocyanin while enzymes such as carbonic anhydrase and alkaline phosphatase are Zn metallozymes (e.g. Coleman and Gettings, 1983). Essential trace metals also manifest potential toxic effects in organisms when present in excess concentrations. However, non-essential trace metals such as cadmium (Cd), mercury (Hg),

lead (Pb) and arsenic (As) do not take part in any biological functions and are toxic to aquatic organisms (e.g. Wood et al., 2011).

In the marine environment, organisms can be exposed to both essential and non-essential metals from the surrounding water, sediment and food. The uptake of metals occurs via water in the dissolved phase and via sediments and food in the particulate phase, both of which can cause lethal effects (e.g. Goulet et al., 2007; Erickson et al., 2011). Waterborne exposure to metals is mainly through the gill of aquatic organisms (e.g. Playle, 2004). The gill is involved in important biological functions such feeding, respiration, ion regulation and excretion in aquatic organisms (Wood, 2001). Exposure to metal contaminants in the water can disrupt any of these biological functions. However, waterborne metal toxicity effects are not strictly influenced by the total concentration of a particular metal present within the environment. The bioavailability and toxicity of metals is dependent on the speciation of the metal.

Water chemistry has a major influence on metal speciation (Allen, 1993). Factors such as temperature, salinity, pH and dissolved organic matter (DOM) can affect speciation and toxicity of metals (Di Toro et al., 2001). For example, presence of DOM or an increase in pH decreases Cu toxicity while high salinity reduces Zn toxicity in aquatic organisms (Rogevich et al., 2008; Sánchez-Marín et al., 2010; Loro et al., 2012). Free ionic concentrations of metals in seawater are lowered by complexation and precipitation (Di Toro et al., 2005). Thus metal speciation influences bioavailability and consequently the toxicity of metals.

Bioavailability can be defined as the metal fraction that is available in free form for absorption by the organism and which has the potential to induce toxicity in aquatic organisms (Di Toro et al., 2005). The bioavailable metals present in the free ionic form in the dissolved phase bind and interact with the sites of toxic action (e.g. gill in fish; Wood, 2001).

However, the physicochemical properties of metals and the physiology of the organism both influence metal uptake, distribution, tissue accumulation, and excretion (Phillips and Rainbow, 1993).

Aquatic organisms also have different handling strategies for essential and non-essential metals (Rainbow, 2002). For example, subcellular and tissue distributions of Cd, Zn and Cu differed in unionid bivalves exposed to these metals (Bonneris et al., 2005). Aquatic organisms have the ability to detoxify and/or eliminate metals to prevent the onset of toxic effects. However, when metal uptake and accumulation exceeds detoxification or elimination rate it results in toxicity (Rainbow, 2007). In such cases, organisms are unable to overcome metal stress, eventually leading to mortality (e.g. Cooper et al., 2013). Exposure to low concentrations of metals can also lead to cellular and metabolic alterations in marine organisms that could have a direct impact on their survival (see *Sections 1.3.5 and 1.6*).

1.3 Cadmium

Cd is naturally found in the Earth's crust, and is also present in air, water and the soil. Cd persists in the environment as it cannot be broken down into less toxic forms. However Cd can be mobilised between environmental compartments (UNEP, 2010). The only known biological function of Cd has been reported in diatoms. Cd has been reported to increase the level of carbonic anhydrase activity to enhance growth in these organisms (Lane and Morel, 2000). Apart from its role in diatoms, Cd is a non-essential metal with no known biological functions. Cd represents one of the major aquatic contaminants reported to be toxic to many aquatic organisms and also poses a risk to human health (McGeer et al., 2011; Storelli, 2011). This justifies the inclusion of Cd on the list of priority pollutants and most hazardous substances (ATSDR, 2012).

1.3.1 Sources of cadmium

The average concentration of Cd in the Earth's crust has been reported to be between 0.1- 0.2 mg kg⁻¹. Natural processes such as leaching of Cd from sediment and soil, weathering of rocks, volcanoes, sea upwelling, wind-blown dust and storm-water runoff release Cd into the aquatic environment (WHO, 1992; Pinot et al., 2000). Cd is an important component used in the manufacture of plasticisers, nickel batteries, alloys, metal coatings, pigment production, cement and fertilisers used in industries and agricultural activities (Thornton, 1995). The major anthropogenic sources that contribute towards Cd contamination are industrial, mining and agricultural activities (ASTDR, 2012). For example, the use of Cd-rich phosphatic fertilisers on agricultural lands is one of the primary sources of Cd contamination in Europe (Pan et al., 2010; see *Section 1.7*). The UNEP (2008) reported that an estimated input of 15,000 tonnes of Cd arising from both natural and anthropogenic atmospheric emissions enters into the aquatic environment annually. However the majority of the global release of Cd arises from anthropogenic sources, with the ratio between anthropogenic and natural sources estimated to be 7:1 (Abel, 1996).

1.3.2 Cadmium concentrations in the coastal environment

Cd concentrations present in the dissolved phase in open ocean waters ranges between 0.001- 1 nmol kg⁻¹ (Sigel et al., 2012). However, the concentrations could increase in coastal waters ranging between 0.2 - 0.9 nmol kg⁻¹ (WHO, 1992; Sigel et al., 2012). The level of Cd bound to particulate matter is significantly lower than the dissolved concentration with values reaching 0.00004 - 0.004 nmol kg⁻¹ (Sigel et al., 2012). In pristine marine sediments, Cd concentrations have been reported to be less than 1 mg kg⁻¹ (Sadiq, 1992; Nordberg et al.,

2007). The Cd concentrations measured in seawater and sediment from different uncontaminated and contaminated regions is tabulated below (Table 1.1).

Table 1.1 *Cadmium concentrations in seawater and sediment from different regions*

Seawater/ sediment	Region	Concentration of Cd	Source
Coastal waters	Atlantic	~0.3	Sigel et al., 2012
Coastal waters	Indian	~0.6 - 0.7	“
Coastal waters	Southern	~0.6 - 0.7	“
Coastal waters	Pacific	~0.8 - 1.0	“
Coastal waters	Sub-Antarctic	0.008 - 0.051	Ellwood, 2004
Coastal waters	New Zealand (Foveaux Strait)	0.02 - 0.59	Frew and Hunter, 1995
Coastal waters	New Zealand (Otago Peninsula)	0.090 - 0.219	Croot and Hunter, 1998
Marine sediments	Atlantic and Pacific	0.1 - 1.0	Thornton et al., 1992
Marine sediments	Italy (Augusta Harbour)	0.12 - 2.98	Romano et al., 2013
Marine sediments	North Sea (Elbe Estuary)	0.1 - 4.0	Wetzel et al., 2013
Marine sediments	Malaysia (Port Klang, Selangor)	0.14 - 2.10	Tavakoly Sany et al., 2013
Marine sediments	Germany (Jade Bay)	0.25 - 0.31	Beck et al., 2013
Marine sediments	Baltic Sea	0.19 - 1.96	Dabrowska et al., 2013
Marine sediments	Mexico (Santa Rosalía Harbour)	1.90 - 6.30	Shumilin et al., 2013
Marine sediments	Spain (Portmán)	0.322 - 0.873	Martínez-Gómez et al., 2012

Cadmium concentrations in seawater are expressed in nmol kg⁻¹ and sediment concentrations are expressed in µg g⁻¹ dry weight.

1.3.3 Regulatory guidelines for cadmium

Australia and New Zealand have set low and high interim guideline values referred as the “trigger values” as part of water and sediment quality guidelines. These interim guideline values are derived from metal toxicity databases by ANZECC & ARMCANZ (2000). The ANZECC water quality guideline trigger values for Cd are presented in Table 1.2. The low and high Interim Sediment Quality Guideline (ISQG) trigger values for Cd in sediments are set at 1.5 and 10 mg Cd kg⁻¹ (ANZECC & ARMCANZ, 2000). In sediments and water, the

ISQG-Low value represents the level below which adverse effects are very unlikely while the ISQG-High value reflects a level at which adverse effects are expected in half of the exposed organisms (Simpson et al., 2005).

Table 1.2 *Trigger values for cadmium ($\mu\text{g L}^{-1}$) at alternative levels of species protection (Australian and New Zealand Water Quality Guidelines).*

Trigger values for Cd	Level of protection (% species)			
	99%	95%	90%	80%
Freshwater	0.06	0.2	0.4	0.8
Marine	0.7	5.5	14	36

Values issued by ANZECC & ARMCANZ (2000)

1.3.4 Sensitivity to cadmium exposure

Cd is absorbed more readily by aquatic organisms when present in its free ionic form (Cd^{2+}) (Di Toro et al., 2001). The levels of Cd toxicity observed in aquatic organisms can be related to the site-specific water constituents present in the environment. For example, water hardness (calcium concentrations), pH and presence of DOM have been reported to influence Cd toxicity in aquatic organisms (e.g. Penttinen et al., 2011). The 96 h acute median lethal concentration (LC_{50}) toxicity values of some of the most commonly used freshwater and marine bioindicator species are presented in Table 1.3. The US EPA (2001) has reported acute LC_{50} values of 0.5 - 73500 $\mu\text{g L}^{-1}$ among freshwater fish species alone. The differences in acute LC_{50} values for Cd are related to the age and size of the organisms tested; tolerance to Cd was observed with an increase in the size of the organism (US EPA, 2001). Water chemistry factors such as hardness and alkalinity used in different laboratory settings could also influence Cd toxicity responses.

Table 1.3 Cadmium LC₅₀ values for different freshwater and marine organisms

Habitat	Species	Common name	Species name	Exposure duration	Chemical	LC ₅₀ values (µg L ⁻¹)	Reference
Freshwater	Cladoceran	Water flea ^a	<i>Daphnia magna</i>	48 h	Cadmium chloride	80	Allen et al., 1995
Freshwater	Fish	Guppy	<i>Poecilia reticulata</i>	48 h	Cadmium nitrate	41900	Slooff et al, 1983
Marine	Bivalve	Blue mussel	<i>Mytilus edulis</i>	96 h	Cadmium chloride	25000	Eisler, 1971
Freshwater	Crustacean	Amphipod	<i>Gammarus pseudolimnaeus</i>	96 h	Cadmium chloride	54.4	Spehar and Carlson, 1984
Freshwater	Fish	Tilapia ^b	<i>Oreochromis mossambicus</i>	96 h	Cadmium chloride	21.4	Chang et al., 1998
Freshwater	Fish	Rainbow trout	<i>Oncorhynchus mykiss</i>	96 h	Cadmium chloride	6.2	Kumada et al., 1980
Freshwater	Fish	Fathead minnow	<i>Pimephales promelas</i>	96 h	Cadmium chloride	135	Spehar, 1982
Marine	Fish	Sheepshead minnow	<i>Cyprinodon variegates</i>	96 h	Cadmium chloride	1230	Hutchinson et al., 1994
Marine	Bivalve	Clam	<i>Macoma balthica</i>	144 h	Cadmium chloride	1710	McLeese and Ray, 1986
Marine	Crustacean	Grass shrimp	<i>Palaemonetes pugio</i>	6 d	Cadmium chloride	300	Middaugh and Floyd, 1978
Marine	Crustacean	Blue crab	<i>Callinectes sapidus</i>	7 d	Cadmium nitrate	50	Rosenberg and Costlow, 1976
Marine	Bivalve	Pacific oyster	<i>Crassostrea gigas</i>	23 d	Cadmium chloride	50	Watling, 1983

Exposure duration presented as h - hours; d - days

Age of the organism – a - 14 d; b - 72 h

1.3.5 Cadmium toxicity and its effects

Cd has been reported to induce several toxic effects at the molecular, cellular, and physiological levels in aquatic organisms (McGeer et al., 2011). Although high Cd concentrations can lead to acute toxicity, adverse effects on physiological, cellular and behavioural processes are also observed at low, environmentally realistic concentrations (e.g. Williams and Gallagher, 2013).

1.3.5.1 Ion regulation

Cd acts as a calcium analogue and inhibits Ca^{2+} uptake by interfering with Ca^{2+} -ATPase (e.g. Hollis et al., 2000). Cd is known to enter cells through calcium channels, compete for calcium binding sites of the mitochondria-rich chloride cells in the gill and intestine of fish (Verbost et al., 1989; Schoenmakers et al., 1992; Klinck and Wood, 2011). For example, Baldisserotto et al. (2005) found that the presence of elevated levels of waterborne and dietary Cd interfered with Ca^{2+} uptake in Cd-exposed rainbow trout. When the Ca pathway is disrupted, it leads to hypocalcaemia and skeletal deformities in fish (e.g. tilapia, *Oreochromis mossambicus*, Wong and Wong, 2000). Competition by Cd for basolateral entry into cells also results in the inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Verbost et al., 1989; Niyogi and Wood, 2004).

Cd-induced decrease in Na^+ , K^+ -ATPase activity was reported in the European eel, *Anguilla anguilla* (Lionetto et al., 2000). The decrease in Na^+ , K^+ -ATPase activity was attributed to Cd binding to the sulfhydryl group in the catalytic active site of this enzyme (Lionetto et al., 2000). Disruption of Na^+ , K^+ -ATPase activity can lead to changes in processes such as epithelial ion transport, Na^+ dependent absorption of nutrients, electrolyte and acid-base balance that could prove to be fatal (McGeer et al., 2000).

1.3.5.2 Oxidative stress

Cd can induce the formation of ROS, and generate oxidative stress in aquatic organisms (e.g. Almeida et al., 2002). The different mechanisms by which Cd induces ROS production include displacement of Fe^{2+} ions which in turn can induce Fenton-type reactions; inhibition of antioxidant enzyme defence and depletion of sulfhydryl binding sites (Stohs and Bagchi, 1995). The formation of ROS induced by Cd is neutralised by increased antioxidant enzyme activity (e.g. Firat et al., 2009). However, when an imbalance between pro-oxidant and anti-oxidant levels occurs, ROS interact with lipids causing lipid peroxidation (e.g. Banakou and Dailianis, 2010; see *Section 1.6.3.2*). The oxidative stress induced as a result of lipid peroxidation can lead to DNA damage (e.g. Regoli et al., 2004).

1.3.5.3 Mitochondrial dysfunction

Mitochondria are the cell organelles involved in ATP production and maintenance of Ca balance within cells (Brookes et al., 2004). Non-specific binding of Cd to enzymes/proteins affects mitochondrial electron transport functions, leading to proton leaks and reduction in ATP synthesis (Sokolova, 2004). Cd can substitute essential cations such as Zn^{2+} and Cu^{2+} , which serve as co-factors in a number of enzymes (e.g. cytochrome C oxidase; Salviati et al., 2002). Mitochondrial enzymes such as citrate synthase and NADP-dependent isocitrate dehydrogenase are involved in the Krebs cycle (Nelson and Cox, 2004). Cd has high affinity towards the sulfhydryl groups present in these enzymes that are involved in maintaining the redox balance. Interference of Cd with the activity of these enzymes has been reported in the oyster, *Crassostrea virginica* (Kil et al., 2006; Ivanina et al., 2008). Consequently, Cd causes mitochondrial dysfunction which could result in major disturbances in tissue energy balance and can eventually cause cell death (Sokolova, 2004).

1.3.5.4 Immune functions

Mitochondrial damage and oxidative stress induced by Cd exposure can also lead to alterations in the immune response of aquatic organisms. Decrease in viability of haemocytes, decrease in total haemocyte cell count, alterations to phagocytic capacity and enzyme activity in haemocytes were reported in the marine gastropod *Haliotis tuberculata* and freshwater crab *Sinopotamon henanense* exposed to Cd (Latire et al., 2012; Qin et al., 2012). Gomez-Mendikute and Cajaraville (2003) reported Cd-induced damage to actin and cytoskeleton structure in the haemocytes of mussels, *Mytilus galloprovincialis*.

Stimulation of apoptosis in aquatic organisms is also known to be an important immunotoxic response to Cd exposure. Cd disrupts cell membrane leading to destabilisation of intracellular organelles (e.g. lysosomes) (Stohs et al., 2001). This causes tissue injury and cell death in the form of necrosis and apoptosis. The induction of apoptosis and genotoxicity due to Cd-induced oxidative stress has been reported in aquatic organisms (e.g. Risso-de Faverney et al., 2004; see *Section 1.3.5.2*).

1.3.5.5 Histopathology

Exposure to Cd can lead to morphological alterations in the gill and digestive gland structure. Hyperplasia and hypertrophy of gill lamellae, lifting of lamellar epithelium and degeneration of pavement cells has been reported in the gill of the teleost fish, *Thalassoma pavo* L. exposed to Cd (Brunelli et al., 2011). Alterations to gill epithelia can lead to disruption of ion regulation and cause depletion of oxygen (Wood, 2011; see *Section 1.2*). Several cellular alterations have been reported in the liver of fish species such as the white bass, *Lates calcarifer* and the European seabass, *Dicentrarchus labrax* (L.) exposed to Cd (Thophon et al., 2003; Giari et al., 2007). The alterations include an increase in lysosomes and autophagolysosomes causing cell injury; dilatation of the endoplasmic reticulum leading to

degeneration of metabolic processes; damage to mitochondria resulting in changes in the osmotic balance within cells and vacuolation associated with energy depletion (e.g. Hinton and Laurén, 1990; Braunbeck, 1998). Najle et al. (2000) reported the occurrence of vacuolisation of basophilic cells, autolysis and loss of basophilia damaging the structure of digestive gland of the Antarctic limpet, *Nacella concinna* exposed to Cd. These structural alterations to the digestive epithelium could impact the digestion, absorption of nutrients and affect hepatocellular homeostasis in Cd-exposed organisms (Giari et al., 2007).

1.3.5.6 Reproductive toxicity

Cd causes reproductive disorders by disrupting endocrine functions in bivalves and fish species (e.g. Vetillard and Bailhache, 2004). Gametogenesis is affected when Cd disrupts metabolism of steroid enzymes such as progesterone, testosterone and estradiol-17 β (e.g. clams, *Ruditapes decussatus*, Ketata et al., 2007). Cd is also known to affect the hypothalamus-pituitary-gonadal axis and affect steroid levels in the Japanese medaka, *Oryzias latipes* (Tilton et al., 2003). Inhibition of oocyte maturation, spermatogenesis, sperm motility, embryo-larval development and hatching of eggs are effects of Cd-induced reproductive toxicity reported in different aquatic organisms (e.g. Sen and Sunlu, 2007; Lizardo-Daudt and Kennedy, 2008; Dietrich et al., 2010).

1.3.5.7 Neurotoxicity and behavioural changes

Cd affects signal transduction and chemoreception mechanisms, processes that rely on Ca²⁺ ions for proper functioning. The underlying mechanism of Cd-induced behavioural changes is based on the antagonist effects of Cd on the Ca²⁺-ATPase pump (see *Section 1.3.5.1*). Cd blocks the Ca²⁺-ATPase pump present in the basolateral neuromast cells of fish, leading to degeneration of sensory and mechanoreceptor functions (Faucher et al., 2008). This could affect sensory functions (Baker and Montgomery, 2001; Wang and Gallagher, 2013)

affecting the escape behaviour (Faucher et al., 2008) and social interactions in fish (Sloman et al., 2003). Exposure to Cd has also been reported to affect locomotor activity (Felten et al., 2008) and reduce growth and survival (Liu et al., 2011).

1.3.5.8 Energy, metabolism and growth

Exposure to Cd can affect the bioenergetics and growth of aquatic organisms, eventually leading to challenges in the survival. Cd is known to induce metabolic alterations similar to those experienced during starvation, such as inhibition of energy yielding mechanisms (Almeida et al., 2001). Exposure to sublethal concentrations of Cd resulted in reduced glycolysis and protein levels in juvenile Nile tilapia, *Oreochromis niloticus* (Almeida et al., 2001). In addition, exposure to a toxic metal such as Cd involves the induction of detoxification and defence mechanisms. The onset of protective mechanisms would significantly alter the energy demand of these organisms thereby affecting their growth. Physiological and metabolic alterations associated with Cd exposure resulted in the depletion of glycogen and protein resources in fish and invertebrates (e.g. Cattani et al., 1996; Moolman et al., 2007). Inhibition of physiological processes can eventually affect the overall fitness and growth of Cd-exposed organisms (e.g. Rose et al., 2006). Manyin and Rowe (2009) reported that Cd induced metabolic depression and also affected growth rate in the grass shrimp, *Palaemonetes pugio*. Studies indicate that exposure to Cd and its accumulation could lead to “extra” energy costs that would affect the metabolism, growth and survival of aquatic organisms.

1.3.5.9 Detoxification mechanism

Metallothioneins (MT) are cysteine-rich, low molecular weight (~6-7 kDa), heat-stable cytosolic metal-binding proteins (Roesijadi, 1992). These proteins are involved in the maintenance of homeostasis and regulation of essential metals such as Cu and Zn (Roesijadi,

1992). MT production is induced to detoxify excess amounts of essential ions such as Cu and Zn, and toxic metals such as Cd (Amiard et al., 2006). MT is involved in the detoxification of Cd and also acts as an oxyradical scavenger protecting against oxidative stress (Amiard et al., 2006; see *Section 1.6.3.1*).

1.3.6 Cadmium food safety levels and effects on humans

Storelli (2011) reported that humans are exposed to Cd through the diet via consumption of seafood, especially intake of shellfish. For example, in China, Cd concentrations in the mussel *Perna viridis* ranged between 0.68 - 1.25 $\mu\text{g g}^{-1}$ dry weight Cd (Yap et al., 2004a) and 0.3 - 1.5 $\mu\text{g g}^{-1}$ dry weight Cd (Ng and Wang, 2005a). Bendell (2009) reported Cd concentrations of 0.35 to 4 $\mu\text{g g}^{-1}$ wet weight in mussels of *Mytilus* species from the Pacific Northwest coast of Canada while average concentrations of Cd reported in the soft tissues of mussel, *Mytilus californicanus* ranged between 0.61 - 6.3 $\mu\text{g g}^{-1}$ dry weight in Mexico (Muñoz-Barbosa et al, 2000). These reports indicate that Cd concentrations in shellfish vary between different mussel species due to differences in uptake and elimination processes. The maximum concentration of Cd intake through the diet set by FSANZ (Food Standards Australia New Zealand) is 2 $\mu\text{g g}^{-1}$ excluding dredge oysters and queen scallops (FSANZ, 2002). The FSANZ provisional tolerable weekly intake level for Cd has been set at 7 $\mu\text{g/kg}$ bw/week (NZTDS, 2009).

The major effects of Cd toxicity in humans include liver damage, renal injury, osteoporosis and reproductive disorders (Alfven et al., 2009; Järup and Åkesson, 2009). In addition, Cd can also cause neurotoxic and carcinogenic effects (IARC, 1993). Chronic Cd poisoning in humans caused Itai-itai disease in people from Toyama Prefecture in Japan. Oral ingestion of Cd-contaminated rice led to bone injury that resulted in skeletal deformities, osteomalacia and renal dysfunction in affected individuals (Ishihara et al., 2001; Inanba et al., 2005).

1.4 Environmental monitoring

Chemical analyses of water and sediment samples from the environment do not provide information on the bioavailable fraction of metals present in the environment. The bioavailable fraction of the metal is responsible for inducing toxic effects in aquatic organisms. Biomonitoring coastal ecosystems using a bioindicator species can provide an integrated assessment of temporal variability of the metal levels in the environment (e.g. Solaun et al., 2013). There are two commonly used biomonitoring strategies by which metal levels in the environment can be assessed (Luoma and Rainbow, 2008). The passive method is followed when an indigenous species is used for understanding accumulation and toxicity responses. The active strategy involves using a species from a reference site and transplanting it to a contaminated site. Caging methods offer a single population of mussels without genetic bias that can be used in regions where indigenous mussels are scarce or absent. For example, Marigómez et al. (2013) highlighted the use of caging mussels, *Mytilus galloprovincialis*, for biomonitoring several contaminant levels in the environment. They recommended that the approach of biomarker application on both native and caged mussels at the same site can help in the assessment of both short- and long-term pollution status of the environment.

The investigation of bioaccumulation levels and estimation of total metal concentration in the tissues and the environment does not provide adequate information about toxicity effects in organisms. Instead it is the “metabolically available” fraction of metal that is neither detoxified nor eliminated that is directly associated with induction of toxic responses in organisms (e.g. Rainbow and Luoma, 2011). The biodynamic modelling approach takes into account metal bioaccumulation as well as its relationship with associated metal toxicity effects thus providing a comprehensive understanding of metal effects in organisms (Bourgeault et al., 2011). The key aspect of the biodynamic approach is the incorporation of environmental metal concentrations together with the measurement of parameters such as

uptake of metals via water and diet, elimination of metals and biological response such as growth rate in the organism. Luoma and Rainbow (2005) have reported the application and usefulness of the biodynamic model by extrapolating metal concentration data from a variety of freshwater and marine organisms.

1.5 Bioindicators

McCarty et al. (2002) defined a bioindicator as “a quantifiable characteristic of biochemical, physiological, toxicological or ecological process or function that has been correlated or causally linked to effects at one or more of the organism, population, community or ecosystem levels of organization”. Additionally, indicators can be used as a sign that can relay a complex message in a simplified and useful manner providing insights about a trend or event that cannot be directly observed (Linton and Warner, 2003). Some of the criteria used for selecting a bioindicator species taken from Boening (1999) are presented in Table 1.4.

Table 1.4 *Characteristics of a bioindicator species (Boening, 1999)*

reflect the health status of the habitat
ability to accumulate metals
exhibit tolerance to metals
respond when exposed to metals
sedentary in nature
long life-span
ease of identification and sampling
relative abundance and availability during all seasons throughout the year
ability to withstand handling and maintenance under laboratory conditions
sufficient availability of tissue samples for chemical analysis

Table 1.5 *Mussel species used in different regions for trace metal contamination and biomonitoring purposes.*

Mussel species	Region	Reference
<i>Mytilus edulis</i>	Canada	Fraser et al., 2011
	China	Fung et al., 2004
	Japan	Szefer et al., 1999
	Russia	Szefer et al., 2006
	USA	O'Connor and Lauenstein, 2006
<i>Mytilus galloprovincialis</i>	Italy	Nesto et al., 2007
	Greece	Tsangaris et al., 2011
	Korea	Szefer et al., 2004
	Romania	Romèò et al., 2005
	Spain	Besada et al., 2008
	Western Mediterranean	Deudero et al., 2009
<i>Mytilus edulis aotenus</i>	New Zealand	Anderlini, 1992
<i>Mytilus edulis chilensis</i>	Argentina	Duarte et al., 2011
<i>Mytilus edulis planulatus</i>	Australia	Richardson et al., 1994
<i>Mytilus edulis trossulus</i>	Canada	Burger and Gochfeld, 2006
<i>Mytilus trossulus</i>	Finland	Turja et al., 2013
	Poland	Szefer et al., 2006
	Sweden	Szefer et al., 2006
<i>Mytilus smaragdinus</i>	Taiwan	Jeng et al., 2000
<i>Mytilus californianus</i>	USA	O'Connor and Lauenstein, 2006
<i>Perna viridis</i>	Malaysia	Yap et al., 2004a
	Hong Kong	Liu and Kueh, 2005
	China	Fung et al., 2004
	India	Vasanthi et al., 2012
	Thailand	Sukasem and Tabucanon, 1993
<i>Perna perna</i>	Africa	Sidoumou et al., 2006
	Brazil	Sáenz et al., 2010
	Yemen	Szefer et al., 2006
<i>Perna canaliculus</i>	New Zealand	Anderlini, 1992
<i>Modiolus barbatus</i>	Greece	Katsikatsou et al., 2011
<i>Mytella guyanensis</i>	Brazil	Szefer et al., 2006
<i>Mytella strigata</i>	Mexico	Szefer et al., 2006

1.5.1 Bivalves as bioindicators

Bivalve molluscs are useful as indicator organisms to assess metal pollution in the aquatic environment (e.g. Szefer et al., 2006). The characteristic features of mussels such as their sedentary nature, filter-feeding behaviour, wide distribution, abundance in coastal and estuarine areas, and ability to accumulate several classes of contaminants, make them an ideal bioindicator species (e.g. Roméo et al., 2005). Goldberg (1975) used mussels to assess the levels of contaminants in coastal environments. Since then, several countries have used different mussel species for coastal biomonitoring as shown in Table 1.5. Mussels have routinely been used in long-term international and national coastal biomonitoring programmes (e.g. UNEP MED POL, Mussel Watch) to assess the level of contaminants.

1.6 Biomarkers

Many marine organisms show signs of stress when they are exposed to trace metals. Organisms exhibit their stress response through different mechanisms. The responses can be measured and are referred as biomarkers. Biomarkers can be defined as any molecular, biochemical, physiological or behavioural changes that can be measured in the cell, tissue, body fluid or at the whole organism level (Depledge et al., 1995).

1.6.1 Classification of biomarkers

Biomarkers can be broadly divided into three classes. The “biomarkers of exposure” reflect the responses that indicate the presence of pollutants while “biomarkers of effect” indicate the magnitude of the organism’s response to presence of pollutants (McCarthy & Shugart 1990). “Biomarkers of susceptibility” are the natural characteristics of the organism to respond to a specific agent that alters the susceptibility of the organism to that exposure (WHO, 1993).

1.6.2 Physiological biomarkers

Physiological responses such as feeding, oxygen consumption and cardiac activity can be integrated to assess the effects of pollutants on the overall physiological status of an organism (Widdows and Staff, 2006). Physiological measurements along with scope for growth (SFG) have been found to be cost-effective, highly sensitive and easy to perform (Widdows et al., 1995). SFG integrates physiological measurements representing energy gain and energy loss to evaluate the cost of maintenance functions, and the amount of ‘excess’ energy that may be applied to processes such as growth and reproduction (Widdows et al., 1995). Physiological biomarkers are relevant at the population and community level and thus considered to be ecologically significant (Widdows and Donkin, 1992). SFG and other physiological biomarkers are used successfully for biomonitoring purposes (e.g. Widdows et al., 2002). Both physiological biomarkers and SFG have been used to assess the effects of environmental stress related to polluted harbours in the mussel, *Mytilus edulis* (Honkoop et al., 2003).

1.6.3 Biochemical biomarkers

Exposure to metals can cause various cellular and biochemical changes in aquatic organisms. Several assays have been developed to measure the biochemical changes associated with metal exposure in aquatic organisms that can be used for biomonitoring purposes (Walker, 1995; see *Section 1.3.5*).

1.6.3.1 Metallothionein production

MT induction in organisms exposed to metals indicates the ability of the organism to prevent binding of metals to sensitive sites of action. The sequestration of metals by MT prevents any “spillover” effects and lessens the toxicity related to metal exposure in exposed organisms (Viarengo and Nott, 1993, see *Section 1.3.6*). MT production in aquatic organisms exposed to

metals has been one of the most widely used biomarkers of exposure (Amiard et al., 2006). Several studies have proved that exposure to metals such as Cd, Hg, Cu and Zn can induce MT production in aquatic organisms (Bebianno and Serafim, 1998; Wu and Chen, 2005).

1.6.3.2 Oxidative stress – Antioxidant enzymes and lipid peroxidation

ROS is produced as a result of the oxidative metabolism of cells. Several metals have been found to induce ROS production through redox cycling and Fenton-type reactions. ROS production occurs when metals act directly on cellular processes or indirectly by inhibiting cellular scavenging mechanisms or by affecting biotransformation processes (Livingstone, 2001; see *Section 1.3.5.2*). Aquatic organisms have antioxidant defence mechanisms that can protect against ROS effects (Valavanidis et al., 2006). The antioxidant defence systems comprising of both enzymatic and non-enzymatic mechanisms (glutathione, ascorbate, vitamin C, β -carotene) have scavenging potential against the formation of ROS (Cajaraville et al., 2000). Catalase, superoxide dismutase, glutathione-S-transferase, glutathione peroxidase, glutathione reductase are anti-oxidant enzymes that protect the cells by neutralising the production of ROS to maintain the redox status of cells. When production of ROS exceeds the organism's scavenging potential, oxidative stress in the form of DNA damage, lipid peroxidation, disruption of membrane permeability and damage to proteins can occur (Regoli et al., 2004; see *Section 1.3.5.2*). The effects of oxidative stress in cells would consequently lead to cell death, tissue injury, aging and related degenerative effects (e.g. Mitchelmore et al., 1998).

The status of oxidative stress in an organism can be assessed by measuring malondialdehyde (MDA) production. Lipid peroxidation occurs when MDA is formed by oxidation of phospholipids. The use of lipid peroxidation as a biomarker for measurement of oxidative stress has been well established (e.g. Valavanidis et al., 2006). Measurement of antioxidant

enzymes and lipid peroxidation has been used as biomarkers to assess metal pollution impacts in mussels (e.g. *Mytilus galloprovincialis*, Vlahogianni et al., 2007) and in fish (e.g. *Solea senegalensis*, Oliva et al., 2012). Antioxidant enzymes and lipid peroxidation represent biomarkers of exposure and damage, both of which reflect stress-related effects in aquatic organisms.

1.6.3.3 Energy index – carbohydrate, protein, lipid

Metal-induced stress in bivalves can lead to a reduction in energy availability and subsequent reliance on energy resources such as glycogen, lipid and protein stores (Smolders et al., 2004). Aquatic organisms are known to utilise their energy reserves in order to maintain physiological integrity during stress (see *Section 1.3.5.8*). Exposure to metals causes high metabolic demand in aquatic organisms. The use of additional energy for detoxification processes can affect the energy cycle in metal-exposed organisms (see *Section 1.3.5.9*). For example, glycogen levels in freshwater mussel, *Lamellidens corrianus* have been reported to decrease on exposure to metals such as Cd, Hg and Cu (Rajalakshmi and Mohandas, 1993). Bivalves are known to utilise protein and lipid reserves for energy after depletion of glycogen (e.g. Camacho et al., 2003). Energy reserves as biomarkers provide information on the effects of metal exposure, before adverse effects are manifested (e.g. Smolders et al., 2005).

1.6.4 Immunotoxic biomarkers

The primary function of immune cells (i.e. the haemocytes) is to protect the organism against pathogens and parasitic infections (Wootton and Pipe, 2003). Haemocytes are responsible for maintenance of homeostasis, cellular defence via phagocytosis and ROS production and transport of nutrients (e.g. Cima et al, 2000). The immune cells of bivalves and fish have been used to investigate the effects of environmental contaminants under both laboratory and field conditions (Galloway and Depledge, 2001). Measurements of haemocyte viability,

phagocytic index, total and differential cell count, ROS production and enzyme activity in haemocytes are important immunotoxic biomarkers in aquatic organisms (e.g. Dyrzynda et al., 1998). Both in vitro and in vivo studies in aquatic organisms have reported alterations in immune functions when exposed to environmental contaminants (e.g. Sauve et al., 2002; Duchemin et al., 2008; see *Section 1.3.5.4*). Immunomodulation in organisms exposed to environmental contaminants could render them susceptible to infection and rapid onset of diseases (e.g. Liao et al., 2006). Thus immunotoxic biomarkers are considered to have ecological importance.

1.6.5 Genotoxic biomarkers

Another class of biomarkers that are ecologically relevant are the genotoxic biomarkers. A range of contaminants including Hg, Cd, As, PAHs, PCBs present in the aquatic environment are genotoxic in nature (e.g. Baršienė et al., 2013). Exposure to genotoxicants can cause DNA mutations and chromosomal aberrations that can alter the genetic makeup of the exposed population (van der Oost et al., 2003). Two assays, namely the micronucleus test and the comet assay, have been used to assess the effects of genotoxic substances under both laboratory and field conditions (Russo et al., 2004; Boettcher et al., 2010). The micronucleus test identifies the formation of chromosomal breakage caused by exposure to environmental contaminants which have a clastogenic effect. Exposure to metals such as Cd, Cu, Cr and Hg are known to induce the formation of micro- and bi-nuclei in the gill and liver cells of several fish species (Cavas et al., 2005; Porto et al., 2007). Nuclear aberrations in cells such as the formation of eight-shaped cells, binuclei, and fragmented apoptotic cells act as an index of cytogenetic damage (Bolognesi et al., 1996). The micronucleus test is simple and easy to perform but is time consuming and is dependent on the skills of the individual scoring the cells (Bolognesi and Hayashi, 2011).

The comet assay is complementary to the micronucleus test and is used to assess single strand, double strand DNA breaks, alkali-labile sites, DNA-DNA and DNA-protein cross links within a cell (Singh et al., 1988). The comet assay can be performed using a small sample size, and helps to detect DNA damage at the single cell level caused by exposure to very low levels of genotoxicants (e.g. Jha et al., 2005). These features make the comet assay an ideal tool to examine the “early” response to toxicity (e.g. Mosesso et al., 2012).

1.7 The New Zealand perspective – metals and bioindicators

This thesis will focus on Cd which is a metal of particular importance in NZ coastal regions. In NZ, the major sources of Cd entering into coastal areas are land-based including agricultural runoff, sewage and stormwater runoff. Extensive use of phosphatic fertilisers in farmlands and horticultural lands is considered to be one of the main sources of Cd (Taylor, 1997; Taylor and Percival, 2001). The phosphate fertilisers used in NZ have been produced from phosphate rocks containing $\sim 450 \text{ mg kg}^{-1}$ Cd, originating from Nauru, an island in the South Pacific Ocean (Taylor, 1997; McDowell et al., 2013). Stormwater runoff associated with galvanised roofing is also a source of Cd input into coastal areas with Cd concentrations often exceeding environmental water quality criteria (Kim and Fergusson, 1993). Effluents from mining activities in NZ may also generate waterborne Cd levels as high as $800 \text{ } \mu\text{g L}^{-1}$ (Craw et al., 2005). The level of Cd along coastal regions in NZ has to be monitored, as any additional Cd in the environment could cause several harmful effects in aquatic organisms. It would be useful to identify suitable biomarkers and an endemic species that can be employed for coastal metal biomonitoring in NZ.

1.8 The green-lipped mussel, *Perna canaliculus*



Figure 1.1 *Green-lipped mussel, Perna canaliculus*

Perna canaliculus is commonly known as the NZ green-lipped mussel (Fig. 1.1). Other local names include kuku and kutai. *P. canaliculus* is an endemic bivalve distributed along the rocky intertidal shores extending to depths of 55 m along NZ's coastline (Powell, 1979; Gardner, 2000). The mussels are the only farmed species of mussels in NZ and are known to reach a maximum size of about 240 mm. Green-lipped mussels have high cultural and economical significance in NZ. The green-lipped mussels are referred by the trademark name of New Zealand Greenshell™ mussels and are exported to more than 70 countries around the world (New Zealand Aquaculture Farm Facts, 2010). Annual production is over 70,000 tons and has been estimated to exceed US \$100 M (NZ Government, 2007). The green-lipped mussels are known to be a source of essential minerals, vitamins, haem iron, omega-3 fatty acids, proteins and lipids (Clark et al., 2009).

The green-lipped mussel, *P. canaliculus* belongs to the family Mytilidae and has the basic characteristics of a bioindicator species provided in Table 1.4. They are abundant along the coastal regions in NZ; available throughout the year; easy to identify, collect and maintain in the laboratory and have a relatively long life-span. In addition, preliminary screening studies

(unpublished data) indicated that *P. canaliculus* was the most sensitive of a number of endemic bivalves (little neck clam, *Austrovenus stuchburyi*; tuatua, *Paphies donacina*; pipi, *Paphies australis*) to metals such as Cd, Cu and Zn. These characteristics favoured the selection of the green-lipped mussels as a potential candidate as bioindicator species for biomonitoring purposes in NZ.

1.9 Objectives

The two main objectives of this thesis are to (a) investigate the toxic effects of Cd in green-lipped mussels using a battery of biomarkers utilising a mechanistic study to understand acute and subchronic effects of Cd in mussels exposed under laboratory conditions and (b) a field study to assess the suitability of employing green-lipped mussels as a bioindicator species for biomonitoring metal contamination in coastal environments. The sediment metal concentrations and bioaccumulation of metals in mussels from different sampling regions were investigated.

Different biomarkers used in this study for assessment of metal toxicity effects in green-lipped mussels

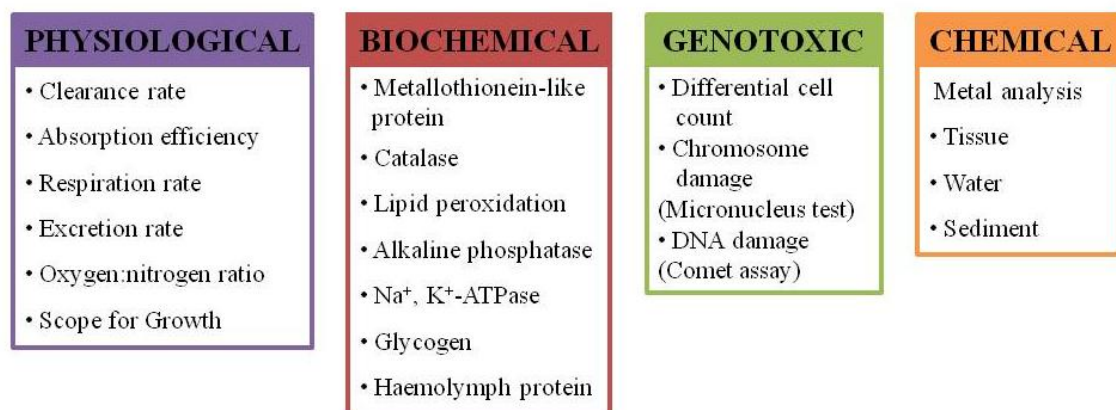


Figure 1.2 List of biomarkers and assays used in this study

1.10 Synopsis of the thesis

This thesis is focussed on the application of a suite of biomarkers including physiological, biochemical, genotoxic and chemical endpoints to investigate the effects of exposure to metals in the green-lipped mussel, *P. canaliculus* (Fig. 1.2). The biomarkers were tested to assess Cd-related toxicity effects in green-lipped mussels under laboratory conditions. Furthermore, a field study was conducted to measure metal concentrations in sediment and in mussels from different sampling sites to assess the potential of green-lipped mussels as bioindicators.

A detailed description of the collection and maintenance of mussels, the different biomarker measurement protocols and metal analysis methods used in this research are presented in *Chapter 2*. Laboratory studies on the effects of Cd exposure in green-lipped mussels are presented in *Chapters 3, 4 and 5*. *Chapter 3* focuses on the effects of Cd on the physiology of green-lipped mussels. This study highlights the differences in the physiological changes of green-lipped mussels influenced by the dose and duration of exposure to Cd under laboratory conditions.

The cellular and biochemical effects of Cd on mussels are presented in *Chapter 4*. A multi-biomarker approach was used to understand the differences in how mussels induce detoxification mechanisms to maintain homeostasis. In addition, the effects of Cd including oxidative stress and energy utilisation processes in Cd-exposed mussels are discussed.

Chapter 5 presents the immunocytotoxic and cytogenotoxic effects in mussels exposed to Cd. This study underlines the damage caused in haemocytes and the gill cells of green-lipped mussels exposed to Cd.

A field study was conducted to assess the feasibility of using green-lipped mussels as bioindicators for coastal metal pollution in NZ and the results are discussed in *Chapter 6*. The

field study involved collection of mussels and sediment samples from nine sampling sites along coastal regions in the South Island, NZ.

The importance of transportation protocol and its effects on the physiology of the mussels are discussed in *Chapter 7*. This study investigates the changes in the physiological responses of mussels when transported from the field to laboratory conditions using four commonly used transportation methods.

The thesis is summarised and future research ideas are discussed in *Chapter 8*. The implications of this research and its contribution to metal toxicology studies are described. This chapter also highlights methodological considerations that can be taken into account for future studies.

2 General Methodology

2.1 Collection and maintenance of mussels

The procedure described in this section was used for collection and maintenance of mussels in *Chapters 3-5*. Green-lipped mussels (70-90 mm shell length) were collected from the rocky intertidal shores of Pigeon Bay (S43° 38.081, E172° 57.156), Banks Peninsula, Canterbury, NZ. The mussels were collected in 300 L polypropylene containers filled with seawater from the collection site and provided with adequate aeration. They were then transported to the aquarium facility at the University of Canterbury, Christchurch on the same day. The mussels were cleaned by gently scraping epibionts off their shells before being maintained in 800 L tanks with circulating seawater under a 12 L:12 D light cycle at 12°C for 2 weeks with algal feed. Prior to experimentation, mussels were transferred to a 15°C controlled temperature room (12 L:12 D light cycle) and maintained in 60 L tanks, with feeding, for one further week. A modified approach was used for mussel collection and maintenance in *Chapters 6-7* and has been detailed in individual chapters.

2.2 Algal culture maintenance

Green-lipped mussels were fed on a daily diet comprising of a monoculture of *Tetraselmis chuii*, throughout the acclimation period and during both acute and subchronic Cd exposure experiments (*Chapters 3-5*). Media preparation and subculturing was conducted under aseptic conditions to avoid bacterial contamination. The algal cultures were grown in filtered seawater containing F/2 media. The F/2 media composition included NaNO₃, NaH₂PO₄, trace metals and vitamins (Guillard and Ryther 1962; Guillard, 1975, see *Appendix 1*). The stock cultures were maintained in 15 L polypropylene bottles while subculture was maintained in a 40 L cylindrical polypropylene tank. Constant aeration and 24 h light conditions were provided and the algal cultures were kept at room temperature (~15°C). The algae cells were maintained in the logarithmic phase by subculturing at regular intervals. The cell

concentration was assessed by both algal cell count using a haemocytometer and measurement of the algal chlorophyll content using a calibrated fluorometer (AquafluorTM). The linear regression between the fluorometer readings and cell counts was used to obtain the corresponding algal cell concentration. Preliminary trials showed that an algal cell concentration of $\sim 5 \times 10^6$ cells ml⁻¹ mussel⁻¹ did not cause any pseudofaeces production in the green-lipped mussels and hence this algal concentration was used in all experiments.

2.3 Waterborne exposure to cadmium: experimental set-up

Waterborne exposure of mussels to Cd consisted of acute (96 h) and subchronic (28 d) exposure treatments. Both toxicity treatments were carried out as static renewal assays and seawater was changed every 48 h. Fresh seawater collected from Lyttelton Harbour was used throughout the experiments presented in *Chapters 3-5*. A cadmium chloride stock solution (1 g L⁻¹ Cd as CdCl₂·2½H₂O in 2% Ultrapure HNO₃ acid) was prepared and appropriate volumes of this solution were added to achieve desired concentrations in the exposures. Water samples from all treatments were collected at the initiation of the exposure, then before and after every water change until the end of the experiment. Seawater samples were analysed using the method described in *Section 2.7*. In *Chapters 3-5*, to determine exposure level, initial and final Cd concentrations within each water change were averaged, and then this value was averaged across all water changes within the exposure. The results are presented in Table 3.1. This mean value was then averaged across all replicates. Prior to use all glassware and polypropylene material was acid-washed using 1% HNO₃. All treatment containers were continually aerated.

2.3.1 Acute and subchronic cadmium exposures

The conditions presented in this section were used for mussels in *Chapters 3-5*. Acute 96 h exposures were performed in 25 L acid-washed polypropylene containers with 10 L of

aerated natural seawater, with $n = 10$ mussels per treatment. Each exposure consisted of a control (without added Cd) and six nominal Cd concentrations of 1000, 2000, 4000, 8000, 16000 and 32000 $\mu\text{g L}^{-1}$. Biomarker responses were measured in mussels from control, 2000, and 4000 $\mu\text{g Cd L}^{-1}$ of the acute Cd treatment. These Cd concentrations represent the highest doses where no mortality of mussels was observed in the acute 96 h exposure (see *Section 3.2.2*). The 28 d subchronic Cd exposure study was conducted in 25 L acid washed polypropylene containers with 20 L of aerated natural seawater. Twenty mussels were randomly selected and placed into each treatment. There were three exposure treatments: control (without Cd), and two Cd concentrations (200 $\mu\text{g L}^{-1}$ and 2000 $\mu\text{g L}^{-1}$), with each being replicated six times. The higher level of Cd chosen (2000 $\mu\text{g L}^{-1}$) was equivalent to the lowest level of Cd used for biomarker assessment from the acute exposure, while the lower value (200 $\mu\text{g L}^{-1}$) was chosen as representing a high environmental exposure scenario (Craw et al., 2005; see *Section 3.2.3*). Throughout the experiment, mussels were fed from a monoculture of *Tetraselmis chuii* once daily ($\sim 5 \times 10^6$ cells ml^{-1} mussel $^{-1}$).

2.4 Physiological biomarkers

Physiological biomarkers were measured in mussels in *Chapters 3 and 7*. All physiological measurements were conducted according to the procedures used by Widdows and Staff (2006), and where appropriate, were expressed in terms of dry weight of the mussel, using a wet weight to dry weight conversion factor (dry weight = (wet weight - 1.18)/29.24; see *Appendix 2*). A modified protocol was used in *Chapter 7* and has been detailed in the methods section of that chapter.

All physiological measurements were made on mussels that were then returned back to the exposure. For this reason the only weight measurement obtained for these mussels was the wet weight (including shell). Preliminary assessments were performed using 30 mussels of

size range between 70 and 90 μm (same size range as those used in Cd treatments) where the whole body weight, wet weight and dry weight of each mussel was measured. The data were used to establish a correlation between weight conversions and was used in the physiological calculations.

2.4.1 Clearance rate

Clearance rate is the estimation of the volume of water cleared of particles by a mussel in a given period of time. Mussels were measured individually in filtered seawater. A series of ceramic, cellulose and polypropylene filters were used to remove all particulate matter from the seawater. Each mussel was placed in a 5 L polypropylene container filled with 2 L of filtered seawater, and after 30 minutes 30,000 cells ml^{-1} of *Tetraselmis chuii* was added to the container. This algal concentration prevented the production of pseudofaeces (see *Section 2.2*). A container without a mussel was used as the blank. Experiments were conducted at 15°C with each container provided with constant aeration. The algal cell concentration in each container was measured every 15 minutes for 1 h. The decline in algal cell concentration was calculated using the equation (modified from Coughlan, 1969):

$$CR (L g^{-1} h^{-1}) = \frac{(V) x (\log_e C_1 - \log_e C_2)}{W/T}$$

where CR is clearance rate; V is the volume of filtered seawater used (2 L); C_1 and C_2 are the algal cell concentrations at beginning and end of the experiment; W is mussel dry weight (g); and T is the time.

2.4.2 Excretion rate

At the end of the clearance rate measurements, mussels were transferred into 750 ml glass containers filled with 500 ml filtered seawater for the determination of excretion rate. Containers were covered with aluminium foil and the mussels were left undisturbed for 1 h at

15°C. A blank (container without a mussel) was also maintained. After 1 h a 5 ml aliquot of seawater was taken and the excretion rate of the mussels was estimated by analysing ammonia concentration using the phenol hypochlorite method (Solórzano, 1969). The rate of ammonia production in the mussels was expressed as $\mu\text{g NH}_4\text{-N g}^{-1} \text{h}^{-1}$ (Widdows, 1985).

2.4.3 Respiration rate

The rate of oxygen consumption of individual mussels was measured by closed respirometry. Respirometers were filled with oxygen-saturated seawater and were maintained at 15°C using a water bath. Oxygen electrodes (Strathkelvin 1302) were fitted to the respirometers to record the decline in partial pressure of oxygen ($p\text{O}_2$) inside the chamber over a period of 1 h. A blank (without mussel) was also maintained. Readings from a Strathkelvin oxygen meter (Model 781) were recorded via a PowerLab (ADI instruments, Version 7) data recording system. The respiration rate of the mussels was then calculated using the following equation (modified from Widdows and Staff, 2006):

$$\text{Rate of } O_2 \text{ uptake } (\mu\text{mol } O_2 \text{ g}^{-1} \text{ h}^{-1}) = \frac{[C(t_0) - C(t_1)] \times V_r \times 60}{W/(t_1 - t_0)}$$

where t_0 , t_1 = start and finish times (min) of the measurement period; $C(t)$ = concentration of oxygen in the water ($\mu\text{mol } O_2 \text{ L}^{-1}$) at time t ; V_r = volume of respirometer minus the animal; and W is mussel dry weight (g).

2.4.4 Absorption efficiency

Absorption efficiency is an estimate of the amount of organic material absorbed by the mussel from the known concentration of algal food ingested. Faecal matter produced by mussels during the excretion and respiration rate experiments was collected in separate test tubes using a pipette. Faeces were then placed on pre-ashed (at 450°C) and pre-weighed 47 mm GFC Whatmann filters. The faecal pellets were washed with 5 ml of 0.5 M ammonium

formate solution to remove all seawater salts from the faeces. The filters with the faeces were then dried at 60°C for 24 h, cooled in desiccators, weighed and ashed at 450°C for 1 h to provide the dry weight and ash-free weight of the filters respectively. A volume of algal culture, equal to that used in the clearance rate experiments, was treated in a similar way to calculate the amount of organic matter in the food that was available to the mussels. Absorption efficiency was calculated using the following equation (Conover, 1966):

$$\text{Absorption efficiency} = \frac{(F - E)}{[(1 - E) F]}$$

where F = ash free dry weight: dry weight ratio for the food; E = ash free dry weight: dry weight ratio for the faeces and expressed as percentage ratio.

2.4.5 Oxygen to nitrogen ratio

Excretion rate and respiration rate values recorded for individual mussels were used to calculate the atomic ratio of oxygen utilised to nitrogen excreted (O:N) using the following equation from Widdows (1985):

$$O:N = \frac{(mg O_2 h^{-1})/16}{(mg NH_4-N h^{-1})/14}$$

2.4.6 Scope for growth

All physiological measurements were converted into their appropriate energy equivalents of J g⁻¹ h⁻¹ using constant values from Widdows and Johnson (1988). SFG was calculated using the equation of Widdows et al. (1995):

$$SFG (J g^{-1} h^{-1}) = A - (U + R)$$

The parameters used in the equation above are defined below, as follows:

$$\text{Energy ingested (C) (J g}^{-1} \text{ h}^{-1}) = \text{Maximum Clearance Rate (L g}^{-1} \text{ h}^{-1}) \times \text{mg POM L}^{-1} \times 23.5 \text{ J mg POM L}^{-1}$$

where POM = Particulate Organic Matter (the algal cell concentration of *Tetraselmis chuii* culture used was 1.33 mg POM L⁻¹);

$$\text{Energy absorbed (A) = Energy ingested (C) } \times \text{Absorption efficiency};$$

$$\text{Energy lost by excretion (U) (J g}^{-1} \text{ h}^{-1}) = \text{ammonia excretion rate (}\mu\text{g NH}_4\text{-N g}^{-1} \text{ h}^{-1}) \times 0.0249$$

where excretion of 1 $\mu\text{g NH}_4\text{-N h}^{-1}$ is equivalent to energy loss of 0.0249 J h⁻¹;

$$\text{Energy respired (R) (J g}^{-1} \text{ h}^{-1}) = \text{respiration rate (}\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}) \times 0.456$$

where 0.456 J μmol^{-1} O₂ is the heat equivalent of oxygen uptake (Gnaiger, 1983). POM values were calculated using a similar method to that used to measure absorption efficiency. Algal culture samples (500 ml) from a stock culture in the logarithmic phase were filtered onto GFC Whatmann filters, and the POM was calculated as the difference in filter weight before and after filtration and ashing.

2.5 Biochemical biomarkers

Different biochemical responses were measured in mussels used in *Chapter 4*. The n values of mussel tissues used for each biochemical biomarker assay are reported in *Section 4.2.1*.

2.5.1 Collection of haemolymph and tissues

Prior to dissection, mussels were transferred to fresh seawater from the aquarium. A sharp knife was inserted between the shells to sever the adductor muscle. The shell was then pulled gently apart to open the mussel. Seawater inside the mussel was drained and the haemolymph was withdrawn from the posterior adductor sinus using a 27 gauge needle with a 1 ml syringe containing 0.2 ml of phosphate-buffered saline (pH 7.4). The haemolymph sample was

transferred into a 1.5 ml microcentrifuge tube and maintained on ice. One haemolymph sample of ~0.1-0.2 ml was withdrawn from each mussel, with an approximate final cell concentration of 10^3 cells ml^{-1} . Fresh haemolymph samples were used for slide preparation (see Section 2.6.1). The gill and digestive gland was collected using clean forceps. Fresh gill samples were used for slide preparation (see Section 2.7.1) while the remaining gill, digestive gland and haemolymph samples were stored at -80°C until further analysis.

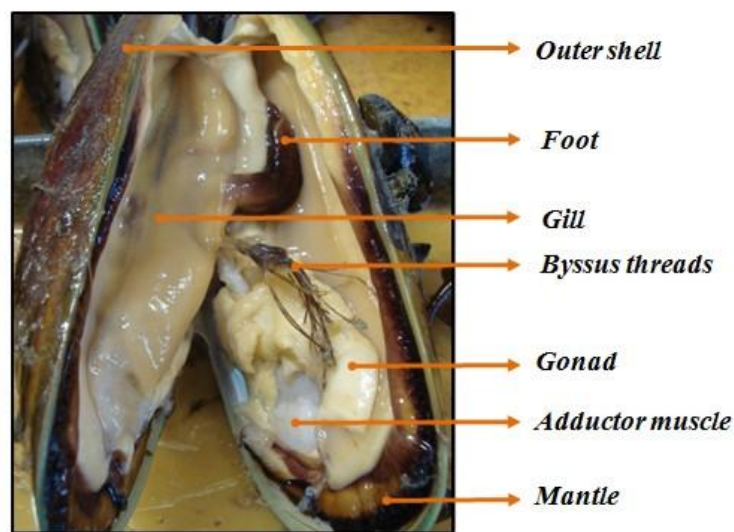


Figure 2.1 Internal structure of the green-lipped mussel, *Perna canaliculus*

2.5.2 Tissue homogenisation for metallothionein-like protein, catalase, and alkaline phosphatase

The gill and digestive gland of mussels (~3 g wet weight pooled from two individuals per replicate) were prepared according to the method of Viarengo et al. (1997). The tissues were homogenised in 1:3 (w/v) ice-cold homogenisation buffer containing 0.5 M sucrose, 20 mM Tris-HCl (pH 8.6), 0.5 mM phenylmethylsulfonyl fluoride and 0.01% β -mercaptoethanol using an Ultra Turrax T25 Basic (IKA Labortechnik). The tissue homogenate was then centrifuged at $30,000 \times g$ for 20 min at 4°C using Beckman Coulter Optima L-90 ultracentrifuge. The supernatant was collected and stored at -80°C until analysis. Prior to

analysis, the protein content of each sample was determined by the Bradford (1976) method. This tissue supernatant was used for MTLP quantitation, catalase and alkaline phosphatase activity determination.

2.5.3 *Metallothionein-like protein estimation*

MTLP content was analysed using the method of Viarengo et al. (1997) with minor modifications. To a 1 ml aliquot of the supernatant 1.05 ml of cold absolute ethanol and 80 μ l chloroform was added, and centrifuged at 6000 x g for 10 min at 4°C. The resultant supernatant was collected, to which 1 ml cold ethanol was added. This sample was then kept at -20°C for 1 h, before being centrifuged again at 6000 x g for 10 min using a swinging rotor Eppendorf 5810 R centrifuge. The supernatant was discarded and the pellet was rinsed with 1 ml of ethanol:chloroform:homogenisation buffer (87:1:12; containing no β -mercaptoethanol) at 6000 x g for 10 min. The pellet was then dried under a nitrogen gas stream, re-suspended in 300 μ l of 5 mM Tris-HCl buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA; pH 7) at room temperature, and then 4.2 ml of 0.43 mM 5,5-dithiobis-2-nitrobenzoic acid (Ellman's reagent) in 0.2 M sodium-phosphate buffer (pH 8) was added. The sample was incubated for 30 min at room temperature and centrifuged at 3000 x g for 10 min. The supernatant developed a yellow colour on addition of Ellman's reagent indicating formation of acid soluble thiols. The absorbance of the supernatant was measured at 412 nm. Reduced glutathione was used as the reference standard to calculate the MTLP content in the sample. The formation of MTLP was expressed as μ g mg protein⁻¹.

2.5.4 *Catalase*

Catalase is an enzyme involved in the decomposition of hydrogen peroxide (H₂O₂) into water and oxygen. Catalase activity was determined using a procedure modified slightly from Aebi (1984). To 50 μ l of the tissue supernatant, 200 μ l of 30 mM H₂O₂ solution was added. The

catalase activity of the samples was measured, via microplate reader, as the decrease in the absorbance at 240 nm due to the decomposition of H₂O₂ at 25°C. Commercial catalase from bovine liver was used as the reference standard. A molar extinction coefficient value of 0.04 mM⁻¹ cm⁻¹ (Aebi, 1984) was used to calculate the catalase activity, which was expressed as μmol of H₂O₂ hydrolysed mg protein⁻¹ min⁻¹.

2.5.5 Alkaline phosphatase

Alkaline phosphatase is an enzyme that catalyses dephosphorylation reactions to generate free phosphates. Alkaline phosphatase activity was measured according to Principato et al. (1985), with minor modifications. A buffer containing 100 mM glycine–NaOH (pH 9), 0.5 mM MgCl₂ and 5 mM p-nitrophenyl phosphate was prepared. To 100 μl of the supernatant, 900 μl of the buffer solution was added and the mixture incubated at room temperature for 15 min. The alkaline phosphatase activity was measured as the formation of p-nitrophenol from p-nitrophenyl phosphate. The enzyme generates a free phosphate from p-nitrophenyl phosphate to form yellow coloured product (p-nitrophenol). A molar extinction coefficient of 18.5 μM cm⁻¹ was used to calculate the amount of p-nitrophenol released at 405 nm using p-nitrophenol as the reference standard. Alkaline phosphatase activity was expressed as nmol mg protein⁻¹ min⁻¹.

2.5.6 Lipid peroxidation

Lipid peroxidation levels were measured using the procedure described by Buege and Aust (1978). Tissues (~0.1 g) were homogenised in 1:3 (w/v) 0.1 M Tris buffer (pH 7.8) and centrifuged at 9000 x g for 10 min at 4°C. Lipid peroxidation levels were measured in the supernatant as MDA equivalents using the reaction mixture consisting of 0.375% (w/v) thiobarbituric acid (TBA), 15% trichloroacetic acid (TCA), and 0.25 N HCl. The tissue supernatant was diluted in 0.1 M Tris homogenising buffer and mixed with 800 μl of

TBA–TCA reagent. The mixture was then heated in a water bath at 100°C for 15 min, cooled and centrifuged at 1000 x g for 10 min to precipitate flocculants. The absorbance of the pink product formed (MDA-TBA) was read at 535 nm to determine the MDA concentration of the tissue samples. An equivalent of MDA, 1,1,3,3-tetramethoxypropane was used as a reference standard. Results were expressed as $\mu\text{mol MDA mg protein}^{-1}$.

2.5.7 *Na⁺, K⁺-ATPase*

NKA activity in gill was measured using the microplate assay protocol devised by McCormick (1993). Mussel gill samples (~0.1 g) were thawed and homogenised in 1:10 buffer containing 150 mM sucrose, 10 mM EDTA, 50 mM imidazole and sodium deoxycholate. The sample was kept on ice and homogenised for 30 s using a pellet pestle, then centrifuged at 5000 x g for 1 min. The supernatant (10 μl) was used to estimate the NKA activity using 150 μl of solution containing 2.8 mM phosphoenolpyruvate, 3.5 mM ATP, 0.44 mM NADH, 50 mM imidazole, 8 units ml^{-1} lactate dehydrogenase, 10 units ml^{-1} pyruvate kinase and 50 μl of salt solution (400 mM NaCl, 10.5 mM MgCl_2 , 100 mM KCl, 50 mM imidazole) with or without ouabain. The enzyme activity was calculated as the difference between the amount of inorganic phosphate liberated in the presence of media containing Mg^{2+} , Na^+ and K^+ (without ouabain) minus the same media containing ouabain. The absorbance of the sample was read every 20 s for over 15 min at 340 nm at 25°C using a microplate reader. The protein content of the supernatant was estimated by the Bradford (1976) method using the microplate reader. The enzyme activity was expressed as $\mu\text{mol ADP mg protein}^{-1} \text{ h}^{-1}$.

2.5.8 *Glycogen*

Glycogen levels in the digestive gland of mussels were determined using the procedure described by Minhorst and Liebezeit (2003) with slight modifications. Mussel digestive gland

(0.2 g wet weight) was homogenised using 3 ml of 30% KOH followed by thorough mixing in a mechanical shaker. The samples were heated in a water bath at 50°C for 1 h. After cooling the tubes on ice, the volume was made up to 10 ml with distilled water. To 50 µl of the digestive gland extract, 3 ml of 0.15% anthrone reagent prepared in 82.4% sulfuric acid was added. The mixture was again heated in a water bath at 90°C for 10 min. The samples were allowed to cool, were incubated for 30 min, and the extinction value of the coloured sample was measured at 620 nm. Commercial glycogen was used as the standard. Glycogen levels were expressed as mg g wet weight⁻¹.

2.5.9 Haemolymph protein

Haemolymph samples were thawed on ice and measured spectrophotometrically at 595 nm after reaction with Bradford reagent (Bradford, 1976). Bovine serum albumin was used as the standard. The protein concentrations were expressed as µg ml⁻¹.

2.6 Immunocytotoxic biomarkers

2.6.1 Differential cell count

The following protocol was used in *Chapter 5* to assess immunocytotoxic biomarkers in mussels. A drop of freshly-collected haemolymph was placed on the centre of a clean frosted microscope slide. The sample was allowed to air dry and then fixed in a methanol:acetic acid (3:1) solution in a Coplin jar for 10 min. Slides were then stained using 5% Wright stain for 10-20 s. Excess stain was removed by rinsing the slides with distilled water, which were then air dried. Slides were wetted in xylene followed by a thin streak of mounting medium and covered with a cover-slip. The slides were coded, placed on a 37°C hotplate and dried for 48 h. About 200 cells per slide were observed and the differential cell count was scored blindly at 100 x magnification using an inverted Leica TCS SP5 confocal microscope. A detailed illustration of different haemocyte cell types is presented in Fig. 5.2.

2.7 Cytogenotoxic biomarkers

2.7.1 *Micronucleus test*

The micronucleus test was performed using the mussel gill and following the protocol of Baršienė et al. (2004) with modifications. A drop of ethanol:acetic acid (3:1) was placed onto a clean frosted microscope slide. A small portion of freshly-collected gill was placed on the drop and cells were smeared onto the slide by nipping the gill with tweezers for about 2 min. The slides were air dried and fixed in methanol for 10 min, followed by staining in 5% Wright stain for approximately 10-20 s. The excess stain was removed by washing the slides in distilled water. The stained slides were wetted with xylene and covered with a cover-slip using a mounting medium. Slides were coded, transferred to a 37°C hotplate, left to dry for 48 h, and then scored blindly by counting 200 cells per slide at 100 x using an inverted Leica TCS SP5 confocal microscope. The frequency of micronuclei, nuclear buds, binucleated cells and fragmented-apoptotic cells was scored for each sample (Fenech et al., 2003). The micronucleus test was used in mussels in *Chapter 5*. The nuclear aberrations observed in the gill cells of mussels are presented in Fig. 5.3.

2.7.2 *Comet assay*

The procedure followed was based on the method of Singh et al. (1988) using haemocytes isolated from the haemolymph samples. Clean frosted microscope slides were coated with a uniform thin layer of 1% Normal Melting Point Agarose (NMPA) and allowed to solidify at 4°C for 15 min. The haemolymph samples were centrifuged at 200 x g for 2 min before the supernatant was discarded and the pellet containing haemocytes was used for the comet assay. For each assay 10 µl of the cell suspension was mixed with 75 µl of 0.7% Low Melting Point Agarose (LMPA) and was coated as a second layer to the slides pre-coated with 1% NMPA. The slides were covered with a cover slip and placed at 4°C until the

agarose layer solidified. The cover slip was then gently removed and a third layer of 85 μ l of 0.7% LMPA was carefully added and allowed to solidify after replacement of the cover-slip. Cover-slips were removed prior to placing the slides in freshly prepared lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 8 mM Tris-HCl, 1% Triton X-100, 10% DMSO; pH 10) at 4°C in the dark for 1 h. After incubation, the slides were removed and rinsed with distilled water. Alkaline DNA unwinding was carried out for 5 min by transferring the slides into a horizontal gel electrophoresis chamber containing freshly prepared electrophoresis buffer (1 mM Na₂EDTA, 300 mM NaOH; pH > 13). Electrophoresis was performed at 300 mA for 15 min. Following electrophoresis, the slides were washed three times in a neutralisation buffer (0.4 M Tris-HCl; pH 7.5) for 5 min. The slides were then stained with 50 μ l of 4',6-diamidino-2-phenylindole (DAPI) and covered with a cover slip, and were observed under fluorescence at 40 x magnification using a Zeiss Axio Imager M1 microscope connected to a camera. Slides were scored blindly and 15-20 cells per slide were analysed using image analysis software (Image J, National Institute of Health, USA). This software calculates the percentage of DNA that occurs in the 'tail' of the 'comet' resulting from electrophoresis, and was used as a measure of DNA damage (Lee and Steinert, 2003). This protocol was used to measure DNA damage in the haemocytes of mussels in *Chapter 5*.

2.8 Metal analysis

In *Chapters 3-5*, metal analysis of seawater and mussel tissue samples from the Cd exposures was carried out using inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500cx, Agilent Technologies, USA). All tubes used for metal analysis were soaked in 2% HNO₃ for 48 h, washed five times using milli-Q water and dried in a clean room. Seawater samples were acidified immediately to pH <2 using concentrated Ultrapure HNO₃ and were diluted using 2% Ultrapure HNO₃ before analysis, whereas gill and digestive gland of mussels from Cd treatments were collected and stored at -80°C until further analysis. After

thawing, 0.2 g wet weight of gill and digestive gland of mussels were dried on aluminium foil overnight at 60°C in a drying oven and the dry weight was determined. The dried tissues were transferred into tubes and acid digested at 90°C on a heating block for 1 h using 5 ml of 50% HNO₃ (Analar grade). Each sample was diluted appropriately using 2% Ultrapure HNO₃. QA/QC was achieved by using certified mussel tissue SRM 2976 (National Institute of Standards and Technology, US). The mean (\pm standard deviation) recovery for Cd was 115 \pm 20%. The method detection limit was determined to be 2 μ g Cd L⁻¹.

A similar protocol was used for analysing metal concentrations in the tissues of the field collected mussels (*Chapter 6*). In addition to the gill and the digestive gland, mantle and foot tissues were collected and stored at -80°C until analysis (see *Chapter 6* for details). The detection limit and recoveries of metals analysed using certified mussel tissue SRM 2976 are presented in Table 6.2. Metal concentrations were also analysed in sediments collected from different field sampling sites (see *Section 6.2.3*).

2.9 Statistical analysis

All experimental and field data (presented as mean \pm SEM unless otherwise noted) were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene's tests, respectively (*Chapters 3-7*). Data that passed these tests were analysed by parametric techniques. For acute Cd exposures this involved one-way ANOVA followed by post hoc LSD (Least Significant Differences) test, while for subchronic Cd exposure a two-way ANOVA was used to test for significant effects of time, treatment and the interaction between these two factors.

Data that failed tests of normality and homogeneity of variance were analysed non-parametrically using Kruskal-Wallis analysis followed by Mann-Whitney U test, or were transformed in order to meet the assumptions of normality and equality of variance. This

statistical approach was used for data presented in *Chapters 3-5*. If the data did not meet the requirements of normality after transformation, the statistical analysis was conducted on the untransformed data in accordance with the hypothesis that ANOVA is robust to violations of this assumption (Schmider et al., 2010). Unless otherwise stated, this statistical assumption was used in *Chapters 3-7*. Any differences in statistical approach are provided in individual chapters (e.g. *Chapters 6 and 7*).

The relationship between Cd exposure and Cd tissue accumulation presented in *Chapters 3-5* was calculated by correlating the mean tissue Cd accumulation in gill and/or digestive gland versus exposure level for each individual replicate within acute or subchronic exposures. This was performed by taking the mean Cd exposure concentration for each replicate up to a given time-point and correlating this to tissue accumulation levels for mussels from the same replicate for the same time period. This relationship was plotted along with all other replicates across all time points (for more detail on time points see *Chapters 3-5*).

3 Impairment of green-lipped mussel (*Perna canaliculus*) physiology by waterborne cadmium: relationship to tissue bioaccumulation and effect of exposure duration[†]

[†]**Chandurvelan, R.**, Marsden, I.D., Gaw, S., Glover, C.N., 2012. Impairment of green-lipped mussel (*Perna canaliculus*) physiology by waterborne cadmium: relationship to tissue bioaccumulation and effect of exposure duration. **Aquatic Toxicology**, 124/125: 114-124.

3.1 Introduction

To maintain vital body functions bivalves acquire energy via feeding, but lose energy through processes such as excretion and respiration (see *Section 1.6.2*). The net energy gain once maintenance of basal physiological processes has been fulfilled is that which is available for reproduction and growth (Bayne and Newell, 1983; Gardner, 2002). However, under stressful conditions bivalves tend to utilise more energy than usual in order to supply vital physiological processes involved in defence or repair (see *Section 1.6.3*). Consequently measures of energy balance such as O:N ratio and SFG may be informative of the whole-body response to environmental stress. Physiological measurements in association with metal bioaccumulation levels in tissues of mussels can provide information regarding the levels of pollutant in the environment and their impacts on individual and ecosystem health (Wang and Rainbow, 2006).

The level of exposure, route of exposure, previous exposure history, and developmental stage are all important factors determining the response of physiological biomarkers to a potential toxicant. Duration of exposure is also a key parameter (e.g. Kraak et al., 1992). Generally, short-term acute studies employ high metal concentrations that can potentially overwhelm the capability of the organism to regulate metal accumulation and/or induce defence mechanisms to survive (Naimo, 1995). Although acute exposures may be reflective of periodic high exposures from a point source, they are unlikely to be indicative of most natural metal exposure scenarios. Nevertheless, studying biomarker responses to acute toxicity gives an overview of the mechanisms of toxicity and/or defence.

A significant advantage of using elevated levels of toxicants is that it better permits the elucidation of toxic mechanisms, a key element in identifying potential biomarker responses. For example, Cd concentrations in the mg L⁻¹ range have been previously used to establish a

role for heat shock proteins in the tolerance of zebrafish embryos to this metal (Hallare et al., 2005). Conversely, chronic or subchronic exposures are likely to be more representative of the environmental exposure scenario (Naimo, 1995). Examining biomarker responses over a longer period, to a lower exposure concentration, elucidates mechanisms that allow the organism to adapt and respond to the presence of a metal pollutant.

Studies on metal accumulation levels in green-lipped mussels, *P. canaliculus* by Whyte et al. (2009) have indicated that human consumers are at risk of exceeding the provisional tolerable weekly intake values for Cd. This is indicative of metal accumulation by mussels from their surrounding environments. The ability of metal burdens to reflect environmental metal levels, suggests the green-lipped mussel may be a valuable tool for coastal monitoring, while increased knowledge regarding metal accumulation in this species will be of direct value for understanding human health effects associated with their consumption.

Overall, the objectives of the present study were to (i) examine the sensitivity of *P. canaliculus* to waterborne Cd; (ii) investigate acute and subchronic effects of Cd on physiology and SFG of green-lipped mussels; (iii) analyse bioaccumulation patterns of Cd in tissues of Cd-exposed mussels as a function of exposure regime; (iv) evaluate the potential for employing *P. canaliculus* as a bioindicator species for biomonitoring NZ's coastal and marine ecosystems.

3.2 Materials and methods

3.2.1 Collection and maintenance of mussels

Adult green-lipped mussels (70-90 mm shell length) were collected and maintained as described in *Section 2.1*. The Cd level in seawater from the collection site was analysed using the ICP-MS and was below the analytical detection limit of $2 \mu\text{g L}^{-1}$ (see *Section 2.8*).

3.2.2 Acute cadmium exposure

The Cd concentrations and conditions used for acute exposure treatment are reported in *Section 2.3.1*. The range of Cd concentrations used, allowed assessment of the acute (96 h) median lethal concentration (LC₅₀). Each treatment was replicated eight times. Mussels were observed every 24 h. If found gaping, mussels were gently poked with a sharp point and those that did not respond were considered dead and removed from the tank. The LC₅₀ values and their 95% confidence limits were calculated using the Trimmed Spearman-Kärber method (Hamilton et al., 1977). The control mussels and the 2000 µg L⁻¹ and 4000 µg L⁻¹ Cd treatments were selected for physiological assessment at 96 h. These two Cd exposure concentrations were the highest tested at which no mortality was apparent.

3.2.3 Subchronic cadmium exposure

The experimental design for subchronic Cd exposure is detailed in *Section 2.3.1* (see *Appendix 3*). A single, randomly-selected, mussel from each replicate (n = 6) for each treatment was assessed for physiological biomarker measurements on Days 5, 10, 15, 20 and 25 of exposure (Fig. 3.1). The days for physiological sampling differed from those for tissue collection (7, 14, 21 and 28) owing to the practical limitations in conducting both physiological assays and tissue sampling at the same time. Statistical analyses showed, however, that there were not significant changes in tissue accumulation over any specific 7 d period for gills or digestive gland, suggesting the small differences in sampling times (a maximum of 3 d) should not have impacted the relationship between physiological markers and the tissue metal burden. Mussels used for physiological measurement were weighed and returned back to their respective treatment containers.

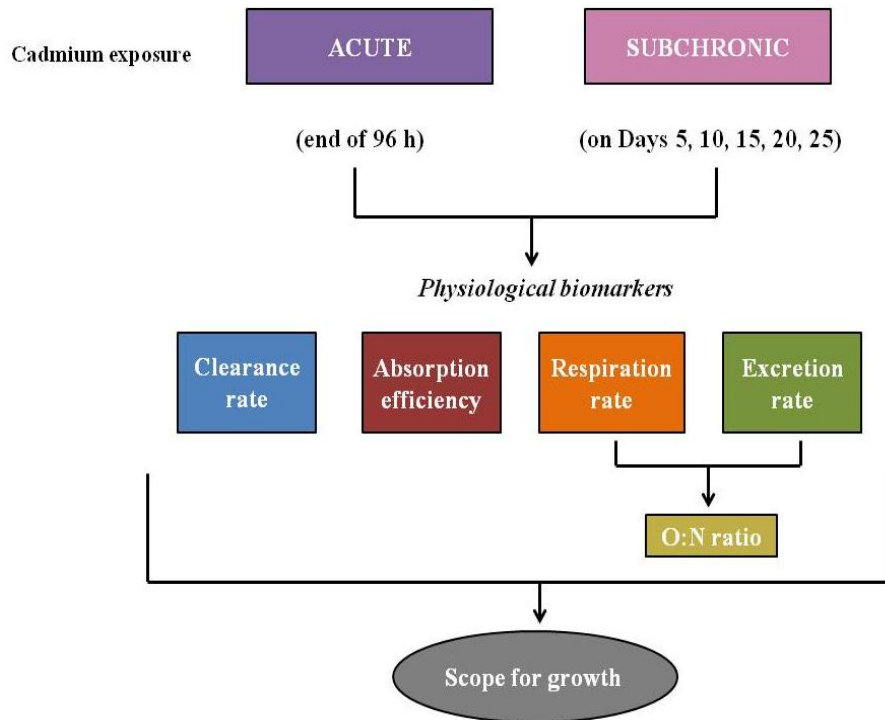


Figure 3.1 Flow chart illustration of cadmium exposure and physiological biomarkers used in this study.

3.2.4 Physiological biomarkers and cadmium analysis

Physiological biomarkers were measured as described in *Section 2.4*. The clearance rate, excretion rate, respiration rate and absorption efficiency of mussels from control and Cd exposure treatments were measured individually ($n = 8$ for acute; $n = 6$ mussels each for subchronic from each exposure treatment). Based on individual physiological measurements the O:N ratio and the SFG of mussels were calculated using equations described in *Section 2.4*. Gills and digestive gland ($n = 4$) were collected at the end of 96 h for acute exposures, and $n = 6$ on Days 7, 14, 21 and 28 for the subchronic exposure for tissue Cd analysis and Cd levels in seawater samples using method described in *Section 2.8*.

3.2.5 *Statistical analysis*

All experimental data were analysed using the statistical software STATISTICA 6 (StatSoft). Data were tested according to the methods detailed in *Section 2.9*. The relationship between Cd accumulation in the tissues and physiological responses following subchronic exposure was determined using the method described in *Section 2.9*. Data across all time points Day 5 physiology vs. Day 7 accumulation, Day 15 physiology vs. Day 14 accumulation, Day 20 physiology vs. Day 21 accumulation and Day 25 physiology vs. Day 28 accumulation were used to model the relationship. Data are presented as mean \pm SEM unless otherwise noted, with statistical significance set at $p < 0.05$.

3.3 Results

3.3.1 *Cadmium exposure levels and mortality*

The nominal and measured concentrations of Cd for each exposure treatment are presented in Table 3.1. Over the course of 48 h there was a small loss of Cd from the solution, likely due to adherence to container walls and/or biomass and absorption by the animals. To account for this the Cd exposure concentration was obtained by calculating average exposure levels over each 48 h period, with these values then averaged across the course of the exposure (see *Section 2.3*). An overall average of 21% (2000 $\mu\text{g L}^{-1}$) and 19% (4000 $\mu\text{g L}^{-1}$) of Cd during acute exposure, and 30% (200 $\mu\text{g L}^{-1}$) and 23% (2000 $\mu\text{g L}^{-1}$) of Cd during subchronic exposure was lost in the 48 h between each water change. Despite the loss of Cd from the exposures, measured Cd concentrations correlated closely to nominal Cd values (Table 3.1).

Table 3.1 Nominal and measured concentrations ($\mu\text{g L}^{-1}$) of Cd analysed in seawater from acute and subchronic exposures for groups undergoing physiological biomarker and Cd accumulation assessment.

Cadmium treatment	Nominal concentration ($\mu\text{g L}^{-1}$)	Measured concentration ($\mu\text{g L}^{-1}$)
Acute (96 h)	0	2.67 ± 0.17
	2000	1955 ± 133
	4000	3844 ± 410
Subchronic (28 d)	0	3.65 ± 5.48
	200	189 ± 28
	2000	1910 ± 278

Measured values represent the mean exposure level (average of initial and final concentrations measured at each water change). Values are expressed as mean \pm SD ($n = 4-8$).

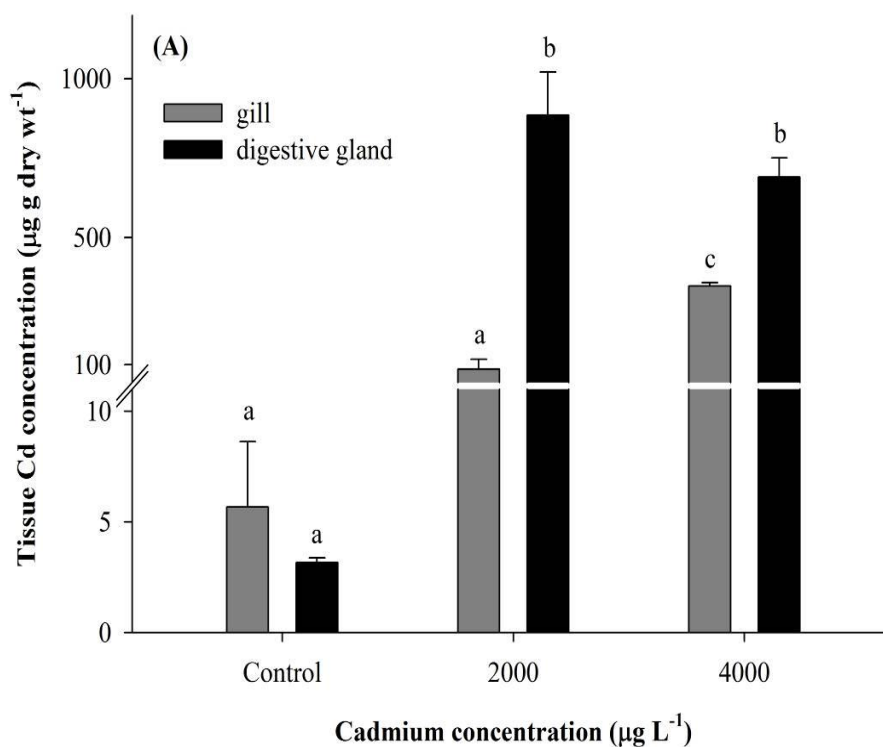
The 96 h LC₅₀ for Cd to *P. canaliculus* was found to be 8160 $\mu\text{g L}^{-1}$. The lower and upper 95% confidence limits ranged between 6380 $\mu\text{g L}^{-1}$ and 10450 $\mu\text{g L}^{-1}$. For subchronic assessment the only mortality observed was at Day 27 in the 2000 $\mu\text{g L}^{-1}$ Cd treatment, where three mussels (15%) perished in a single replicate.

3.3.2 Cadmium accumulation

Acute 96 h exposure to 4000 $\mu\text{g L}^{-1}$ Cd resulted in a significant increase in the accumulation of Cd in both gill and digestive gland of mussels relative to the Cd-free control (Fig. 3.2A). Overall, the gill showed 15.4-fold and 61.4-fold higher Cd in the 2000 and 4000 $\mu\text{g L}^{-1}$ treatments relative to the control. The level of Cd accumulation in the digestive gland of mussels in the 4000 $\mu\text{g L}^{-1}$ was 218-fold higher than the control digestive gland, while the 2000 $\mu\text{g L}^{-1}$ group showed a significantly higher 279-fold elevation in Cd level relative to the control. There was no significant difference in digestive gland Cd accumulation between the

2000 and 4000 $\mu\text{g L}^{-1}$ treatments, in contrast to the significant difference between these two exposure concentrations with respect to gill accumulation.

Cd concentration in gills of mussels exposed to subchronic Cd showed a significant effect of treatment, time and also a significant interaction between these two factors. The subchronic Cd exposure resulted in significantly elevated gill Cd levels (Fig. 3.2B) throughout the exposure in both Cd concentrations relative to the time-matched control. In the digestive gland (Fig. 3.2C) there were no significant interaction effects between the two factors although significant effects of treatment and of time were observed. Within each sampling time, both Cd exposure concentrations exhibited tissue accumulation levels that differed significantly from the unexposed control.



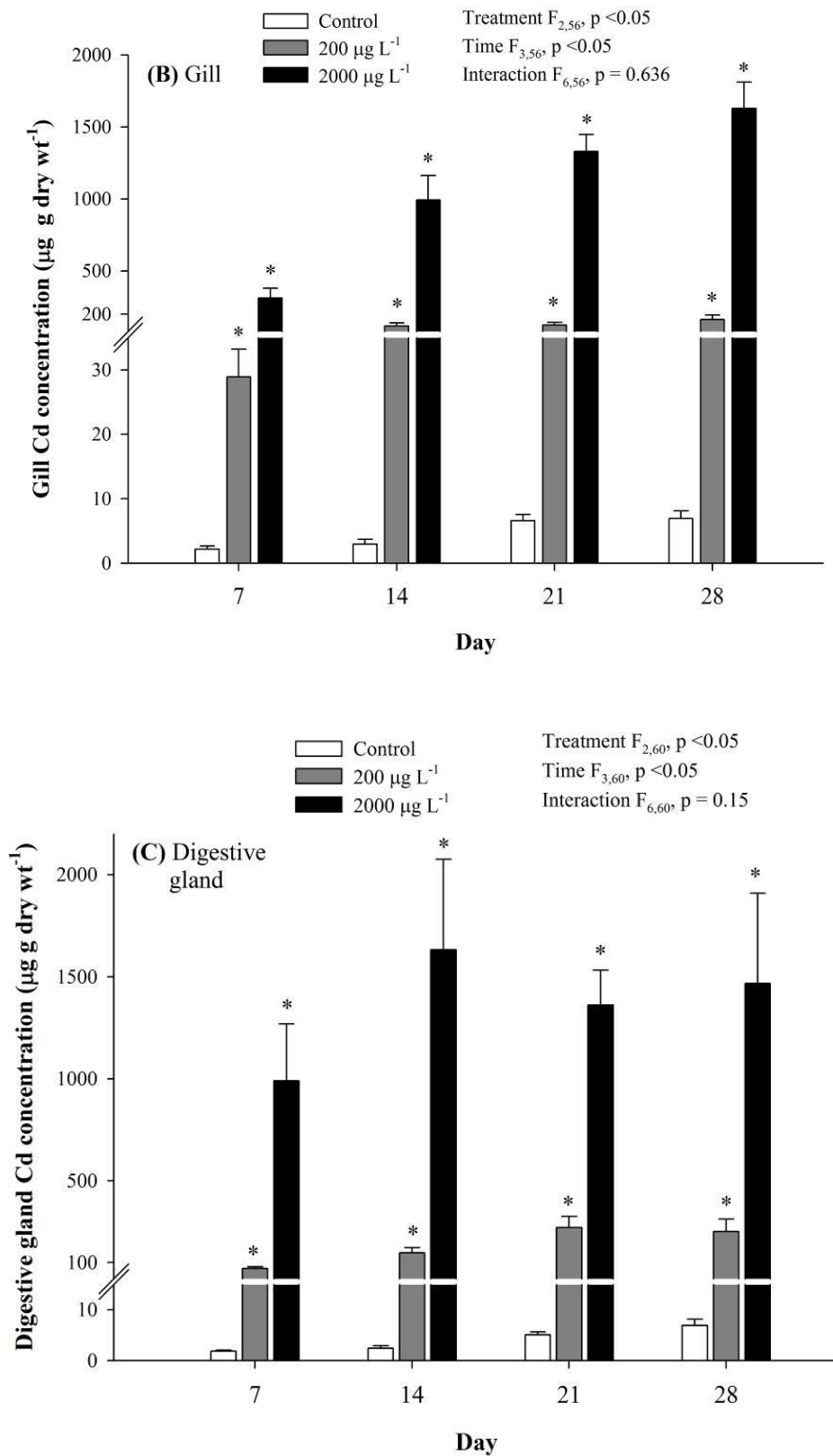


Figure 3.2 Cd accumulation in *Perna canaliculus* gill and digestive gland following acute 96 h exposure (A), in gills during subchronic 28 d exposure (B) and in digestive gland during

subchronic 28 d exposure (C). Values are expressed as mean \pm SEM ($n = 4$ for acute, and 5 or 6 for subchronic exposures). In panel A plotted points sharing letters are not statistically significant ($p < 0.05$) as determined using Kruskal-Wallis ANOVA followed by Mann Whitney U test. In panels B and C data were analysed by two-way ANOVA with exposure concentration (treatment) and time as factors. Data in panel C were log transformed for analysis. The results of the two-way ANOVA analysis are reported on the figures. Asterisks indicate a significant difference between the exposure concentration and the control within a day, as determined by post hoc LSD analysis ($\alpha = 0.05$).

Cd accumulation in gill was significantly linearly related to Cd exposure level at the end of a 96 h acute exposure (Table 3.2). Conversely, accumulation of Cd in the digestive gland exhibited saturation and was best modelled using a logarithmic function (Table 3.2). The relationship between exposure concentration and tissue accumulation was also significant and linear for both tissues during subchronic exposures, although the correlation for this relationship was stronger for gill than for the digestive gland.

Table 3.2 Relationship between Cd exposure level and Cd accumulation in gill and digestive gland of mussels after acute (96 h, 0-4000 $\mu\text{g Cd L}^{-1}$) and subchronic (28 d, 0-2000 $\mu\text{g Cd L}^{-1}$) exposure to waterborne Cd.

Cadmium treatment	Tissue	Correlation coefficient (R)	Equation of the line of best fit
Acute	Gill	0.430	$y = 0.0878x - 23.73$
	Digestive gland	0.394	$y = 105.75\ln(x) - 57.282$
Subchronic	Gill	0.447	$y = 0.7512x + 71.931$
	Digestive gland	0.272	$y = 0.6815x + 58.33$

All of these correlation analyses were statistically significant ($p < 0.001$).

The relationship between Cd accumulation in the two tissues and the physiological biomarkers of Cd-exposed mussels from the subchronic exposure is shown in Table 3.3. A strong and highly significant linear correlation between tissue Cd accumulation and clearance rate was determined. Similar significant linear correlations, but with a reduced coefficient of determination, were calculated for SFG, O:N, respiration rate and excretion rate, although only the relationship to digestive gland accumulation was significant in the latter. The relationship between absorption efficiency and tissue accumulation was not significant.

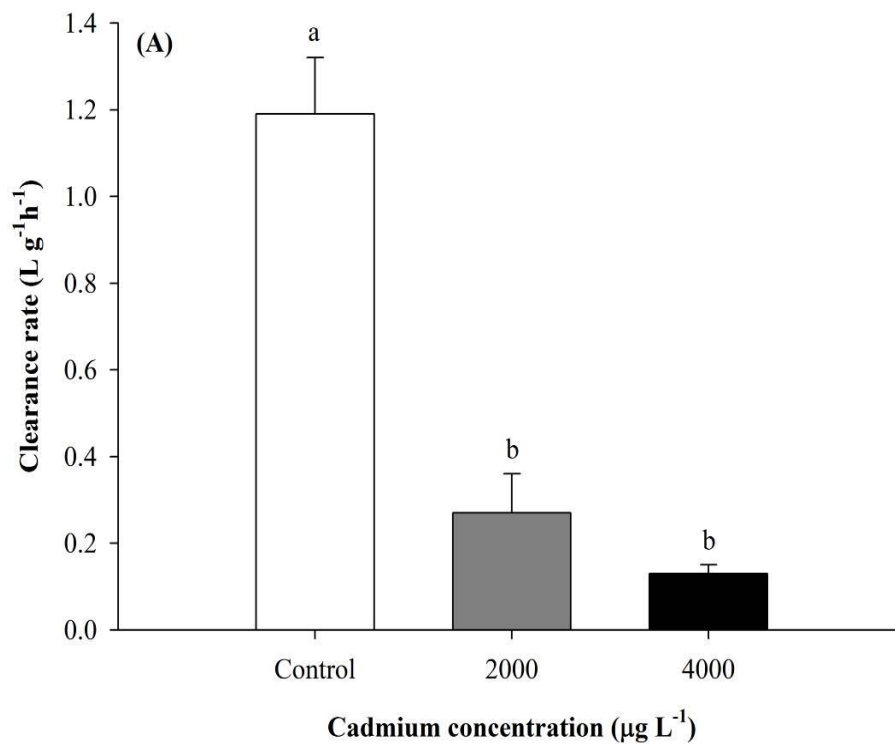
Table 3.2 Relationship between Cd accumulation in gill and digestive gland of mussels and physiological biomarkers after subchronic (28 d, 0-2000 $\mu\text{g Cd L}^{-1}$; $n = 6$) exposure to waterborne Cd.

Physiological measure	Tissue	Correlation coefficient (R)	Equation of the line of best fit	<i>p</i> value
Clearance rate	Gill	0.403	$y = -0.0006x + 0.8729$	<0.001
	Digestive gland	0.438	$y = -0.0005x + 0.9141$	<0.001
Absorption efficiency	Gill	0.080	$y = -0.0001x + 0.5912$	0.172
	Digestive gland	0.029	$y = -8\text{E-}05x + 0.5748$	0.427
Respiration rate	Gill	0.170	$y = -0.0078x + 17.987$	0.037
	Digestive gland	0.176	$y = -0.0071x + 18.464$	0.033
Excretion rate	Gill	0.047	$y = 0.0147x + 50.612$	0.311
	Digestive gland	0.164	$y = 0.0245x + 44.368$	0.041
O:N ratio	Gill	0.159	$y = -0.0073x + 12.866$	0.045
	Digestive gland	0.224	$y = -0.0076x + 13.827$	0.013
Scope for growth	Gill	0.186	$y = -0.0064x + 5.3601$	0.027
	Digestive gland	0.245	$y = -0.0065x + 6.1012$	0.008

Statistically significant correlations ($p < 0.05$) are indicated in bold

3.3.3 Clearance rate

Figure 3.3A and 3.3B illustrate the clearance rate of mussels exposed to acute and subchronic Cd exposure. Acute Cd exposure resulted in 78% (2000 $\mu\text{g L}^{-1}$) and 89% (4000 $\mu\text{g L}^{-1}$) lower clearance rates relative to control values. A significant effect of treatment, time and interaction was noted. The decline in clearance rate was observed in mussels exposed to 2000 $\mu\text{g L}^{-1}$ Cd after 5 d of subchronic exposure, and this effect strengthened and persisted throughout the course of the exposure. There were no significant changes in the 200 $\mu\text{g L}^{-1}$ group clearance rate relative to control.



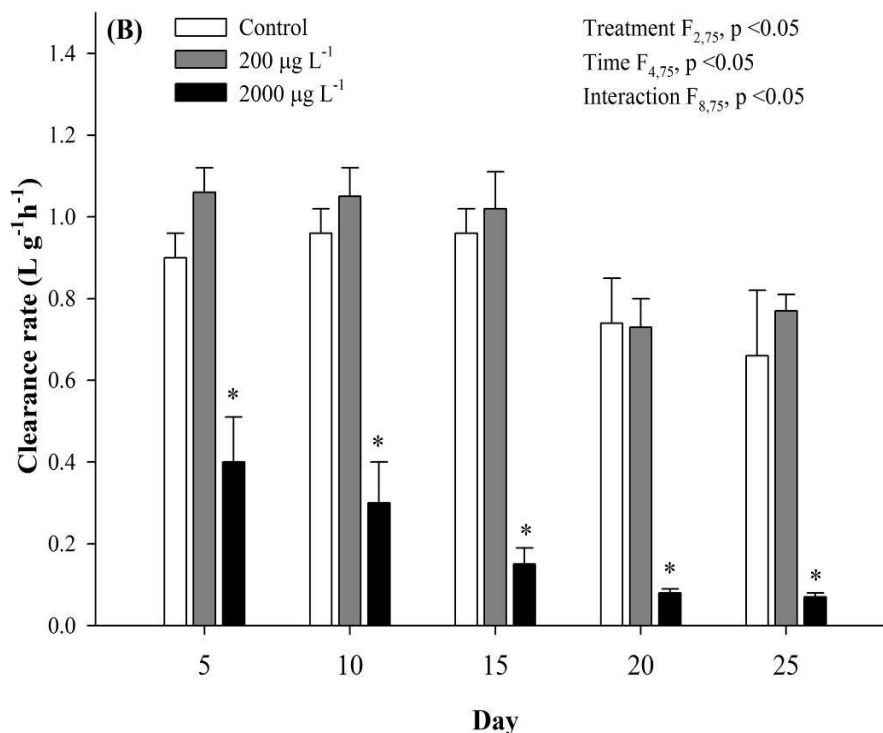
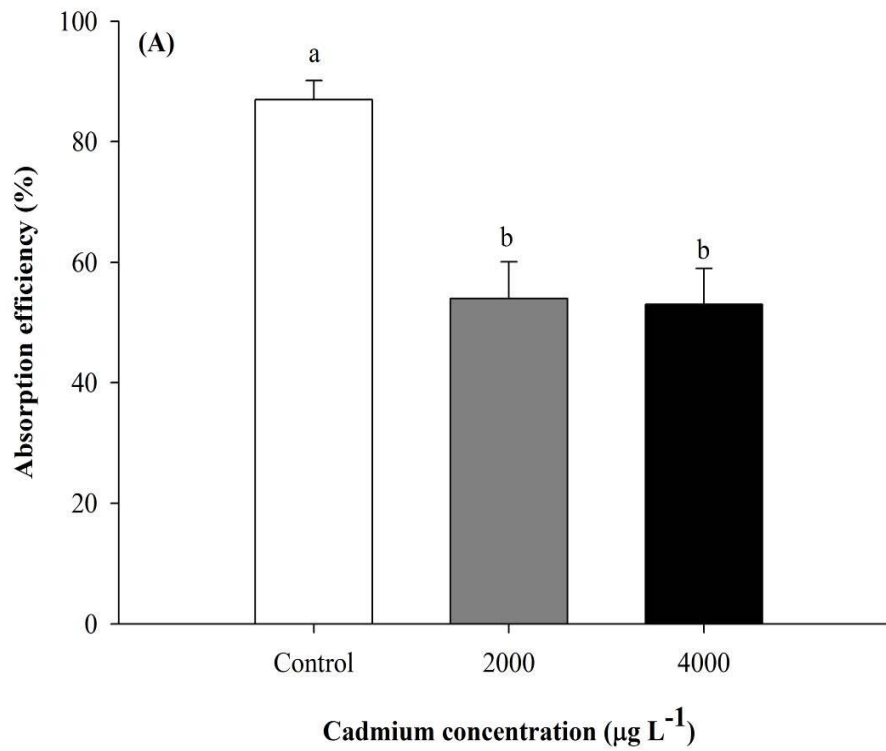


Figure 3.3 Clearance rate measurements of green-lipped mussels (*Perna canaliculus*) during acute (A; 96 h: 0, 2000 and 4000 $\mu\text{g L}^{-1}$, $n = 8$) and subchronic (B; 28 d: 0, 200 and 2000 $\mu\text{g L}^{-1}$, $n = 6$) exposures to Cd. Data are presented as mean \pm SEM. In panel A plotted points sharing letters are not statistically significant ($p < 0.05$) as determined using Kruskal-Wallis ANOVA followed by Mann Whitney U test. In panel B data were analysed by two-way ANOVA with exposure concentration (treatment) and time as factors using log-transformed data. The results of the analysis are reported on the panel. Asterisks indicate a significant difference between the exposure concentration and the control within a day, as determined by post hoc LSD analysis ($\alpha = 0.05$).

3.3.4 Absorption efficiency

A significant reduction in the absorption efficiency of mussels was observed at the end of the acute Cd exposure. Control mussels displayed an absorption efficiency of 87% while the 2000 $\mu\text{g L}^{-1}$ and 4000 $\mu\text{g L}^{-1}$ Cd-exposed mussels recorded significantly inhibited absorption

efficiencies of 54% and 53% (Fig. 3.4A). Although there was a significant overall effect of time on absorption efficiency in the subchronic exposure, the effect of Cd treatment, and the interaction between time and Cd treatment were not significant (Fig. 3.4B).



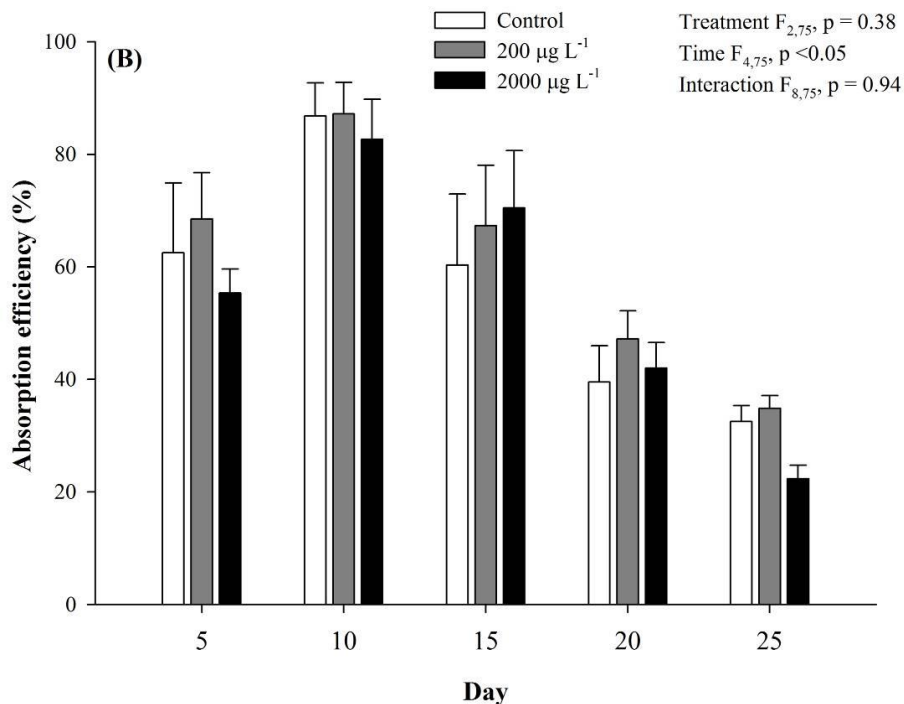


Figure 3.4 Absorption efficiency measurements of green-lipped mussels (*Perna canaliculus*) during acute (A; 96 h: 0, 2000 and 4000 $\mu\text{g L}^{-1}$, $n = 6-8$) and subchronic (B; 28 d: 0, 200 and 2000 $\mu\text{g L}^{-1}$, $n = 6$) exposures to Cd. Data are presented as mean \pm SEM. In panel B data were analysed by two-way ANOVA with exposure concentration (treatment) and time as factors using non-transformed data. See legend of Figure 3.3 for further details.

3.3.5 Respiration rate

Acute Cd exposure did not have any significant effect on the respiration rate of mussels (Fig. 3.5A). However significant differences were recorded during subchronic Cd exposure (Fig. 3.5B). Two-way ANOVA on effects of subchronic Cd exposure on respiration rates of mussels showed significant effects of treatment, time and interaction between these two factors. Specifically, mussels exposed to 2000 $\mu\text{g Cd L}^{-1}$ showed a significant decline in oxygen uptake compared to control mussels at all days throughout the exposure, with the exception of Day 15. In contrast, the respiration rate of mussels in the 200 $\mu\text{g L}^{-1}$ treatment

were first significantly inhibited on Day 15, an effect that persisted on Day 20, but which had dissipated on Day 25.

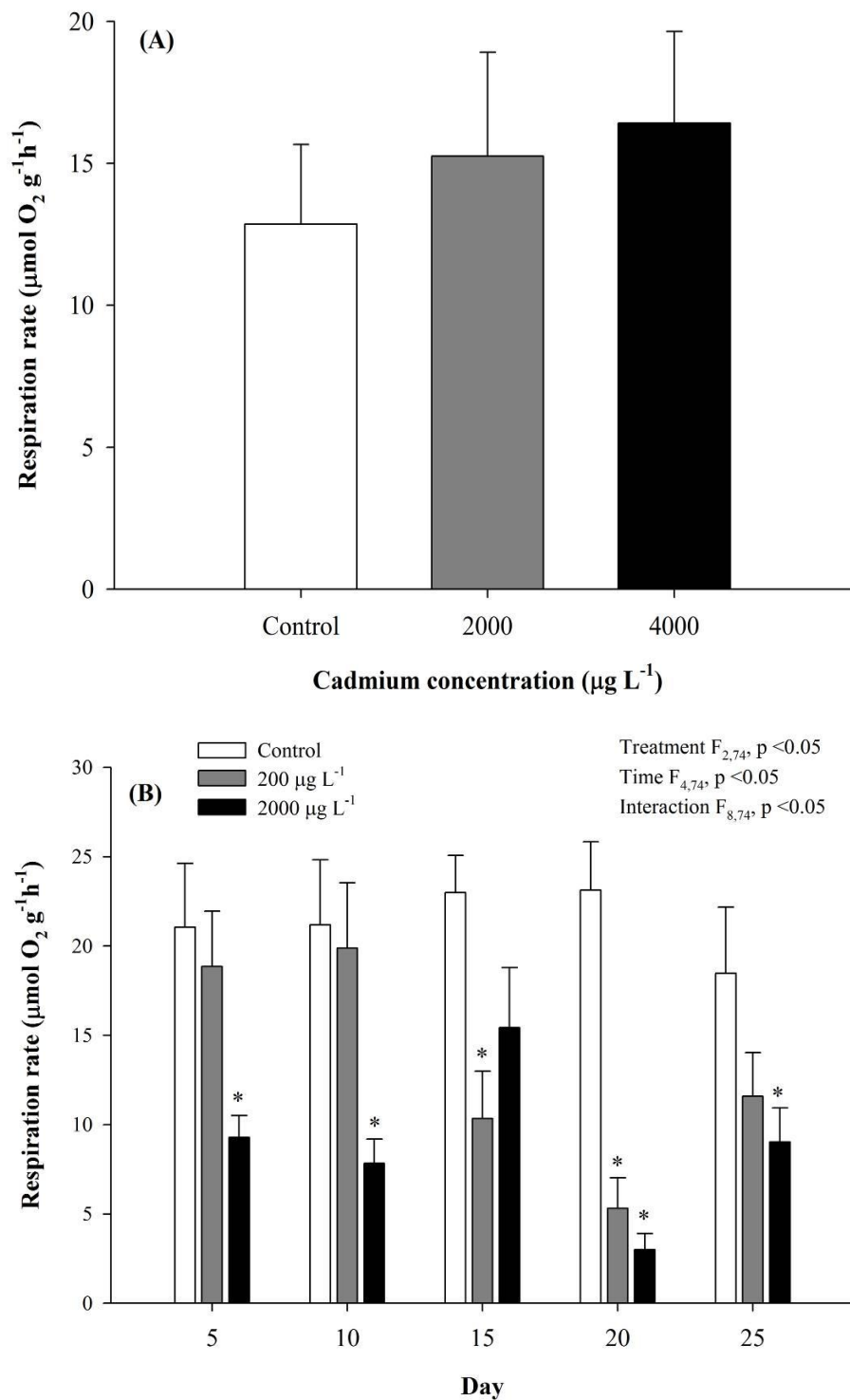
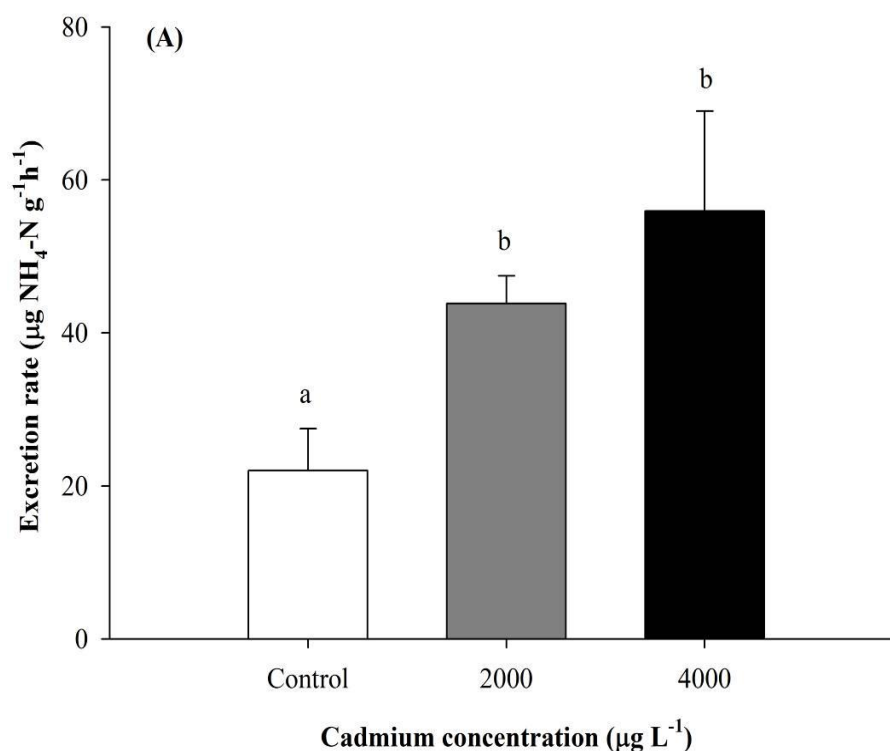


Figure 3.5 Respiration rate measurements of green-lipped mussels (*Perna canaliculus*) during acute (A; 96 h: 0, 2000 and 4000 $\mu\text{g L}^{-1}$, $n = 8$) and subchronic (B; 28 d: 0, 200 and

2000 $\mu\text{g L}^{-1}$, $n = 5-6$) exposures to Cd. Data are presented as mean \pm SEM. In panel B data were analysed by two-way ANOVA with exposure concentration (treatment) and time as factors using non-transformed data. See legend of Figure 3.3 for further details.

3.3.6 Excretion rate

The mussels subjected to acute Cd exposure (Fig. 3.6A) recorded 99% (2000 $\mu\text{g L}^{-1}$) and 160% (4000 $\mu\text{g L}^{-1}$) higher ammonia excretion rates than control mussels. These increases were statistically significant. Subchronic Cd exposure resulted in significant changes based on effects of treatment and time as shown on Fig. 3.6B, with the mussels exposed to 2000 $\mu\text{g Cd L}^{-1}$ displaying excretion rates significantly elevated to the control at all time intervals monitored.



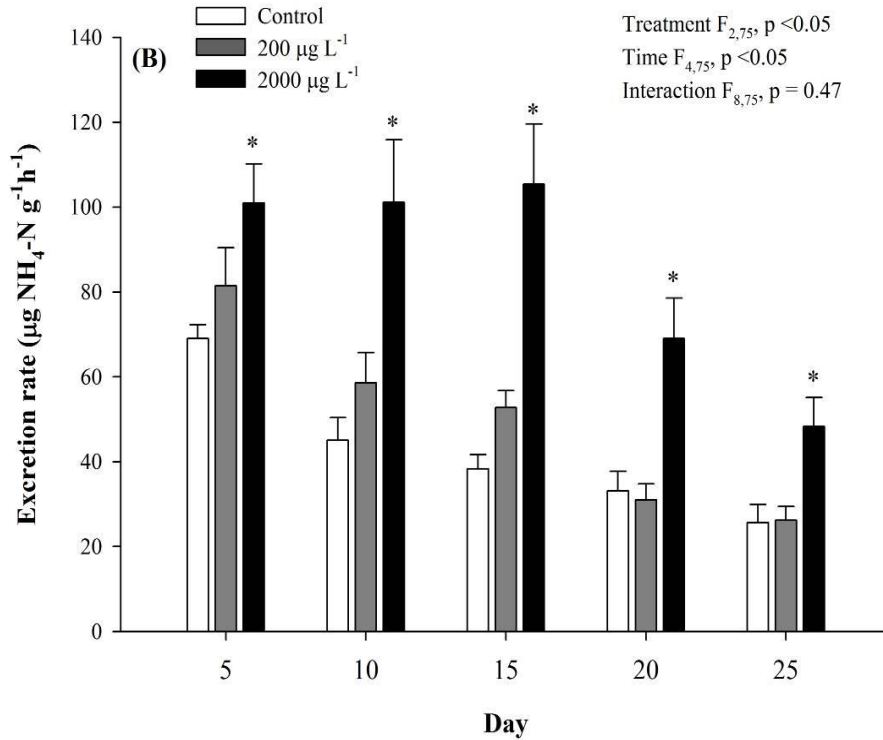


Figure 3.6 Excretion rate measurements of green-lipped mussels (*Perna canaliculus*) during acute (A; 96 h: 0, 2000 and 4000 $\mu\text{g L}^{-1}$, $n = 8$) and subchronic (B; 28 d: 0, 200 and 2000 $\mu\text{g L}^{-1}$, $n = 6$) exposures to Cd. Data are presented as mean \pm SEM. In panel B data were analysed by two-way ANOVA with exposure concentration (treatment) and time as factors using log-transformed data. See legend of Figure 3.3 for further details.

3.3.7 Oxygen:nitrogen ratio

No significant differences were noted in the O:N ratios calculated for mussels from the acute exposure (Fig. 3.7A). Two-way ANOVA on effects of subchronic Cd exposure on O:N ratio (Fig. 3.7B) of mussels showed significant effects of treatment and time. However, the interaction between treatment and time was not statistically significant. Compared to control mussels, a significant decline in O:N ratios of the 2000 $\mu\text{g L}^{-1}$ Cd concentration was recorded throughout the exposure, while significantly lower ratios were also observed for the 200 $\mu\text{g L}^{-1}$ Cd concentration on Days 15, 20 and 25.

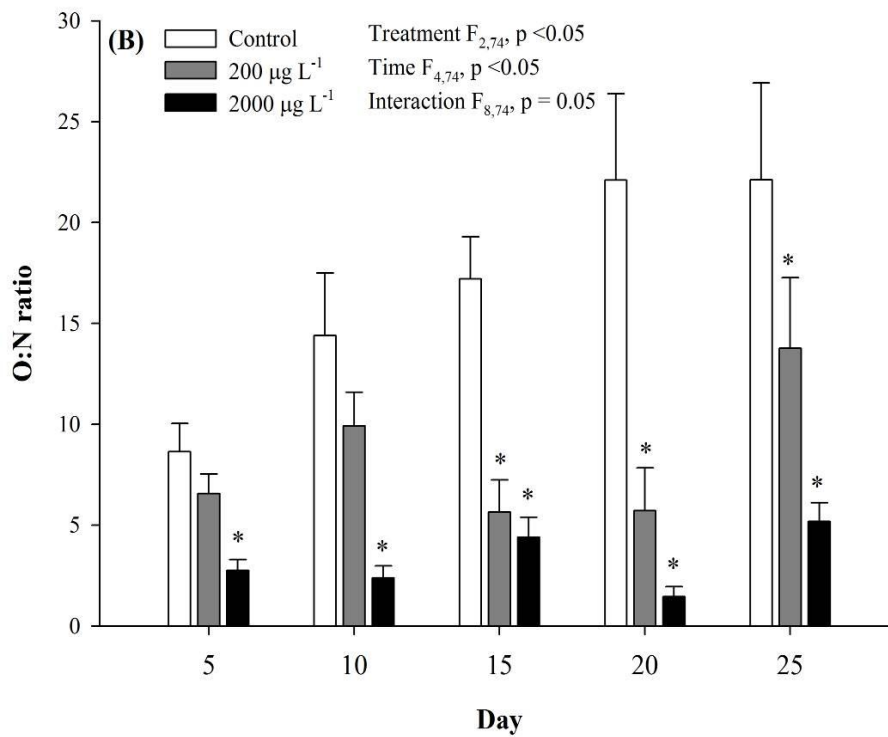
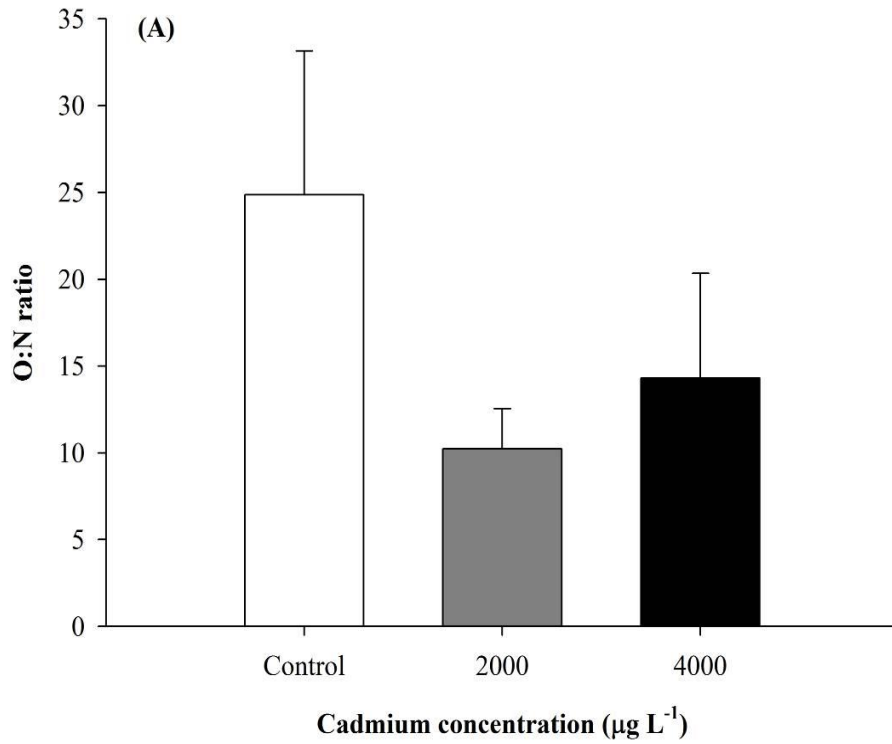
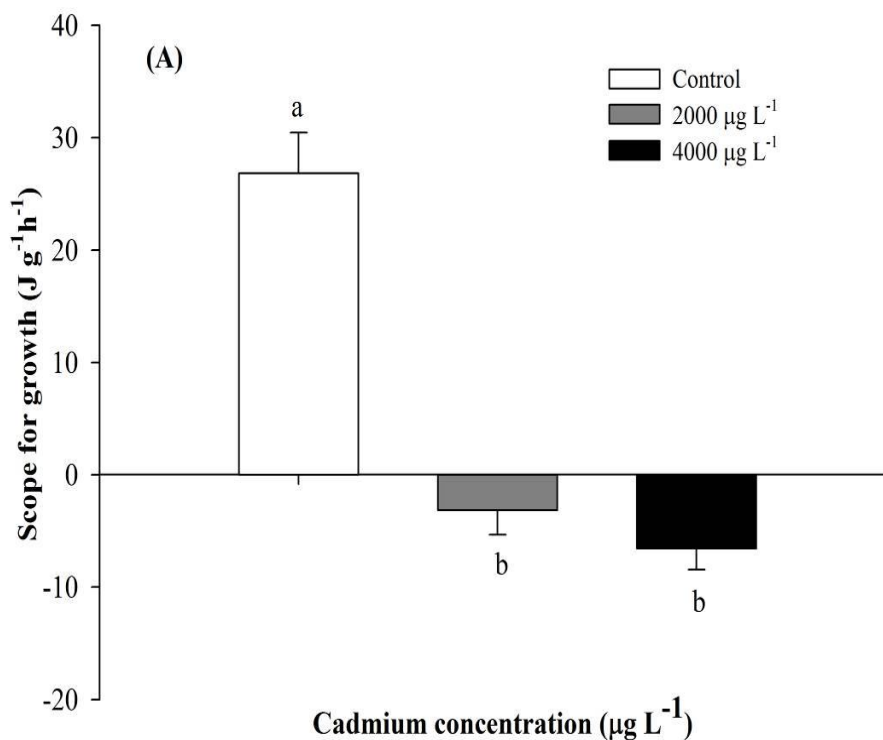


Figure 3.7 Oxygen:nitrogen ratio of green-lipped mussels (*Perna canaliculus*) during acute (A; 96 h: 0, 2000 and 4000 $\mu\text{g L}^{-1}$, $n = 8$) and subchronic (B; 28 d: 0, 200 and 2000 $\mu\text{g L}^{-1}$, n

= 5-6) exposures to Cd. Data are presented as mean \pm SEM. In panel B data were analysed by two-way ANOVA with exposure concentration (treatment) and time as factors using square-root transformed data. See legend of Figure 3.3 for further details.

3.3.8 Scope for growth

A negative SFG was recorded for mussels acutely exposed to Cd (Fig. 3.8A). SFG of mussels from the subchronic Cd exposure showed significant differences in the effect of treatment, time and interaction between these two factors. The 2000 $\mu\text{g L}^{-1}$ Cd exposed mussels displayed significantly inhibited SFG values on Days 10, 15 and 20, while a significant increase in SFG on Day 15 was observed in mussels exposed to 200 $\mu\text{g Cd L}^{-1}$ (Fig. 3.8B).



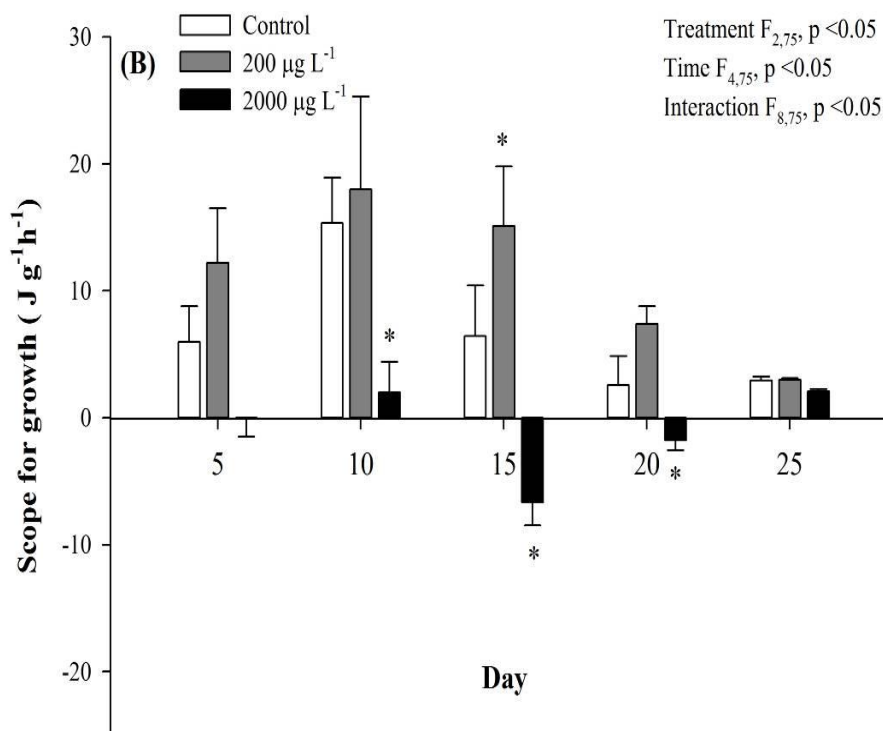


Figure 3.8 Scope for growth measurements of green-lipped mussels (*Perna canaliculus*) during acute (A; 96 h: 0, 2000 and 4000 $\mu\text{g L}^{-1}$, $n = 6-8$) and subchronic (B; 28 d: 0, 200 and 2000 $\mu\text{g L}^{-1}$, $n = 5-6$) exposures to Cd. Data are presented as mean \pm SEM. In panel B data were analysed by two-way ANOVA with exposure concentration (treatment) and time as factors using non-transformed data. See legend of Figure 3.3 for further details.

3.4 Discussion

3.4.1 Acute toxicity

This is the first study detailing the sensitivity of NZ green-lipped mussels to Cd. The 96 h LC₅₀ of *P. canaliculus* was 8160 $\mu\text{g L}^{-1}$. Although the sensitivity of *P. canaliculus* to Cd was high relative to a number of endemic shellfish species that were tested during preliminary experiments (data not shown), it is intermediate relative to other mussel species. For example, smaller (30 - 40 mm) *Perna viridis* assayed at 20 °C displayed a 96 h LC₅₀ value of 1570 $\mu\text{g L}^{-1}$ (Chan, 1988). A 96 h LC₅₀ value of 1620 $\mu\text{g L}^{-1}$ Cd was recorded in *Mytilus edulis*

planulatus (Ahsanullah, 1976) in assays conducted at 18.5°C, while Eisler (1971) has reported *Mytilus edulis* (48 mm) as having a 96 h LC₅₀ value of 25000 µg L⁻¹ at 20°C. Factors such as size, assay temperature and inherent differences in defence pathways (Rana and Singh, 1996) are likely to explain these variations in LC₅₀ values.

3.4.2 Cadmium accumulation

In the current study, Cd accumulation in the digestive gland exhibited evidence of saturation over acute time-frames, but accumulation over the subchronic exposure was linear (Table 3.2). This suggests that acute Cd exposures overwhelmed the capacity of digestive gland to accumulate metal, which would have generated a ‘spillover’ of metal and resulted in toxic impacts. Conversely over longer exposures to lower levels a linear relationship between exposure level and tissue accumulation was discerned, suggesting an ability to adequately sequester Cd in this exposure scenario. These findings are consistent with previous studies in mussels (Erk et al., 2005; Long et al., 2010).

In contrast to the digestive gland, gill accumulation was linear over both exposure durations. In the gill Cd is likely to initially bind to sequestering proteins such as MT, or to be incorporated into lysosomes (Viarengo and Nott, 1993). This is usually a temporary storage before it is then released into the circulation. Once in the haemolymph Cd can be transported to excretory tissues, or ends up sequestered in the digestive gland tissue. In the digestive gland Cd is eventually incorporated into specific organelles, in specific cell types, to remove it from interacting with sensitive cellular processes (Marigómez et al., 2002). This difference in metal handling likely explains the linear relationship between exposure concentration and gill accumulation (a temporary storage and slow release into the circulation), and the differences in burdens between these two tissues (digestive gland for long-term storage, so burden increases over subchronic exposures). Measures of gill Cd accumulation might

therefore be a fast and relatively simple surrogate measure for determining elevated environmental Cd levels in natural settings, while the digestive gland could be indicative of the longer exposure history. Both of these parameters may be of some utility with respect to biomonitoring.

It is important to note that during subchronic exposures mussels were fed, and consequently Cd uptake or adsorption by algal cells may have contributed to Cd exposure in the mussels. Therefore it is possible that the accumulation and effects observed are the result of both waterborne and dietary exposure. However, despite the significant decreases in clearance rate (i.e. feeding) observed at the highest Cd exposure level, this group still accumulated the most Cd, indicating that the dietary contribution to tissue burden was not likely to be significant.

3.4.3 Physiological biomarkers – does tolerance to cadmium come at a cost?

Clearance rate was sensitive to Cd exposure. The feeding rate showed impairment that was linearly correlated to tissue metal accumulation and was consistently inhibited throughout the course of the 28 d subchronic exposure at a concentration of 2000 $\mu\text{g Cd L}^{-1}$. Many of the other physiological parameters measured showed more complex relationships with tissue Cd accumulation. This was particularly the case for measures such as SFG, which exhibited recovery over the exposure period despite an increasing tissue Cd burden. Therefore, in terms of utility as a physiological indicator of high Cd exposure levels, clearance rate offers significant promise. The weakness of clearance rate as an indicator is associated with the fact that effects were only observed at the highest Cd level during subchronic exposure. Further research would be required to determine whether the relationship between tissue accumulation and clearance rate holds for exposures to common environmental levels of exposure.

The Cd-induced inhibition of clearance rate may represent a behavioural impairment, rather than a physiological response per se. On exposure to an environmental stressor mussels tend to close their shell valves thereby minimising exchange with the outside environment (Akberali and Trueman, 1985). This acts as a protective mechanism to isolate their important internal structures from the stressor. In the case of Cd this would reduce accumulation in soft tissue, and thus limit the toxicological impacts of exposure. A consequence of this behaviour is that mussel feeding declines, as demonstrated by the decrease in clearance rate.

The decrease in clearance rate could, however, be explained by direct physical changes at the gill. It has been reported that presence of metals leads to mucus secretion that coats the gill epithelium thereby hindering ciliary activity that is essential to the feeding process (Sze and Lee, 1995). Studies have also reported that Cd can cause tissue damage to the gill (Sunila and Lindström, 1985). These changes might impair the ability of the mussel to adequately clear and ingest food. If damage were to occur to the feeding apparatus, then it would be reasonable to expect that absorption efficiency would also be impacted. Such an effect was clearly observed over acute time-frames, but significant impairment of absorption efficiency as a result of Cd exposure was not observed during the subchronic experiment. It is therefore possible that the decrease in clearance rate in subchronic exposure was behaviourally-mediated, but over an acute exposure period direct physical perturbation of feeding may be the more important inhibitory mechanism.

As subchronic Cd exposure progressed, food intake and the overall energy availability decreased as a consequence of effects on clearance rates. This impacted the utilisation of energy resources, and was reflected in the significant effect of Cd exposure on O:N ratios. Ikeda (1977) stated that O:N ratios of 8 and less (as reported here for both subchronic Cd exposure groups) represented the use of protein as the sole source of energy. Values higher

than that represented utilisation of both proteins and lipids, with values higher than 24 relating to exclusive use of lipids as an energy source. This clearly indicates that during subchronic Cd exposure mussels relied exclusively on protein as an energy source, and suggests that the deficiencies in nutrient uptake and utilisation demonstrated by impaired clearance rate forced the mussels to metabolise endogenous sources of protein. This switch in energy resources is a last resort for animals during prolonged starvation, and has been noted previously in mussels (Sprung and Borcharding, 1991). A metabolic breakdown of protein also likely explains the increases in ammonia excretion observed in Cd-exposed mussels (*this chapter*; Aldridge et al., 1995).

The other factor involved in energy balance, and which could also have impacted O:N ratio, is oxygen consumption (respiration rate). In the subchronic experiment respiration rate was significantly affected. Although the responses of respiration rate to Cd exposure vary according to length of exposure, Cd concentration, and species tested, most literature supports the findings of the present study. For example, in the mussel *Perna viridis* a significant decrease in oxygen consumption was observed after one week of Cd exposure (Cheung and Cheung, 1995).

SFG is a predicted measure that elucidates the physiological health status of mussels (see *Section 1.6.2*). In the present study, the negative SFG values obtained for the Cd-exposed mussels indicated an imbalance in metabolic activity over the period of exposure. Under normal conditions, energy acquisition through feeding and food absorption is expended via respiration and waste removal (excretion) to maintain normal functioning of the mussel. The residual energy available is then utilised for growth and reproduction (Widdows and Donkin, 1992). A negative SFG value is considered a severe stress indicator. The drastic reduction in clearance rate measurements along with the increase in excretion rates as a result of Cd

exposure are the primary factors generating the negative SFG values recorded. It is, however, worth noting that SFG had recovered by the conclusion of the subchronic study. An improved SFG value must reflect either an increase in energy absorption, or a decrease in energy expenditure. In the present study it is likely that the significantly inhibited respiration rate in mussels exposed to $2000 \mu\text{g Cd L}^{-1}$ is driving the return to positive SFG. The observed decrease in respiration rate could be explained as interplay between several complex factors such as damage to respiratory organs, valve closure, mucus production and other compensatory mechanisms of survival, such as reduction of energy cost (Anandraj et al., 2002). The pattern of other physiological markers strongly suggests that the effect on respiration rate, and hence SFG, is likely to be a pathological response, rather than a controlled physiological regulation of respiration. As such the recovery of SFG may be misleading. This, coupled with the relatively poor correlation of SFG with tissue accumulation indicates that this index of impairment is not a reliable marker of metal exposure and/or effect, at least over the duration of exposures tested in the present study.

3.4.4 Importance of duration of exposure

There is limited information available regarding the physiological changes of organisms subjected to short-term and high concentrations of metal exposure. The current study showed that although acute and subchronic exposures may differ in terms of metal exposure concentrations and duration of exposure they can have consistent impacts on the physiology of the exposed organisms.

In contrast to the consistent effect of measurements such as clearance rate, respiration rate showed variability between acute and subchronic Cd exposures, being significantly impacted only during the latter. Research examining the impacts of PAH exposure on shellfish have noted variability in respiration rate as a function of contaminant concentration, with elevated

respiration at low concentrations, and inhibited respiration at high concentrations (Kim et al., 2007). These authors attributed this to a valve closure response at high levels. This could explain the decrease in respiration rate in subchronic exposures to Cd, as the effect is similar to that observed for clearance rate, but could not explain the lack of decline during acute exposures. Instead the lack of change in respiration rate over acute time-frames may represent a combined effect of decreased oxygen availability, and also the increased metabolic costs associated with the acute exposure to a high level of metal. Over acute periods animals may enact mechanisms to deal with the toxicant that increase metabolic rate, whereas over chronic periods of exposure conservation of resources becomes more important, and metabolic costs and respiration rates decline. This pattern has been observed for acute *versus* chronic nickel exposure in the freshwater crustacean *Daphnia magna* (Pane et al., 2003).

Most exposures to metals in the natural environment occur over long time-frames to relatively low levels, however pollutant inputs via point sources can result in high levels of metals over acute exposure periods (e.g. Brix et al., 2010). For this reason it is important that biomonitoring tools offer consistent information over the concentration range of potential exposures that may threaten the ecosystem.

3.5 Conclusions

The current study highlights the utility of employing green-lipped mussels as a sentinel species to monitor coastal ecosystems in NZ. *P. canaliculus* is widespread around NZ's coastal waters, and is an important component of the NZ diet (Whyte et al., 2009). These factors suggest it is a convenient organism to assess for environmental contamination, and that impairments related to Cd level will yield potentially important information regarding human consumption risk. The findings show that these mussels are relatively tolerant to Cd exposure, accumulate Cd in key tissues, and show physiological impairment in response to

Cd exposure. For example, clearance rate, a simple physiological measure, was inhibited in response to both acute and subchronic Cd exposure. Furthermore, the magnitude of clearance rate inhibition was correlated directly and linearly with gill Cd accumulation, which was in turn correlated to environmental Cd exposure. The use of clearance rate could yield cost-effective information on the health status of mussels under natural conditions. Consistent with the current findings, previous studies in other bioassay organisms have also identified the value of feeding rates as a sensitive sublethal indicator of toxicity (Barata et al., 2008).

4 Biochemical biomarker responses of green-lipped mussel, *Perna canaliculus*, to acute and subchronic waterborne cadmium toxicity[†]

[†]**Chandurvelan, R.**, Marsden, I.D., Gaw, S., Glover, C.N., 2013. Biochemical biomarker responses of green-lipped mussel, *Perna canaliculus*, to acute and subchronic waterborne cadmium toxicity. **Aquatic Toxicology**, 140/141: 303-313.

4.1 Introduction

Cd is known to induce a number of changes in the biochemical profile of exposed organisms that reflect either exposure to, or a toxic impact from, Cd. For instance, Cd is known to induce the production of MT. This is a low molecular weight, high sulfhydryl content protein that exhibits strong affinity for Cd (Viarengo and Nott, 1993; see *Section 1.6.3.1*). MT-bound Cd cannot interact with sensitive components of the cell, thereby limiting Cd-induced toxicity. However, if MT binding capacity is exceeded toxic effects may present. These include the production of ROS and resulting oxidative stress (Company et al., 2010; see *Section 1.3.5.2*), the inhibition of key enzymes in cellular homeostasis (e.g. alkaline phosphatase and NKA; Lionetto et al., 1998), and even indirect effects resulting from the added costs of defence and damage repair (e.g. changes in metabolic substrate utilisation; Ngo et al., 2011; see *Section 1.3.5.8*). Any changes in mussel biochemistry could have a negative effect on the well-being of the organism and, if prolonged, could ultimately lead to death (see *Section 1.3.5*).

Multiple biochemical biomarkers have been previously used to study the effects of metal exposure in mussels under laboratory conditions (e.g. Al-Subiai et al., 2011). Galloway et al. (2004) highlighted the benefits of employing multiple biomarkers to assess pollution impacts on organisms, where the use of different biochemical biomarkers, along with chemical analyses, provides a time-integrated assessment of the environmental pollution levels in a particular region of interest (see *Section 1.6.3*).

The main objective of this chapter was to investigate biochemical changes in green-lipped mussels to provide an integrated assessment of the responses of this species to acute and subchronic Cd exposure. In the present chapter concentrations of 2000 and 4000 $\mu\text{g Cd L}^{-1}$ were used to investigate the effects of this metal over acute exposures, as these

concentrations are known to cause sublethal effects without causing mortality to green-lipped mussels over 96 h (*Chapter 3*). In the current chapter, potential biochemical mechanisms of Cd toxicity (e.g. MTLP, catalase, lipid peroxidation, alkaline phosphatase, NKA, glycogen and haemolymph protein) were examined in this species for the first time, and clear differences in the effect of exposure concentration and duration were discerned. Biochemical changes were correlated with tissue Cd accumulation to investigate the relationship between these parameters, and identify potential biomarkers of Cd exposure that may be of use in a biomonitoring strategy.

4.2 Materials and methods

4.2.1 Biochemical biomarkers

The acute and subchronic Cd exposure treatments have been detailed in *Section 2.3*. Mussels were randomly collected from exposure tanks at the end of the 96 h acute exposure and on Days 7, 14, 21 and 28 during the subchronic exposure to Cd. The gill, digestive gland and haemolymph tissues were collected and stored at -80°C until processed. All biochemical biomarkers were measured using the protocols detailed in *Section 2.5*.

The n values for each of these analyses was $n = 6$ for the acute study and $n = 8$ for the subchronic study, with each of these values representing mussel tissue pooled from two individuals sampled from a single exposure chamber. Similarly, the n values of tissues obtained from individual mussels for the other biochemical biomarker measurements were as follows: lipid peroxidation ($n = 6$ for acute; $n = 6$ for subchronic), NKA activity ($n = 6$ or 8 for acute; $n = 5$ or 6 for subchronic), glycogen ($n = 5$ for acute; $n = 6$ for subchronic) and haemolymph samples ($n = 4$ or 5 for acute; $n = 6$ for subchronic). Fig. 4.1 shows the biochemical biomarkers used in this study.

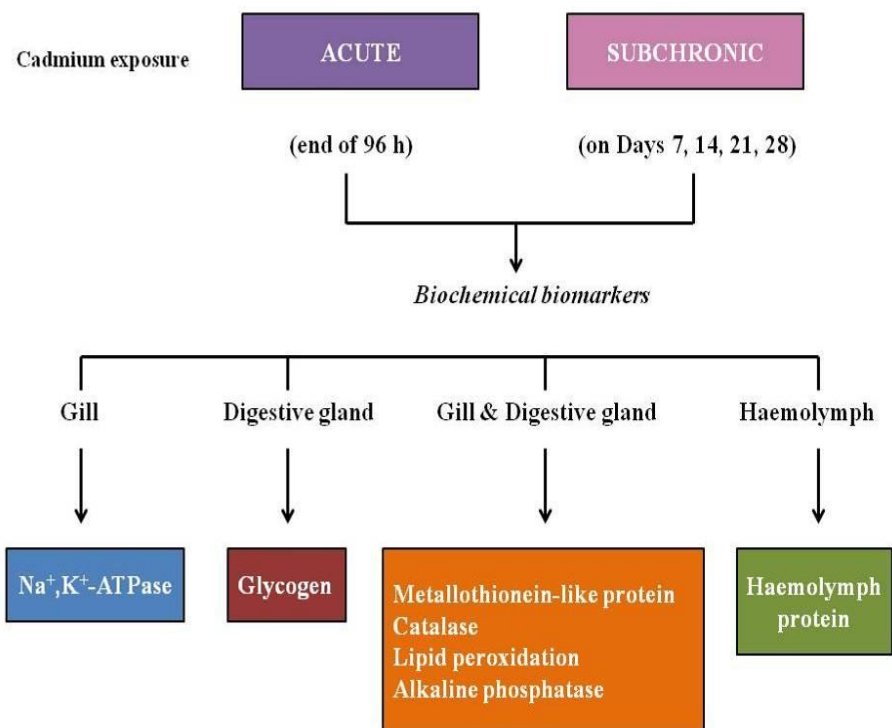


Figure 4.1 Flow chart illustration of cadmium exposure and biochemical biomarkers used in this study

4.2.2 Statistical analysis

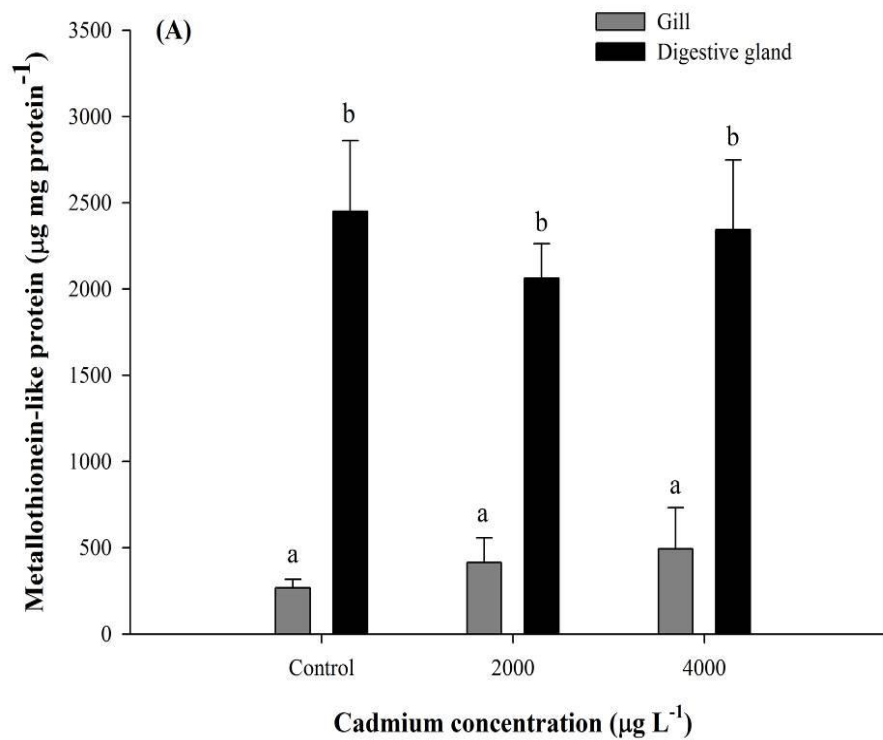
Biochemical biomarker data were tested using the statistical methods detailed in *Section 2.9*. The relationship between Cd accumulation and each biochemical biomarker response in gill and digestive gland on Days 7, 14, 21 and 28 was modelled using the method described in *Section 2.9*.

4.3 Results

4.3.1 Metallothionein-like protein

Digestive gland MTLP levels were significantly higher than those measured in the gill in all mussel groups (Fig. 4.2A). However, exposure to acute Cd concentrations did not result in any significant changes in the gill or digestive gland relative to the unexposed control. Conversely, a significant increase in MTLP levels in gill (Fig. 4.2B) and digestive gland (Fig.

4.2C) was observed from Day 7 in 2000 $\mu\text{g L}^{-1}$ Cd-exposed mussels and from Day 14 in the 200 $\mu\text{g L}^{-1}$ Cd-exposed mussels. The elevation in MTLP levels in both the gill and the digestive gland persisted throughout the 28 d subchronic Cd exposure, and in both tissues this effect was strongly and significantly correlated with tissue Cd accumulation (gill $R = 0.957$; digestive gland $R = 0.964$, both $p < 0.0001$; Table 4.1).



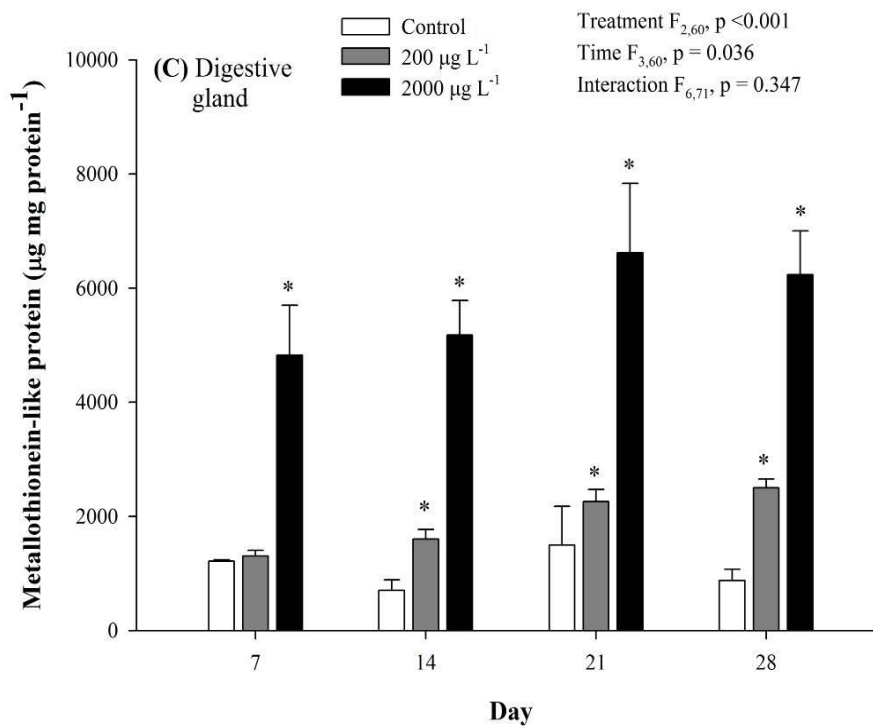
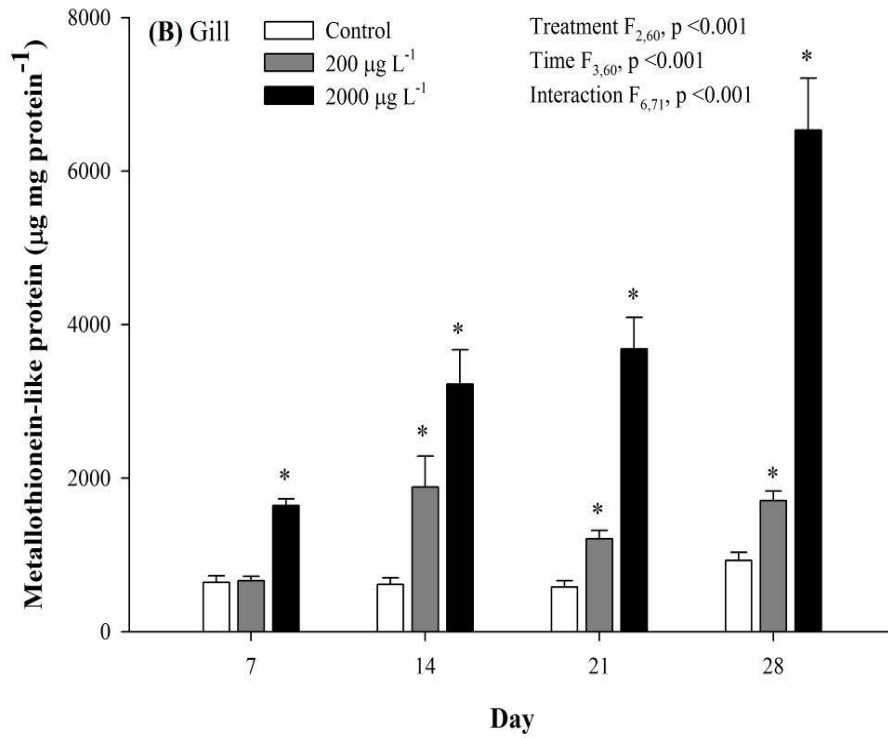
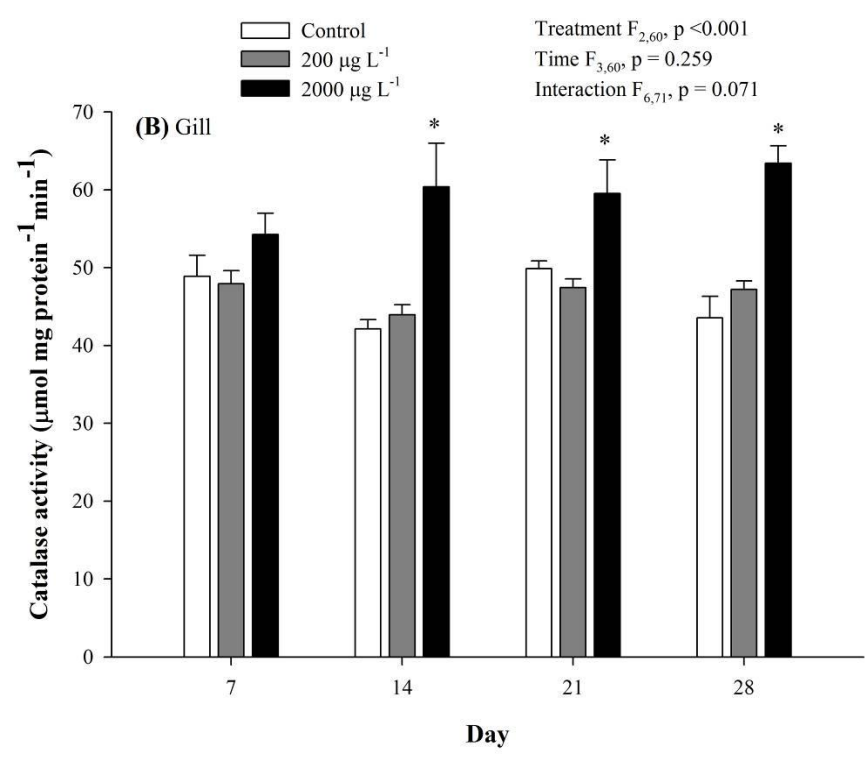
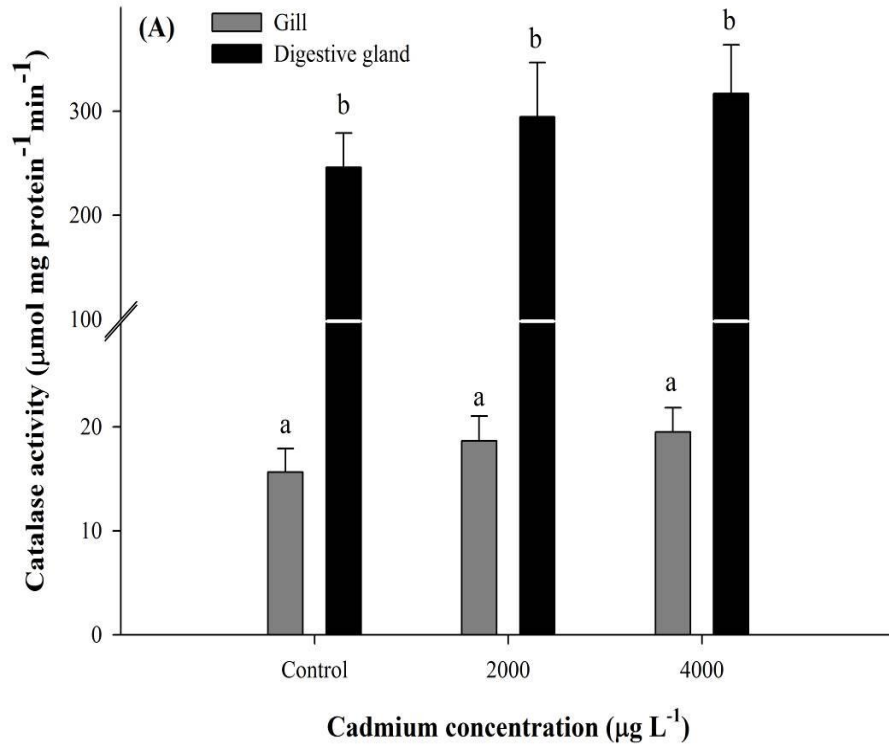


Figure 4.2 Metallothionein-like protein levels in green-lipped mussel (*Perna canaliculus*) gill and digestive gland after acute Cd exposure (A; 96 h: 0, 2000 and 4000 $\mu\text{g L}^{-1}$, $n = 8$), and in gill (B) and digestive gland (C) during subchronic Cd exposure (28 d: 0, 200 and 2000 $\mu\text{g L}^{-1}$)

¹, $n = 6$). Values are expressed as mean \pm SEM. In panel A, plotted points sharing letters are not significantly different ($p < 0.05$), determined using post hoc LSD analysis. Data in panel (A) were square-root transformed prior to statistical analysis. In panels B and C data were analysed by two-way ANOVA with exposure concentration (treatment) and time as factors. Data in panels B and C were log-transformed for analysis. The results of the two-way ANOVA analysis are reported on the panels. Asterisks indicate a significant difference between the exposure concentration and the control within a day while plotted points with different letters indicate significant difference with respect to day within an exposure concentration as determined by post hoc LSD analysis ($\alpha = 0.05$).

4.3.2 Catalase

Catalase activity in mussels subjected to acute Cd exposure did not show any significant changes in either the gill or the digestive gland (Fig. 4.3A). Catalase levels in the digestive gland were significantly higher than in the gill of mussels. However, after 14 d of subchronic Cd exposure a significant increase in gill catalase activity of mussels exposed to $2000 \mu\text{g L}^{-1}$ Cd relative to time-matched controls was noted (Fig. 4.3B). In the digestive gland, catalase activity was increased in the $2000 \mu\text{g L}^{-1}$ Cd-exposed mussels throughout the 28 d of subchronic Cd exposure, an effect that was also evident in the $200 \mu\text{g L}^{-1}$ Cd-exposed mussels from Day 21 (Fig. 4.3C). Moreover, catalase activity in the digestive gland was significantly affected by time as a factor in both the 200 and $2000 \mu\text{g Cd L}^{-1}$ exposure groups. As noted with MTLP, there was a significant positive correlation between Cd accumulation in the tissue and the magnitude of the biochemical response (Table 4.1). This relationship was stronger for the gill ($R = 0.907$, $p < 0.0001$) than for the digestive gland ($R = 0.708$, $p = 0.0100$).



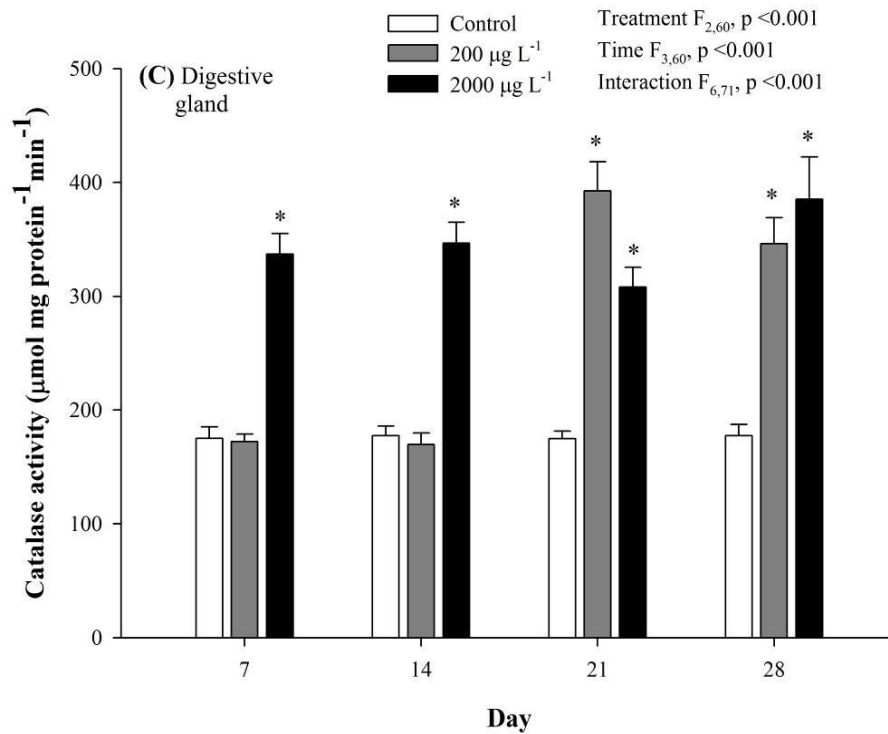
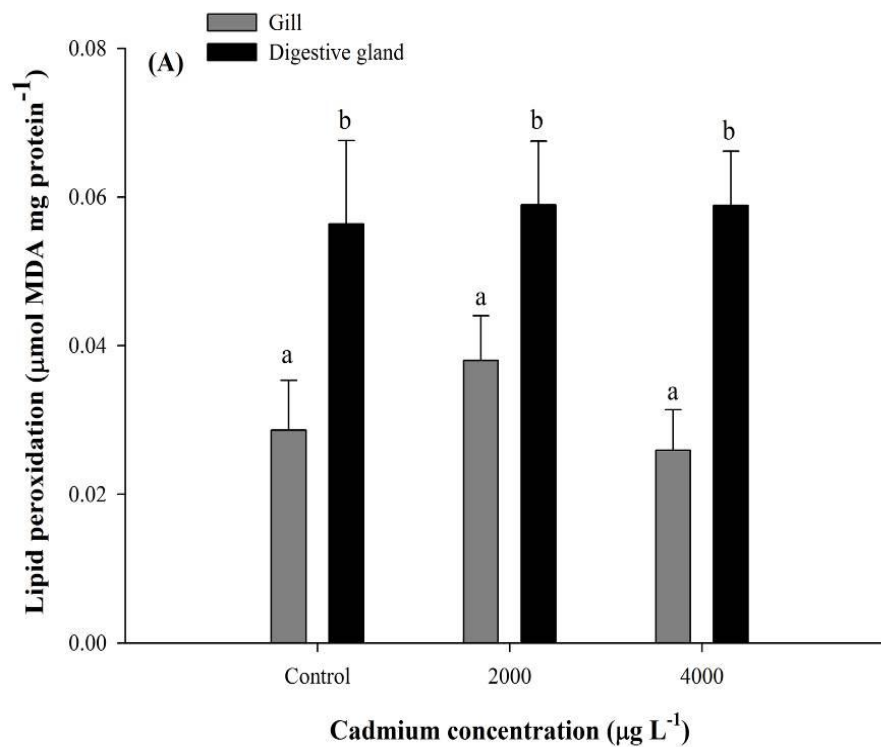


Figure 4.3 Catalase activity (mean \pm SEM) in green-lipped mussel (*Perna canaliculus*) gill and digestive gland after acute Cd exposure (A; $n = 8$) and in gill (B; $n = 6$) and digestive gland (C; $n = 6$) during subchronic Cd exposure. All other details are identical to those in the Figure 4.2 legend, except that only the data in panel B were log-transformed prior to statistical analysis.

4.3.3 Lipid peroxidation

No significant differences in lipid peroxidation levels were found between control and Cd-exposed mussels at the end of the 96 h acute exposure, although significant differences between gill and digestive gland levels were noted at all exposure concentrations (Fig. 4.4A). During the subchronic Cd exposure, lipid peroxidation levels in the gill were unchanged in Cd-exposed mussels relative to their time-matched control, with the exception of Day 21 in the 2000 $\mu\text{g Cd L}^{-1}$ exposure group, which exhibited a significantly lower level of oxidative stress (Fig 4.4B). Conversely, the lipid peroxidation levels in the digestive gland of Cd-exposed mussels were significantly higher than that of the control mussels (Fig. 4.4C). This

effect was present in both treated groups over the first 14 d of exposure, but diminished with time. Subsequently no significant differences were observed at Day 21, and only the 2000 $\mu\text{g Cd L}^{-1}$ mussels exhibited elevated peroxidation levels relative to unexposed mussels at Day 28. Lipid peroxidation in the digestive gland did not correlate with Cd accumulation in this tissue (Table 4.1). A significant correlation was, however, noted for the gill where increased Cd accumulation resulted in decreased lipid peroxidation ($R = 0.634, p = 0.0274$; Table 4.1).



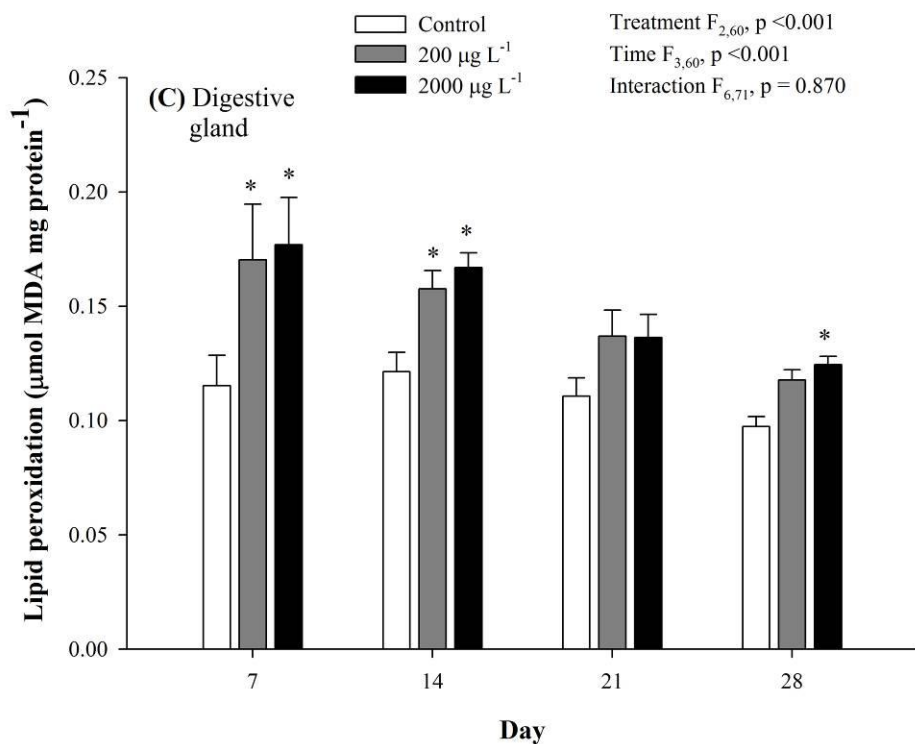
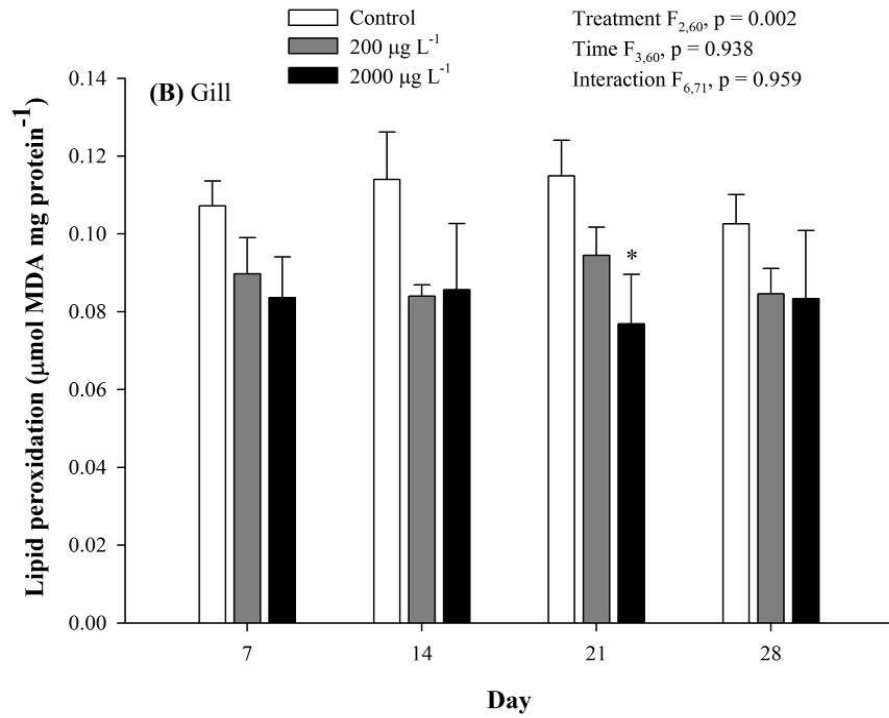
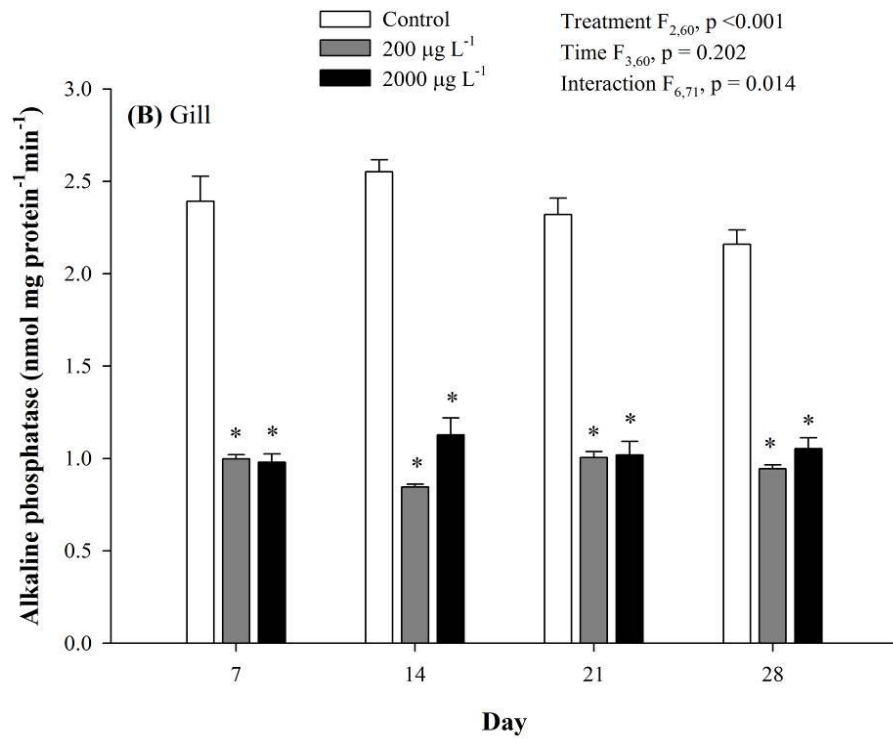
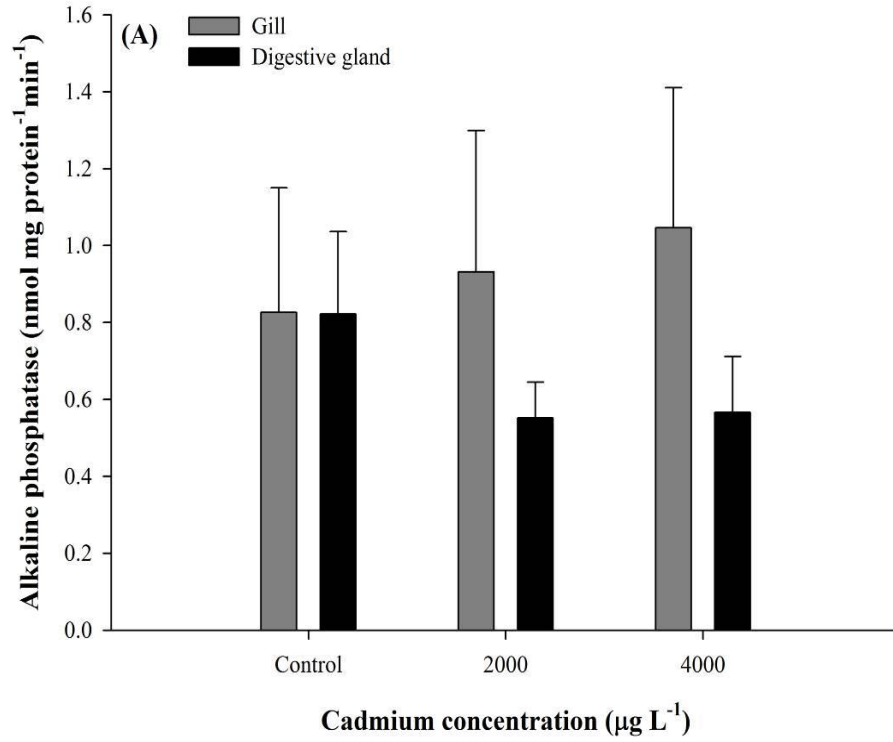


Figure 4.4 Lipid peroxidation (mean \pm SEM) in green-lipped mussel (*Perna canaliculus*) gill and digestive gland after acute Cd exposure (A; $n = 6$) and in gill (B; $n = 6$) and digestive

gland (C; n = 6) during subchronic Cd exposure. All other details are identical to those in the Figure 4.2 legend, except that only the data in panel C were log-transformed prior to statistical analysis.

4.3.4 Alkaline phosphatase

Compared to control mussels, no significant changes in alkaline phosphatase activity were observed in the gill and digestive gland of mussels acutely exposed to Cd (Fig. 4.5A). However, subchronic Cd exposure resulted in a significant reduction of alkaline phosphatase activity in the gill of 200 and 2000 $\mu\text{g L}^{-1}$ Cd-exposed mussels, an effect that was seen after 7 d and which persisted throughout the 28 d exposure (Fig. 4.5B). The digestive gland of mussels exposed to 2000 $\mu\text{g L}^{-1}$ Cd exposure had significantly lower enzyme activity than its time-matched control while no such changes were observed for the 200 $\mu\text{g L}^{-1}$ Cd mussels (Fig. 4.5C). Only the decrease in digestive gland activity was significantly correlated to tissue accumulation (Table 4.1).



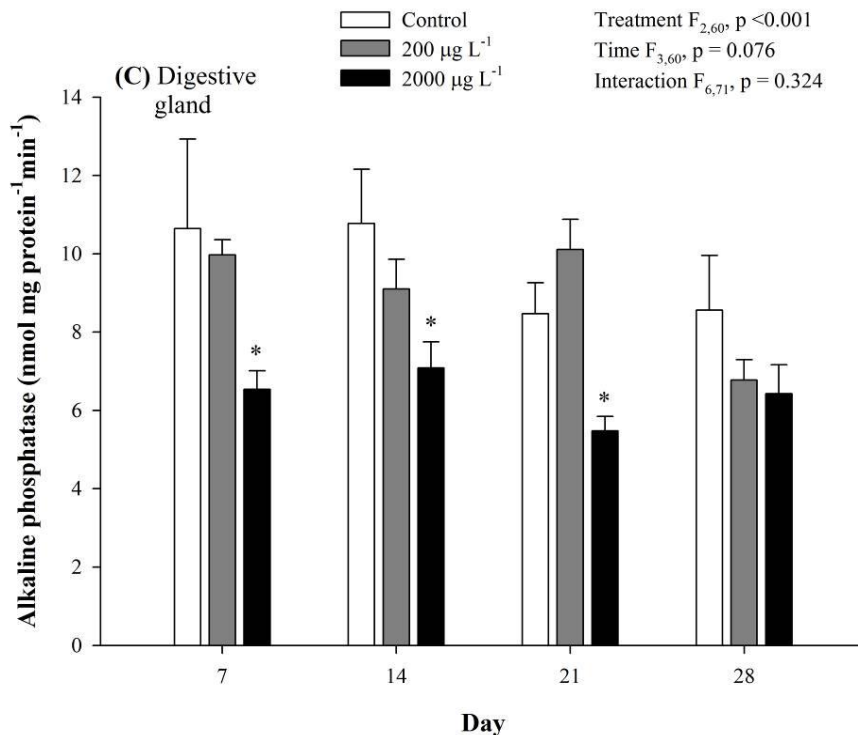


Figure 4.5 Alkaline phosphatase activity (mean \pm SEM) in green-lipped mussel (*Perna canaliculus*) gill and digestive gland after acute Cd exposure (A; $n = 8$) and in gill (B; $n = 6$) and digestive gland (C; $n = 6$) during subchronic Cd exposure. All other details are identical to those in the Figure 4.2 legend, except that only the data in panel C were log-transformed prior to statistical analysis.

4.3.5 Na^+ , K^+ -ATPase

The 2000 and 4000 $\mu\text{g Cd L}^{-1}$ acute exposure groups had significantly lower NKA activity in their gill than control mussels (Fig. 4.6A). Compared to the time-matched control, the NKA activity was significantly higher in the subchronic 200 $\mu\text{g L}^{-1}$ Cd group on Days 7, 14 and 28 with a significant decline observed on Day 21 (Fig. 4.6B). In contrast, the 2000 $\mu\text{g L}^{-1}$ Cd-exposed mussels recorded no significant changes with the exception of Day 28 when significantly higher enzyme activity was observed. These results did not correlate significantly with tissue Cd accumulation (Table 4.1).

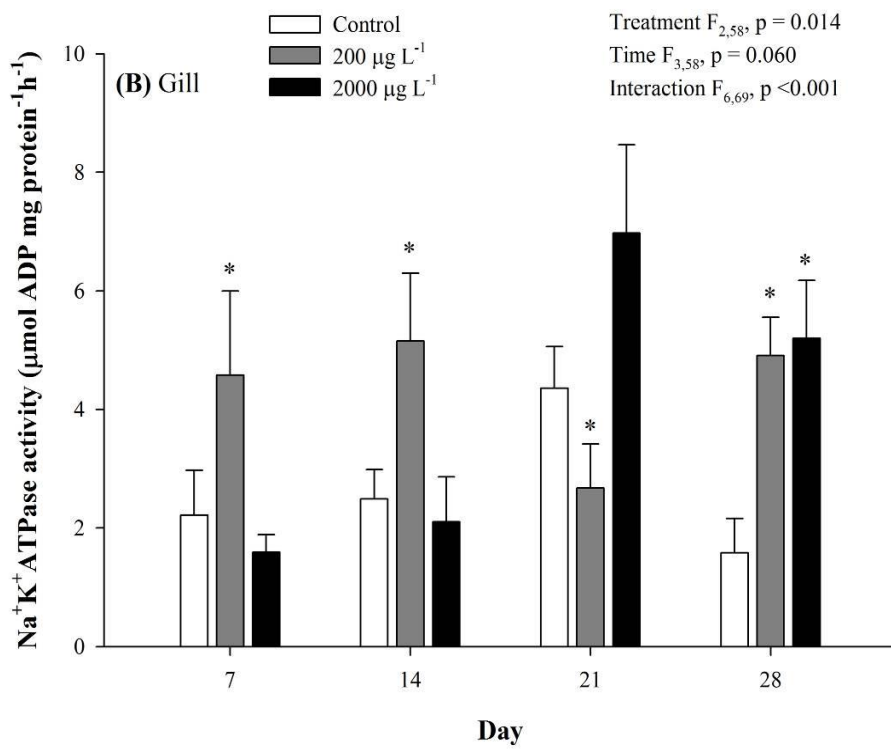
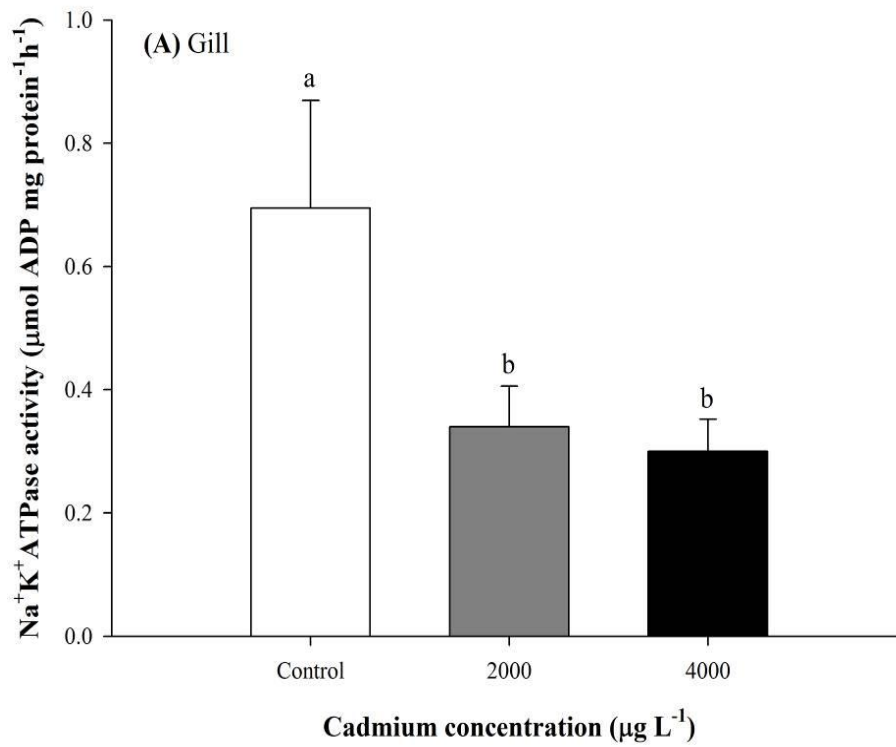
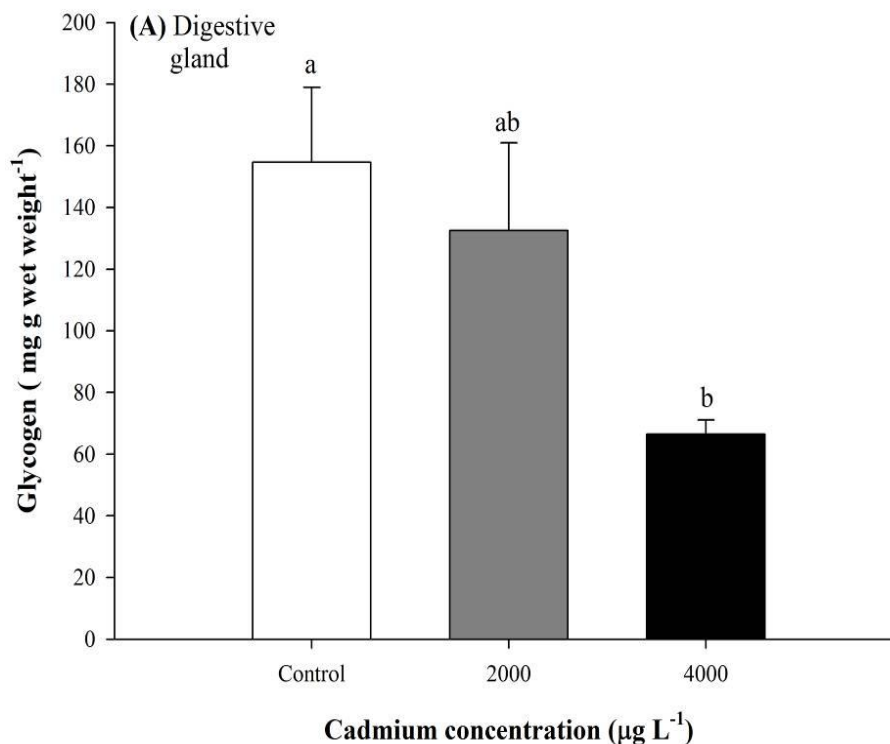


Figure 4.6 Na^+ , K^+ -ATPase activity (mean \pm SEM) in green-lipped mussel (*Perna canaliculus*) gill after acute (A; $n = 6-8$), and during subchronic (B; $n = 5-6$) Cd exposure. All other details are identical to those in the Figure 4.2 legend, except that only the data in panel B were square-root transformed prior to statistical analysis.

4.3.6 Glycogen

Significantly reduced levels of glycogen were found in the digestive gland of the $4000 \mu\text{g L}^{-1}$ Cd-exposed mussels compared to the control at the end of the acute 96 h exposure (Fig. 4.7A). However, no significant changes were observed in the $2000 \mu\text{g L}^{-1}$ Cd-exposed mussels. The 28 d subchronic Cd exposure resulted in significantly lower levels of glycogen in the digestive gland of the $2000 \mu\text{g L}^{-1}$ Cd exposed mussels, an effect that was present only at Day 14 and Day 21 (Fig. 4.7B). This reduction in glycogen was correlated significantly with tissue Cd accumulation ($R = 0.733$; $p = 0.0067$; Table 4.1).



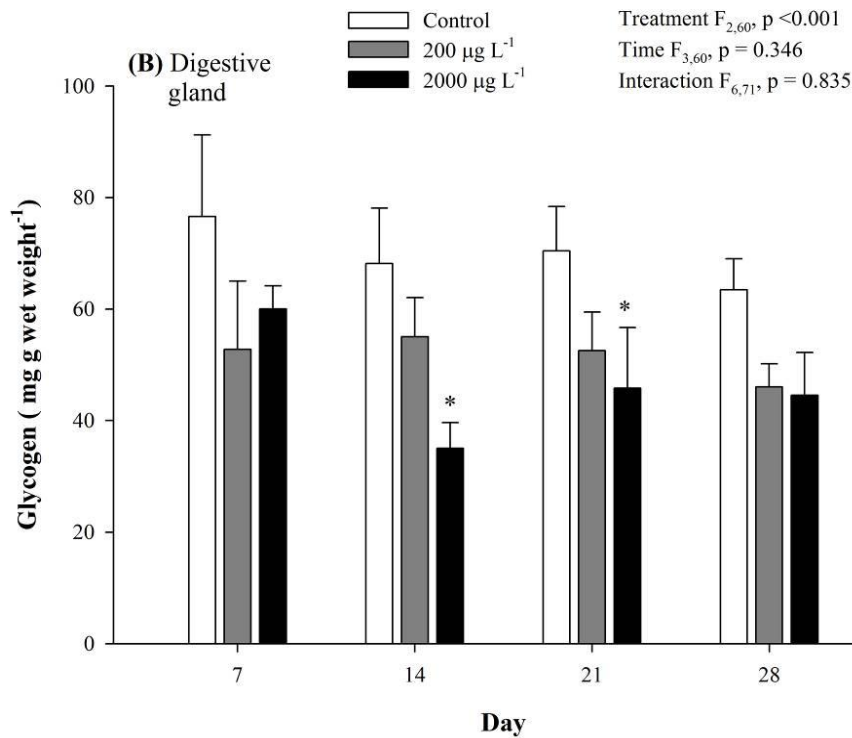


Figure 4.7 Glycogen levels (mean \pm SEM) in green-lipped mussel (*Perna canaliculus*) digestive gland after acute (A; $n = 5$), and during subchronic (B; $n = 6$) Cd exposure. All other details are identical to those in the Figure 4.2 legend, except that only the data in panel B were not transformed prior to statistical analysis.

4.3.7 Haemolymph protein

Acute Cd exposure did not induce any significant changes in haemolymph protein levels of mussels (Fig. 4.8A). In contrast, subchronic exposure produced significantly higher levels of protein in both Cd groups compared to the time-matched control group throughout the 28 d exposure (Fig. 4.8B). The protein levels were also found to differ significantly within exposure treatments, based on the time of the exposure.

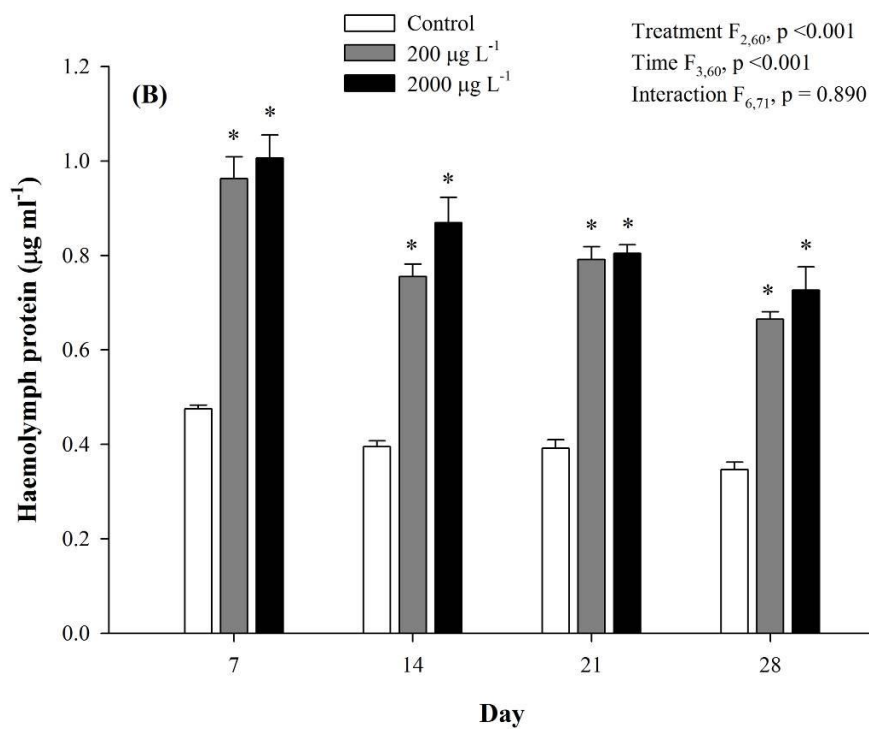
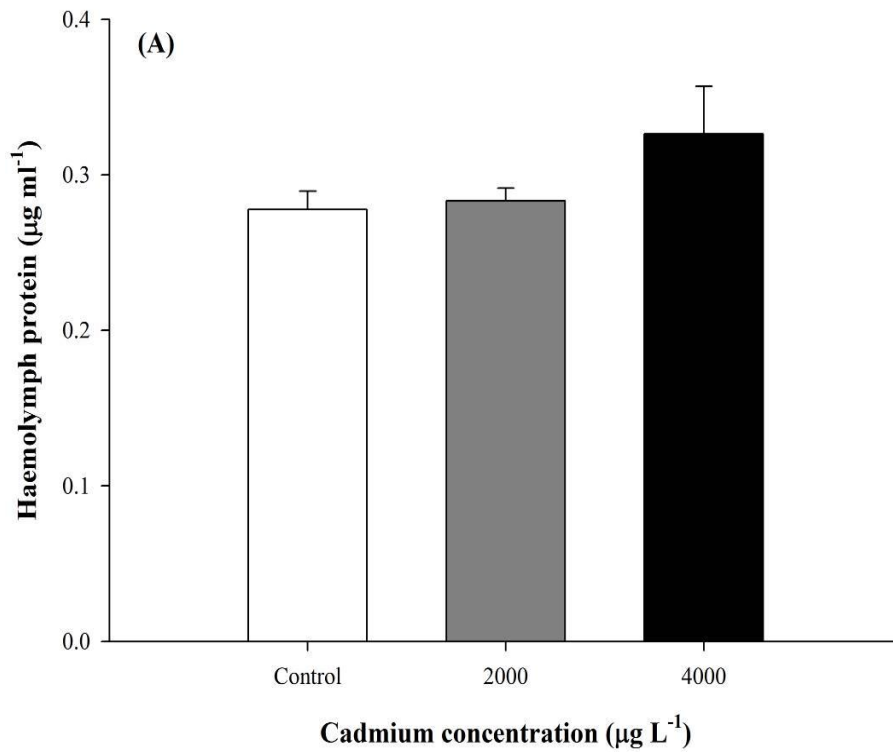


Figure 4.8 Haemolymph protein levels (mean \pm SEM) in green-lipped mussel (*Perna canaliculus*) haemolymph after acute (A; $n = 4-5$), and during subchronic (B; $n = 6$) Cd

exposure. All other details are identical to those in the Figure 4.2 legend, except that only the data in panel B were log-transformed prior to statistical analysis.

Table 4.1 Relationship between Cd accumulation in gill and digestive gland of mussels and biochemical biomarkers after subchronic (28 d, 0-2000 $\mu\text{g Cd L}^{-1}$; $n = 6$) exposure to waterborne Cd.

Biochemical measure	Tissue	Correlation coefficient (R)	Equation of the line of best fit	<i>p</i> value
Metallothionein-like protein	Gill	0.957	$y = 2.932x + 761.240$	<0.0001
	Digestive gland	0.964	$y = 3.254x + 1214.019$	<0.0001
Catalase	Gill	0.907	$y = 0.011x + 46.223$	<0.0001
	Digestive gland	0.708	$y = 0.105x + 209.332$	0.01
Lipid peroxidation	Gill	0.633	$y = -1.428x + 0.099$	0.0274
	Digestive gland	0.408	$y = 1.652x + 0.127$	0.1877
Alkaline phosphatase	Gill	0.453	$y = -0.0005x + 1.664$	0.1391
	Digestive gland	0.778	$y = -0.0022x + 9.468$	0.0029
Na⁺, K⁺-ATPase	Gill	0.464	$y = 0.0014x + 3.085$	0.1286
Glycogen	Digestive gland	0.733	$y = -0.0139x + 63.093$	0.0067

Statistically significant correlations ($p < 0.05$) are indicated in bold. Accumulation data was taken from Chapter 3, in mussels that were exposed concurrently with those used for biochemical analyses in the present chapter.

4.4 Discussion

4.4.1 Cadmium detoxification

Subchronic exposure to Cd led to a gradual increase in MTLP levels in both the gill and digestive gland of mussels during the 28 d exposure. An induction of MT over time has been previously noted in Cd-exposed bivalve species (e.g. Serafim and Bebianno, 2007). This induction is known to play an important role in the sequestration and detoxification of toxic metals such as Cd. Cd binds to the multiple high affinity sulfhydryl sites within MTLP, and can thus be stored in a non-reactive form until excreted (Hogstrand and Haux, 1991).

Consequently, the ability of MTLP to sequester Cd is considered to confer tolerance to mussels (Rainbow, 2002). The importance of this response was demonstrated by the strong and significant correlation between the levels of MTLP in the tissues and the level of tissue Cd accumulation (Table 4.1).

The induction of MTLP was only observed in the subchronic exposure, and not following acute exposure to Cd. Ma et al. (2008) observed similar results in the freshwater crab, *Sinopotamon henanense*, where acute exposure to Cd was not accompanied by an increase in MT production. In ectotherms such as mussels MT induction can be a relatively slow process. For example, G eret and Cosson (2002) found that MT protein levels in the blue mussel, *Mytilus edulis*, did not increase after a 4 d exposure to 200 $\mu\text{g L}^{-1}$ Cd, however MT levels increased significantly after 21 d of exposure. Similarly, MT mRNA transcript levels in digestive gland of zebra mussels were not elevated until Day 7 of Cd exposure (Navarro et al., 2011). This suggests that the lack of a measurable MTLP induction response in green-lipped mussels after acute exposure may have been a consequence of the short duration of the exposure.

An alternative explanation for the lack of acute response is that Cd inhibited the induction of MTLP. A toxic effect of Cd on MT induction has been previously observed in amphipods exposed to a similar dose to that used in the present study (2000 $\mu\text{g L}^{-1}$; Martinez et al., 1996), and has also been observed in the haemocytes of the oyster *Crassostrea virginica* (Roesijadi et al., 1997). It is therefore possible that the high doses (~25-50% of *P. canaliculus* 96 h LC₅₀; Chapter 3) used in the present study, caused a general toxic effect that inhibited the ability of the mussels to generate a MTLP response.

4.4.2 Oxidative stress

MT acts as a first line of defence, but if the levels of MT are insufficient to sequester cellular Cd, then toxic effects may develop. A commonly noted effect of Cd toxicity is oxidative stress (Stohs et al., 2001). In tissues oxidative stress can be measured using a number of different endpoints, most commonly markers of oxidative damage (e.g. lipid peroxidation; *Section 1.6.3.2*), or changes in oxidative defence mechanisms (e.g. enzymes such as catalase that convert H₂O₂ into water and oxygen, thereby protecting the cells against oxidative stress; *Section 1.3.5.2*). In the present study, as observed for MTLP levels, there were no significant changes in either catalase or lipid peroxidation following acute exposure. In the case of catalase this could reflect the time taken for induction of this protective mechanism. For example, Viarengo et al. (1999) observed a minor increase in catalase activity only after 7 d of exposure to 200 µg Cd L⁻¹. Similar results were also observed in *Perna perna* exposed to 200 µg Cd L⁻¹ (de Almeida et al., 2004) where catalase levels increased only after 120 h of exposure.

An increase in lipid peroxidation is the standard reported impact in mussels exposed to Cd (e.g. Pytharopoulou et al., 2011). Although there are other reports of high acute waterborne Cd exposures causing no increase in tissue lipid peroxidation (e.g. Japanese flounder; Cao et al., 2012), the hypothesis is that the lack of effect may be related to an unusually potent anti-oxidant defence in *P. canaliculus*. Lipid peroxidation will only occur when the pro-oxidant effects of Cd overwhelm the anti-oxidant defences. Lipid extracts of the green-lipped mussel have well-described anti-inflammatory effects, attributed to the presence of furan fatty acids with a very potent ROS-scavenging ability (Wakimoto et al., 2011). Consequently it may be that the lack of lipid peroxidation in this species, at least over short-term exposures, relates to this rare and significant anti-oxidant defence mechanism. It is also important to note that other anti-oxidant defence mechanisms such as enzyme activities of superoxide dismutase,

glutathione peroxidase, and glutathione transferase were not measured in this study. Thus the understanding of acute Cd-induced oxidative stress responses in *P. canaliculus* remains incomplete.

Markers of oxidative stress were, however, significantly impacted over the longer 28 d exposure to Cd. The results showed a significant increase in catalase activity in both the gill and digestive gland of mussels subchronically exposed to Cd, an effect that correlated significantly with tissue Cd accumulation. This suggests an induction of catalase activity in response to Cd-mediated oxidative stress, a commonly recorded response in aquatic biota (e.g. Bebianno et al., 2004).

Cd exposure usually results in an increase in oxidative damage in exposed bivalves (e.g. Pytharopoulou et al., 2011). Contrary to expectation, lipid peroxidation levels were relatively unchanged in Cd-exposed gills (except for a decrease at Day 21 in the 2000 $\mu\text{g Cd L}^{-1}$ group). Although unexpected, similar findings have been observed in the digestive gland of the unionid bivalve, *Pyganodon grandis* (Bonneris et al., 2005) and in gills of the oyster *Crassostrea gigas* (Géret et al., 2002) exposed to Cd. These authors attributed the lack of lipid peroxidation to intensified anti-oxidant enzyme activity and MTLP production, mechanisms that would not only limit Cd-induced oxidative damage, but could also reduce constitutive levels of peroxidation. This explanation is consistent with the subchronic patterns of branchial MTLP in the present study.

Increases in MTLP and catalase were also found in the digestive gland, however the pattern of lipid peroxidation in this tissue was quite distinct from that in the gill. An increase at both tested subchronic concentrations was recorded, although this was an effect that diminished over the course of the exposure. This reduction in peroxidation levels with time is an effect noted previously in bivalve digestive gland, and has been attributed to upregulation of anti-

oxidant defence mechanisms (de Almeida et al., 2004). As these changes over time in the current study did not appear to correlate with MTLP and catalase in this tissue, it suggests that there was a delay in the induction of alternative mechanisms either preventing or repairing oxidative damage in the digestive gland.

Tissue differences in lipid peroxidation responses to waterborne metal exposure have been recorded previously. For example, Bonneris et al. (2005) recorded an increase in gill lipid peroxidation, but a decrease in digestive gland lipid peroxidation associated with cellular Cd accumulation in a freshwater mussel. It is likely that differences in responses relate to tissue-specific differences in anti-oxidant defence. As mentioned above, in the current study only a single measure of anti-oxidant defence was measured, catalase activity. While catalase activity in mussel gill and digestive gland is relatively insensitive to seasonal changes (Power and Sheehan, 1996), other measures may fluctuate with season. For example, glutathione and the activity of glutathione-S-transferases, the enzymes that conjugate glutathione to electrophilic substrates, can vary significantly with seasons in mussels (Power and Sheehan, 1996). Most importantly, glutathione-S-transferase activity is consistently low and constant in digestive gland relative to activity in the gill, which is higher and fluctuates more than two-fold over an annual cycle (Power and Sheehan, 1996). The lack of a complete understanding of anti-oxidant defences in the green-lipped mussel currently limits interpretation as to why the responses between gill and digestive gland differ to Cd exposure.

It is, however, pertinent to note that lipid peroxidation levels in the gill, but not in the digestive gland, correlated significantly with tissue Cd accumulation (Table 4.1). This suggests that a factor other than the Cd accumulation itself was responsible for the lipid peroxidation pattern in digestive gland. Reduced food intake and changes in energy metabolism were noted in green-lipped mussels exposed to Cd in *Chapter 3*, and these are

parameters known to influence lipid peroxidation in mussels (Viarengo et al., 1991). It is therefore possible that metabolic changes in the digestive gland related to the presence of Cd, generated the lipid peroxidation effects observed in this study.

4.4.3 Effects of cadmium on cellular homeostasis enzymes

Alkaline phosphatase is involved in transphosphorylation of monoesters (Coleman and Gettings, 1983). It is also known to be highly sensitive to the presence of metals (Regoli and Principato, 1995). Subchronic exposure to Cd resulted in a consistent and significant decline in alkaline phosphatase activity in the gill of mussels exposed to both Cd concentrations, while an inhibitory effect of alkaline phosphatase activity in the digestive gland was observed only in the 2000 $\mu\text{g L}^{-1}$ Cd-exposed mussels. Inhibitory effects of Cd on alkaline phosphatase activity have previously been reported in the pearl oyster (*Pinctada fucata*; Xiao et al., 2002) and clam (*Scrobicularia plana*; Mazzora et al., 2002). These effects can be attributed to the high affinity of Cd for sulfhydryl groups and the subsequent displacement of zinc from the alkaline phosphatase cofactor-binding sites (Grintzalis et al., 2012). Alkaline phosphatase dysfunction could impair processes such as membrane transport, shell formation, and immune function (El-Demerdash and Elagamy, 1999; Chakraborty et al., 2010), and thus could be a key mechanism for organism-level effects of Cd in mussels.

NKA is a key transmembrane enzyme involved in the maintenance of cellular ion homeostasis (MacGregor and Walker, 1993). Acute Cd exposure in green-lipped mussels resulted in a significant decrease in NKA activity. Cd exposure in the freshwater mussel, *Anodonta cygnea*, was found to have an inhibitory effect on NKA as Cd substituted itself for the cofactor magnesium (Pivovarova and Lagerspetz, 1996). This could explain the decrease in NKA activity seen in the acute Cd-exposed mussels (Fig. 4.6A). Alternatively, a general impairment of ion transport (be it an action on other transporters and/or on the integrity of the

gill cell membrane itself) might result in a compensatory increase in NKA activity (Issartel et al., 2010). Such an effect was noted in response to subchronic Cd exposure. A similar increase in NKA activity has also been observed in the freshwater gammarid, *Gammarus pulex* exposed to Cd (Felten et al., 2008).

4.4.4 Effects of cadmium on energy substrates

Subchronic Cd exposure in green-lipped mussels resulted in a significant transient reduction in digestive gland glycogen levels, an effect that was correlated with Cd accumulation in this tissue (Table 4.1). A similar effect on digestive gland glycogen was observed after acute exposure to 4000 $\mu\text{g L}^{-1}$ Cd. Bivalve molluscs store energy as carbohydrates in the form of glycogen in their tissues, and this can be utilised as an energy resource during nutritional stress. In *Chapter 3* it was shown that Cd induced a significant reduction in the feeding rate of mussels, which may indicate that a lack of adequate energy could have forced the Cd-exposed mussels to rely on their glycogen stores to meet the costs associated with Cd detoxification mechanisms (e.g. MT production). A similar decrease in feeding and glycogen levels was also reported in the estuarine bivalve, *Macoma balthica* exposed to sublethal Cd concentrations (Duquesne et al., 2004). This depletion of stored energy may come at a cost, however, as this will reduce available energy for functions such as growth and reproduction (Ngo et al., 2011). For this reason tissue glycogen levels are considered to be an indicator of organism fitness (e.g. Ansaldo et al., 2006).

Proteins are a potential energy resource during stress in mussels (Hawkins and Bayne, 1991). Subchronic exposure to Cd resulted in an increase in haemolymph protein levels, suggesting that the mussels had started to utilise proteins as an energy source. Spann et al. (2011) reported that exposure of the Asian clam, *Corbicula fluminea*, to low doses of sediment spiked with Cd for 1 week led to an increase in free amino acids and energy metabolism in

the tissues of clams. Similar increases in free amino acids in response to other environmental pollutants have been reported in the mussel, *Mytilus galloprovincialis* (Jones et al., 2008). Confirming the idea that protein is used as a fuel, low O:N ratios and high ammonia production rates, characteristic of protein catabolism (Bayne et al., 1985), were evident in *Chapter 3*.

The subchronic Cd exposure is likely to be more representative of the natural conditions experienced by the animal, both in terms of dose and duration. A number of the endpoints examined during this exposure were strongly correlated with tissue accumulation, which in turn was shown to correspond to Cd levels in the exposure medium in *Chapter 3*. Among these endpoints, MTLP in particular, but also catalase activity, were strongly positively correlated with tissue accumulation in both the gill and the digestive gland. In combination with measurement of clearance rate (*Chapter 3*), these biochemical endpoints may have value as indicators of metal impairment in field-exposed mussels. These findings support the concept of integrating physiological and biochemical biomarker responses, along with tissue bioaccumulation measures, in *P. canaliculus* as a potential “bioindicator” species for metal contamination in NZ coastal waters. However it is clear that additional work is required to better understand the responses to lower environmental metal levels, especially over acute time-frames.

4.5 Conclusions

In the present chapter significant differences in the response of green-lipped mussels to Cd exposure were noted as a function of exposure duration. Most commonly the literature reports an increase in MT levels, accompanied by increased markers of oxidative stress (e.g. antioxidant enzyme activity and lipid peroxidation) following Cd exposure (e.g. Bebianno et al., 2004; Serafim and Bebianno, 2007; Pytharopoulou et al., 2011). To a large extent this

was observed over the subchronic exposure period. However, contrary to expectation, almost no change in measures of the metal detoxification pathway and oxidative stress were observed after acute exposure. This may be indicative of a number of factors including enhanced anti-oxidant defence mechanisms, speed of induction of these mechanisms, and/or Cd inhibition of cellular pathways of defence (*see Sections 4.4.1 and 4.4.2*). Consequently, measures such as gill NKA activity and digestive gland glycogen, which showed significant dose-dependent decreases in response to acute Cd exposure, may be more appropriate indicators of organism stress in acute exposure scenarios.

Importantly the present study also identifies key mechanisms of subchronic Cd toxicity. Catalase, a key marker of oxidative stress, increased in both studied tissues, but lipid peroxidation, a marker of oxidative damage, increased only in the digestive gland. This latter effect was not correlated directly to Cd accumulation in this tissue. This suggests that while Cd induces oxidative stress it is not a direct contributor towards toxicity. Instead, changes in metabolism (decrease in glycogen, accompanied by increased haemolymph protein) suggest that the animal is under energetic stress. This is consistent with the change in fuel use (increase in protein metabolism) reported in *Chapter 3*. These effects may stem from both an increase in energy demand associated with detoxification processes (e.g. induction of MTLP and catalase), and a decrease in energy input, via clearance rate inhibition likely due to behavioural avoidance in the form of shell valve closure (*Chapter 3*).

5 Waterborne cadmium impacts immunocytotoxic and cytogenotoxic endpoints in green-lipped mussel, *Perna canaliculus*[†]

[†]**Chandurvelan, R.**, Marsden, I.D., Gaw, S., Glover, C.N., Waterborne cadmium impacts immunocytotoxic and cytogenotoxic endpoints in green-lipped mussel, *Perna canaliculus*.
Submitted to Aquatic Toxicology.

5.1 Introduction

The gill and the haemolymph of mussels are excellent candidates for assessing toxic impacts of metal exposure. The mussel gill is in direct contact with the environment and is thus susceptible to any toxic metals therein. Furthermore, the gill is the pathway by which most metal enters the mussel, be it by direct uptake from water, or via the feeding mechanism (Naimo, 1995). Once absorbed, metals enter the haemolymph. The haemolymph of molluscs is comprised of haemocytes that are responsible for humoral and cellular immune defence in bivalves (Galloway and Goven, 2006). Haemocytes also play a key role in transferring metals to tissues for detoxification and/or accumulation, and by virtue of this exposure to the absorbed toxicant, they are susceptible to toxic impacts (see *Section 1.6.4*).

Mussels are also considered to be sensitive to the genotoxic effects of metals (Bolognesi et al., 1996). The micronucleus test and comet assay are reliable and cost-effective assays that detect nuclear abnormalities and DNA damage in cells (see *Section 1.6.5*). One metal known to cause the type of genetic damage detected by the micronucleus and comet assays is Cd (e.g. Bertin and Averbeck, 2006). At the cellular level Cd is known to affect cytoskeletal structure (Chora et al., 2009), cell adhesion and shape (Brousseau et al., 2000), haemocyte viability (Olabarrieta et al., 2001) and to cause changes in haemocyte populations (Cheng, 1988). Cd is also known to cause oxidative stress (e.g. lipid peroxidation; *Chapter 4*) and interfere with DNA repair processes (Pruski and Dixon, 2002). Cd exposure can lead to single strand DNA breaks (Hartwig, 1994), and the kinds of abnormalities detected using the micronucleus test and comet assay.

The objectives of the present chapter were to investigate the immunocytotoxic and cytogenotoxic effects of acute and subchronic waterborne Cd on the haemocytes and gill cells of the green-lipped mussel in order to identify additional biological impacts of Cd exposure

that may be of relevance to the use of this species as a bioindicator, and to further delineate mechanisms of waterborne Cd toxicity in this organism.

5.2 Material and methods

5.2.1 Immunocytotoxic and cytogenotoxic biomarker measurements

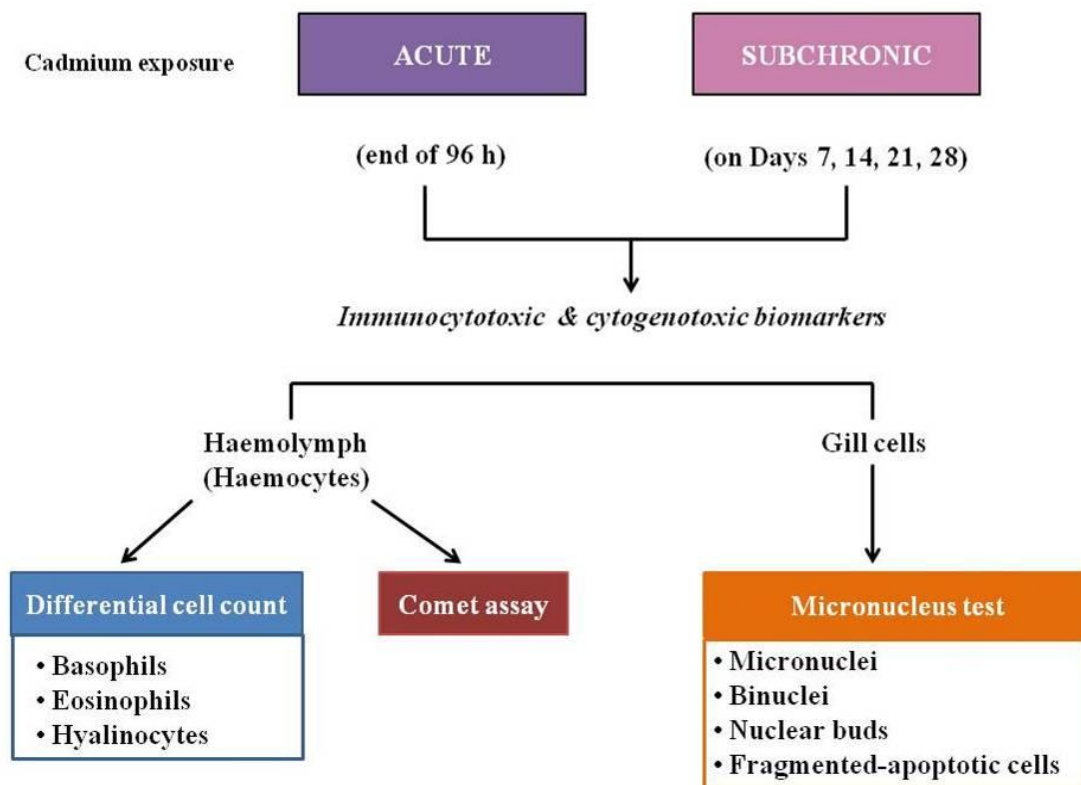


Figure 5.1 Flow chart illustration of cadmium exposure and immunocytotoxic and cytogenotoxic biomarker assays used in this study.

The Cd exposure treatments and experimental set-up have been provided in *Section 2.3*. Differential cell count, the micronucleus test and the comet assay were conducted based on the protocol detailed in *Sections 2.5 and 2.6*. The immunocytotoxic and cytogenotoxic biomarkers used in this study are presented in Fig. 5.1.

Haemocytes were classified based on differential staining characteristics (Fig. 5.2). Eosinophils were identified as large, pink-stained cells with granules in their cytoplasm while basophils had a granular cytoplasm and stained blue. Hyalinocytes also stained blue, but had very few, or no, granules in the cytoplasm.

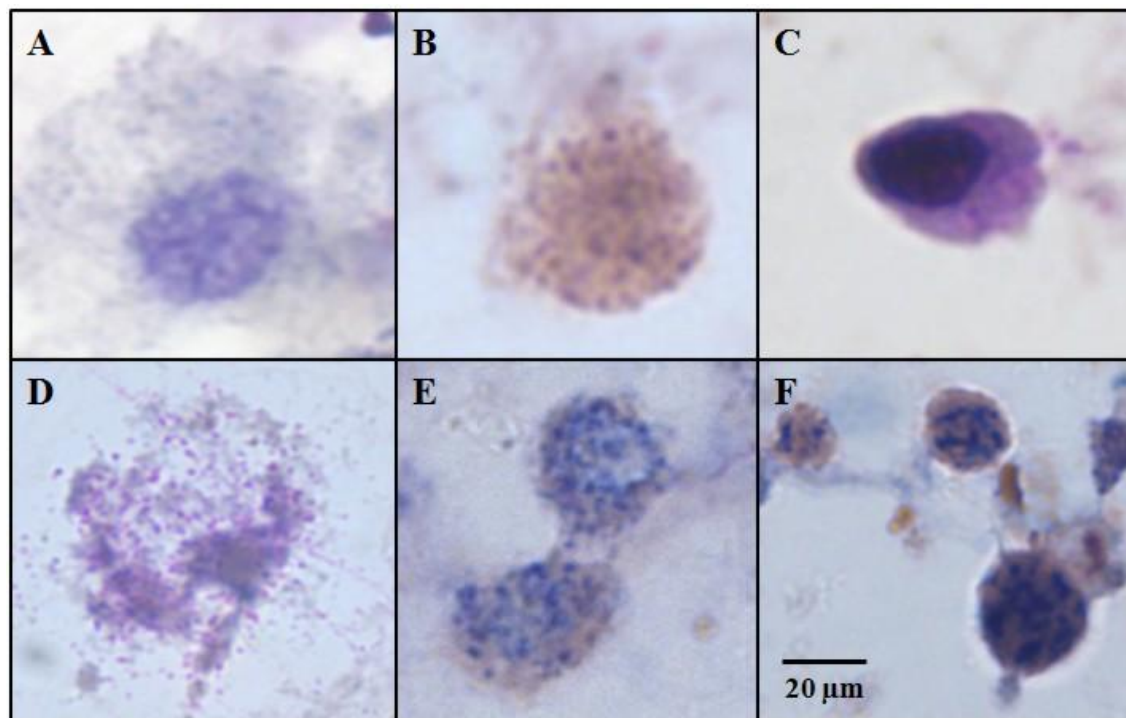


Figure 5.2 *Haemocytes observed in the haemolymph of green-lipped mussels. (A) Basophil (B) eosinophil (C) hyalinocyte (D) eosinophilic basophil (E) basophilic eosinophil (F) brown-orange cell.*

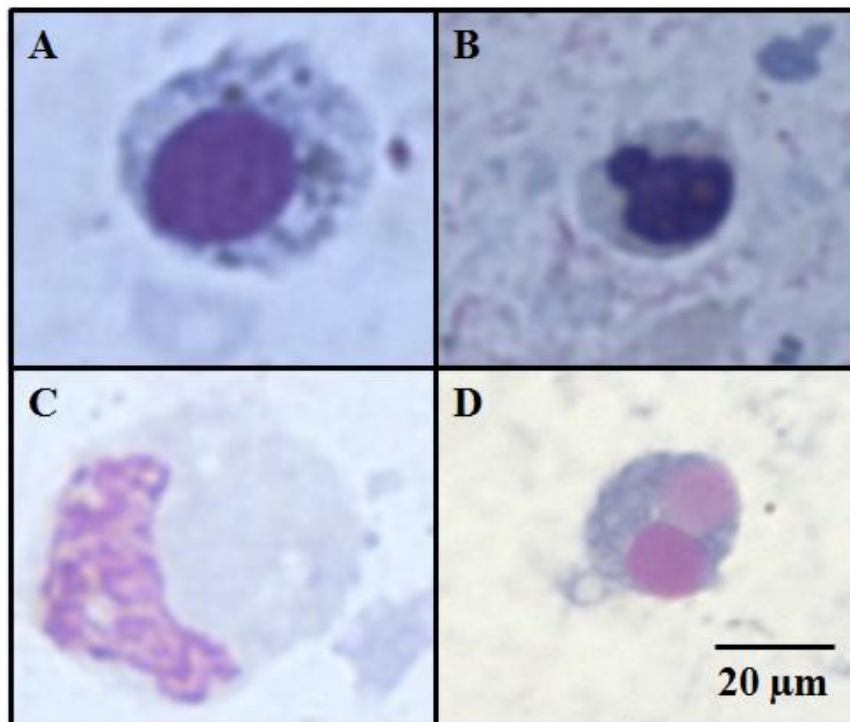


Figure 5.3 Nuclear abnormalities observed in the gill cells of green-lipped mussels. (A) Micronucleus (B) nuclear bud (C) fragmented-apoptotic cell (D) binucleus.

Micronuclei were identified as round or ovoid structures detached from the nucleus with a diameter 1/3 to 1/20 of the main nucleus (Fig. 5.3A). Nuclear buds were identified as an extrusion of nuclear material from the nucleus similar to a micronucleus but connected to the main nucleus by a nucleoplasmic bridge (Fig. 5.3B). Cells with an intact cell membrane containing condensed or fragmented nuclear material with slightly higher staining intensity than normal cells were identified as fragmented-apoptotic cells (Fig. 5.3C). Cells comprising two nuclei with intact nuclear membranes touching each other, which were of similar size and staining characteristics, were identified as binucleated cells (Fig. 5.3D).

5.2.2 Statistical analysis

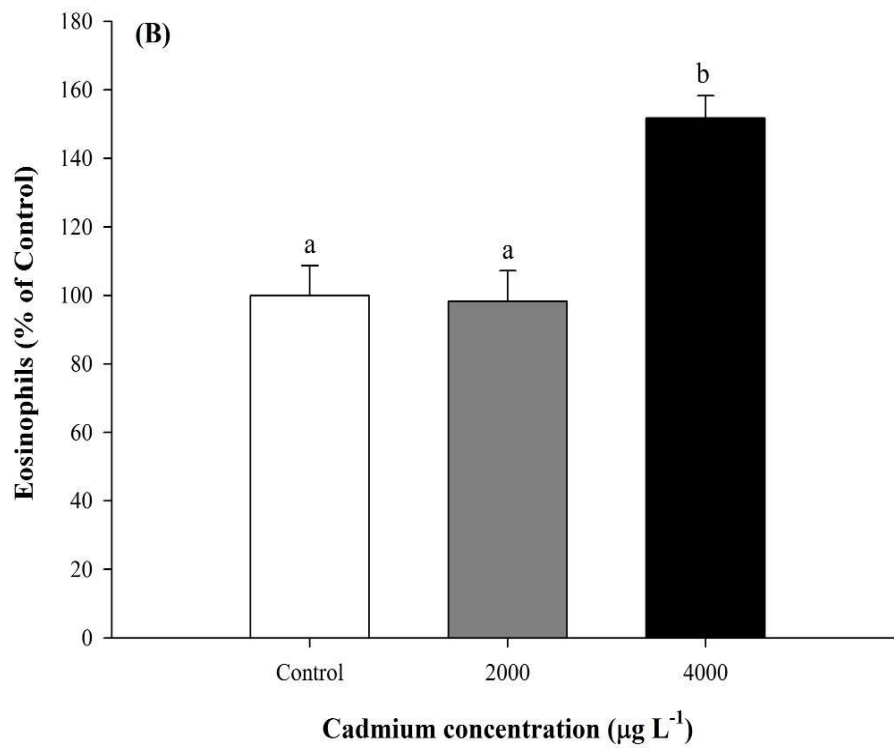
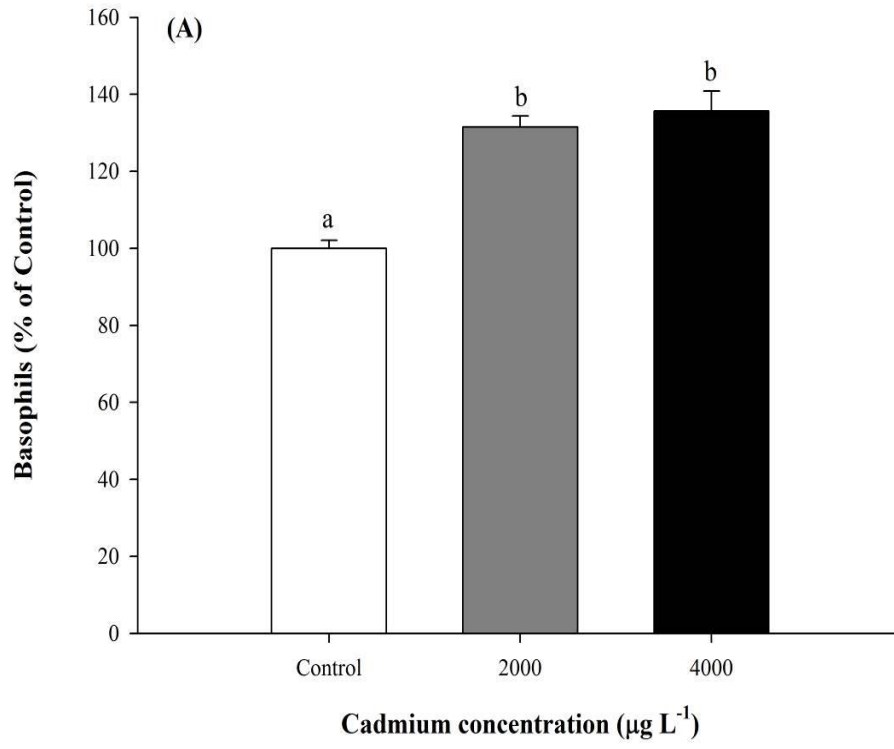
Data were tested statistically using methods described in *Section 2.9*. Additionally apart from parametric analysis, acute exposure data were also analysed by non-parametric method using

Kruskal-Wallis ANOVA followed by Tukey test (non-parametric). The mean value of each nuclear aberration measured using the micronucleus test was correlated with the corresponding mean gill Cd accumulation level on Days 7, 14, 21 and 28 during the subchronic exposure to Cd as described in *Section 2.9*. Data are presented as mean \pm SEM with statistical significance set at $p < 0.05$.

5.3 Results

5.3.1 Differential cell count

A total of six distinct haemocyte types were identified in green-lipped mussels. In accordance with the criteria outlined in *Section 2.3*, basophils were identified as large, granular, blue-stained cells (Fig. 5.2A), eosinophils were of similar morphology but stained pink (Fig. 5.2B), and hyalinocytes were blue-stained, with minor or absent granulation (Fig. 5.2C). Three other haemocyte types were identified that did not fall into these criteria. A basophilic eosinophil was characterised as a haemocyte with a blue-stained cytoplasm and pink-stained granules (Fig. 5.2D) while eosinophilic basophils were identified as cells with a pink-stained cytoplasm and blue-stained granules (Fig. 5.2E). The other cell type identified was an orange-brown cell, which displayed a characteristic orange to brown-coloured cytoplasm and a blue nucleus (Fig. 5.2F). These latter three cell types were not taken into account as part of the differential cell count assessment, as they occurred only rarely in scored slides.



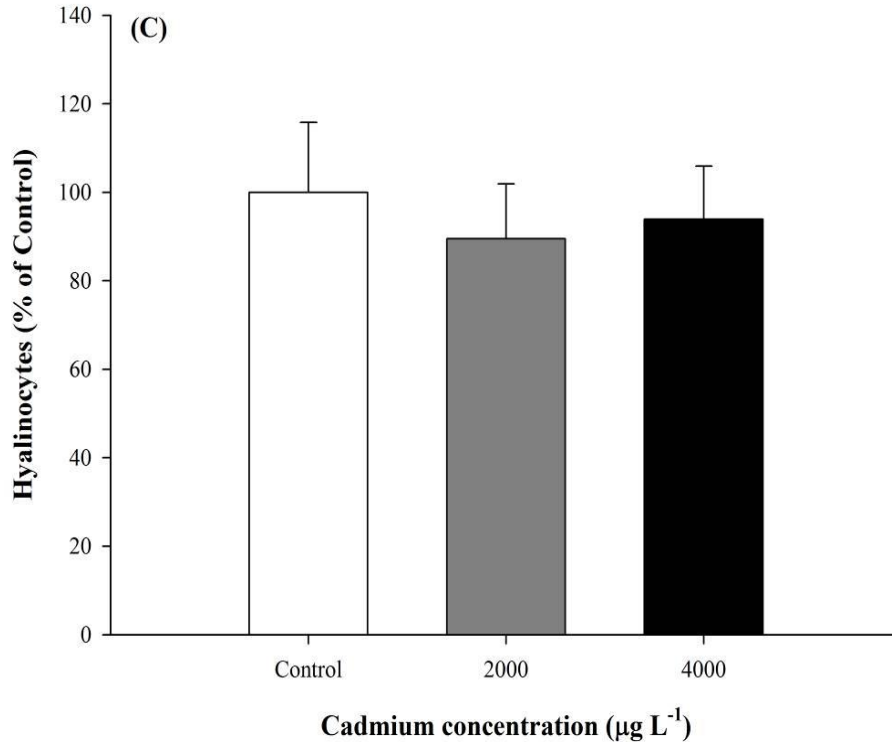
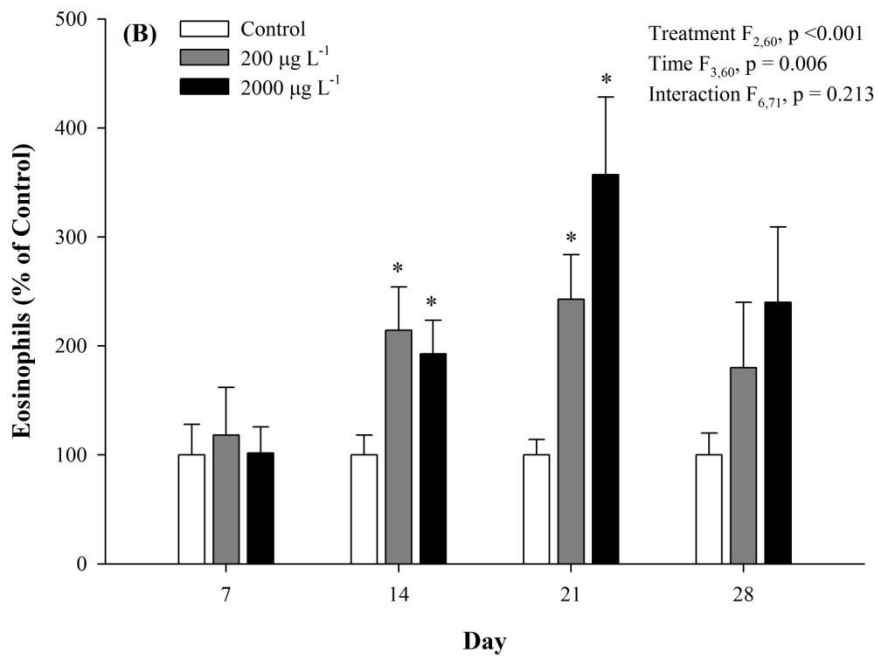
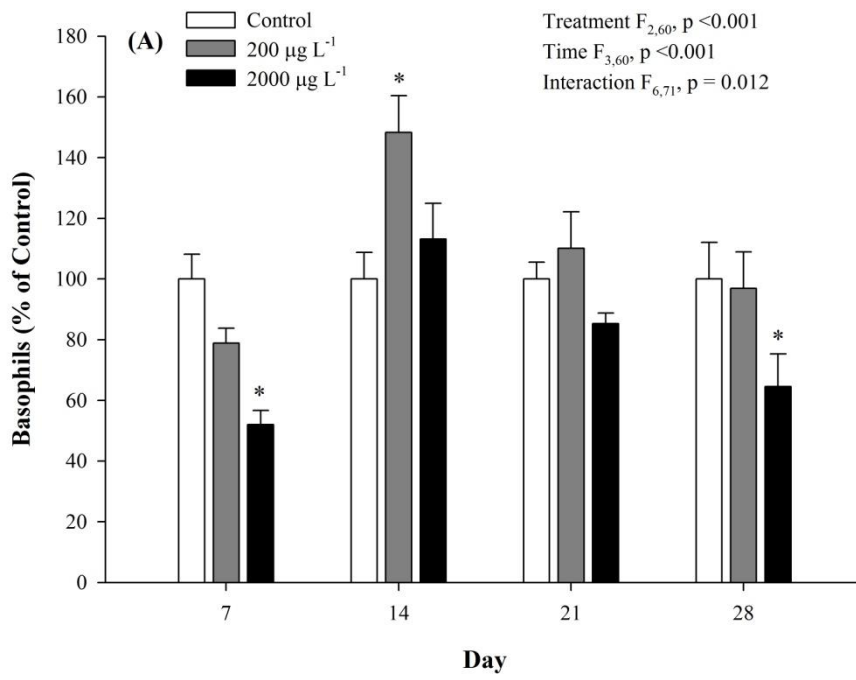


Figure 5.4 *Basophil (A), eosinophil (B) and hyalinocyte (C) count in haemolymph of green-lipped mussels (*Perna canaliculus*) following acute (96 h) exposure to Cd (0 (control), 2000 and 4000 $\mu\text{g L}^{-1}$; $n = 8$). Data are presented as a percentage of the control (mean \pm SEM). Plotted points sharing letters are not significantly different ($p < 0.05$) as determined using one-way ANOVA followed by post hoc LSD test (all panels). Data in panel A were log-transformed prior to statistical analysis.*

Compared to controls, a significantly higher percentage of basophils were recorded in mussels acutely-exposed to both 2000 and 4000 $\mu\text{g L}^{-1}$ Cd (Fig. 5.4A), while a significantly higher percentage of eosinophils were also observed in the haemolymph of 4000 $\mu\text{g L}^{-1}$ Cd-exposed mussels (Fig. 5.4B). Conversely, the relative numbers of hyalinocytes did not show any significant changes following the 96 h acute Cd exposure (Fig. 5.4C).



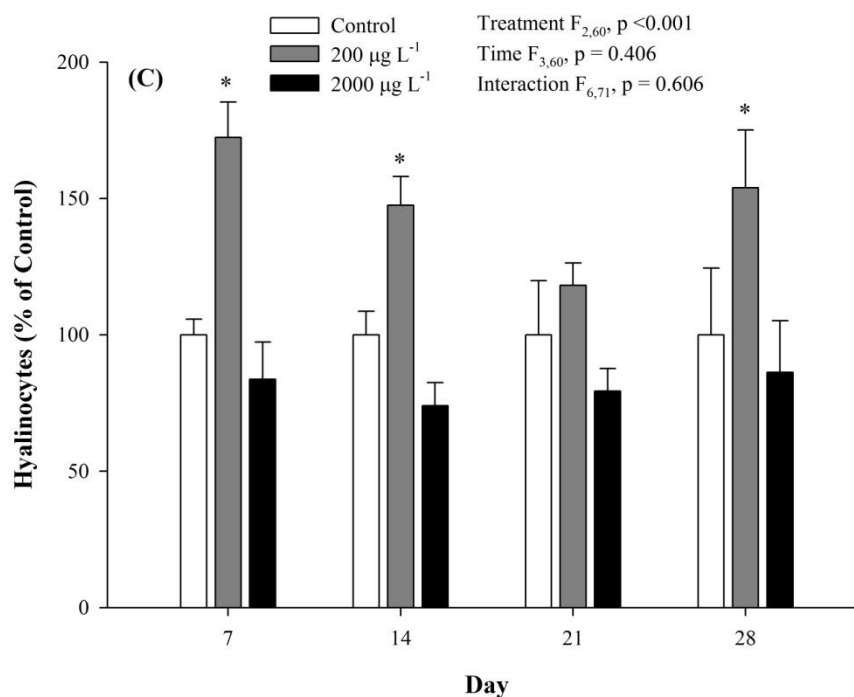


Figure 5.5 Basophil (A), eosinophil (B), and hyalinocyte (C) count in haemolymph of green-lipped mussels (*Perna canaliculus*) during subchronic (28 d) exposure to Cd (0 (control), 200 and 2000 $\mu\text{g L}^{-1}$; $n = 6$). Data are presented as percentage of control (mean \pm SEM). Data were analysed by two-way ANOVA with exposure concentration (treatment) and time as factors (all panels). The results of the analysis are reported on the panel. Asterisks indicate a significant difference between the exposure concentration and the control within a day, as determined by post hoc LSD analysis ($\alpha = 0.05$). Data in panel B were log-transformed prior to statistical analysis.

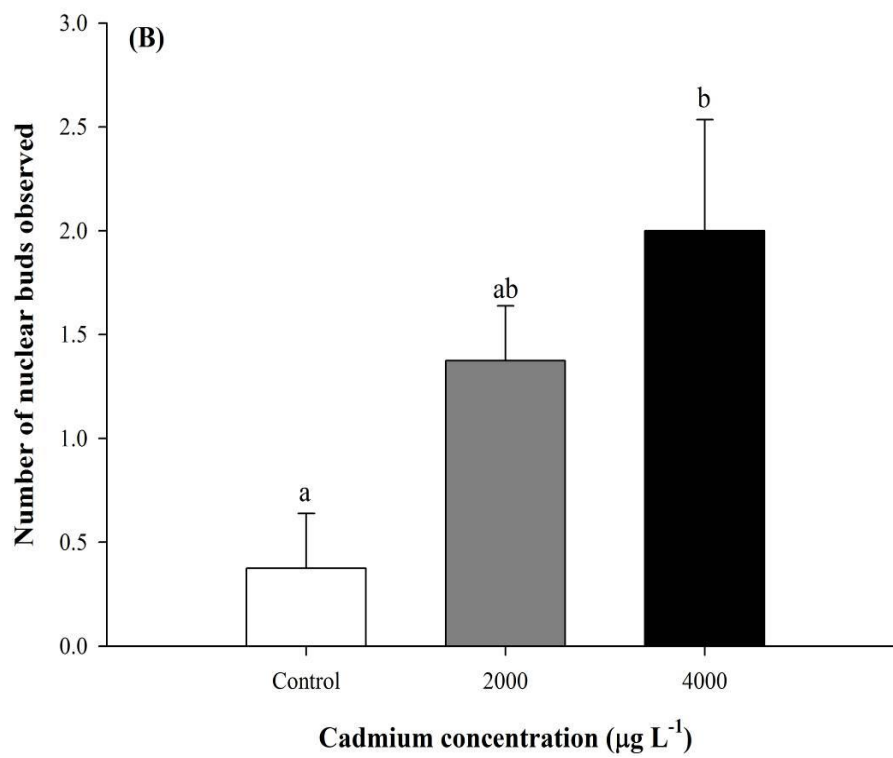
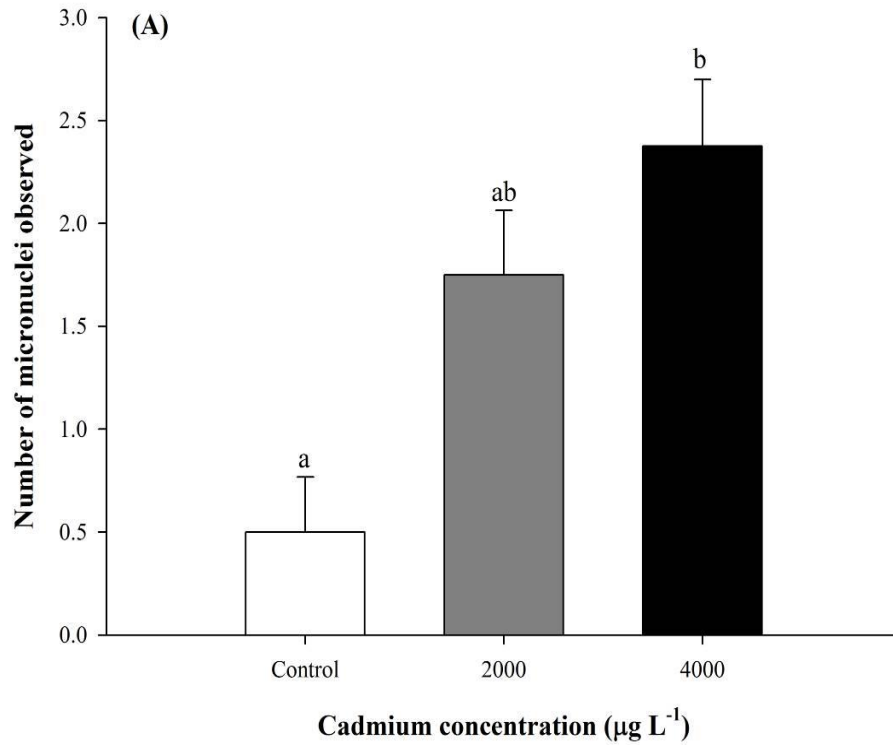
Subchronic exposure to Cd resulted in a significant effect of concentration (treatment), time, and interaction between these factors, on the relative percentage of basophils in the Cd-exposed mussels (Fig. 5.5A). The 2000 $\mu\text{g L}^{-1}$ Cd-exposed mussels showed a significant decline in the percentage of basophils on Days 7 and 28 compared to the time-matched control mussels. In contrast the 200 $\mu\text{g L}^{-1}$ Cd-exposed mussels exhibited a significant

increase in the relative numbers of basophils, an effect that was, however, only present on Day 14 (Fig. 5.5A).

A significant increase in eosinophils in Cd-exposed mussels relative to the unexposed control was observed on Days 14 and 21 during subchronic exposure to Cd. This increase occurred in both the 200 and 2000 $\mu\text{g Cd L}^{-1}$ concentrations (Fig. 5.5B). Subchronic exposure to Cd also led to a significant increase in the relative percentage of hyalinocytes in the 200 $\mu\text{g L}^{-1}$ Cd-exposed mussels compared to the control at all time-points investigated (with the exception of Day 21), but there was no effect of exposure at the higher level (Fig. 5.5C).

5.3.2 *Micronucleus test*

Compared to the control mussels, a significant increase in numbers of micronuclei (Fig. 5.6A) and nuclear buds (Fig. 5.6B) was observed in the gill cells of 4000 $\mu\text{g L}^{-1}$ Cd-exposed mussels at the end of 96 h. Acute Cd exposure also resulted in a significant increase in the formation of fragmented-apoptotic cells in gill of mussels exposed to 2000 and 4000 $\mu\text{g L}^{-1}$ Cd (Fig. 5.6C). Binucleated cells exhibited a trend whereby the number of these cells observed relative to the control increased with Cd exposure concentration over 96 h. However this trend narrowly eluded statistical significance ($p = 0.050$; Fig. 5.6D).



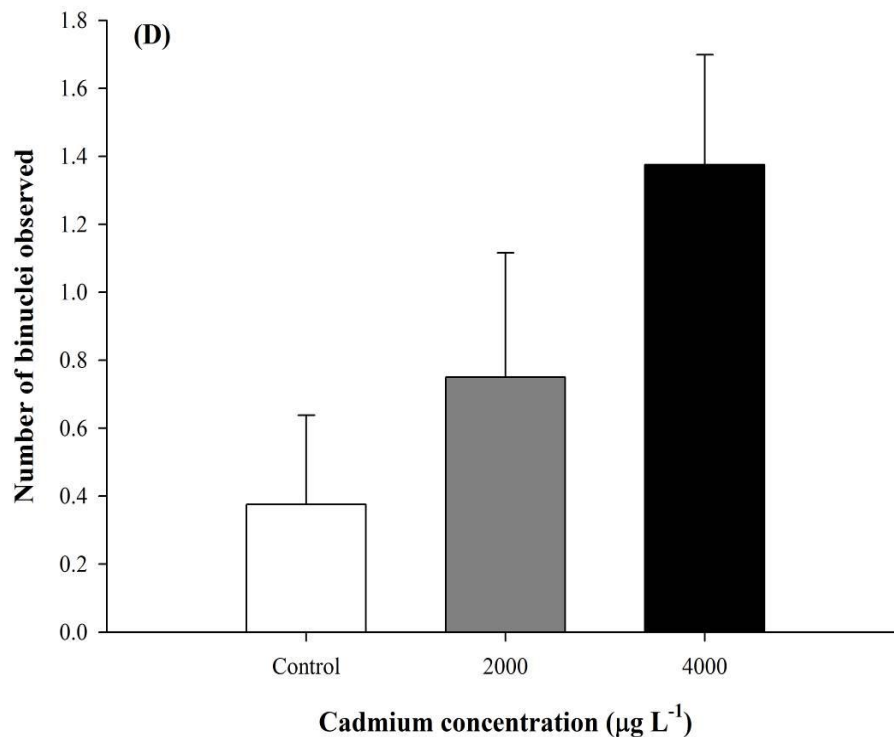
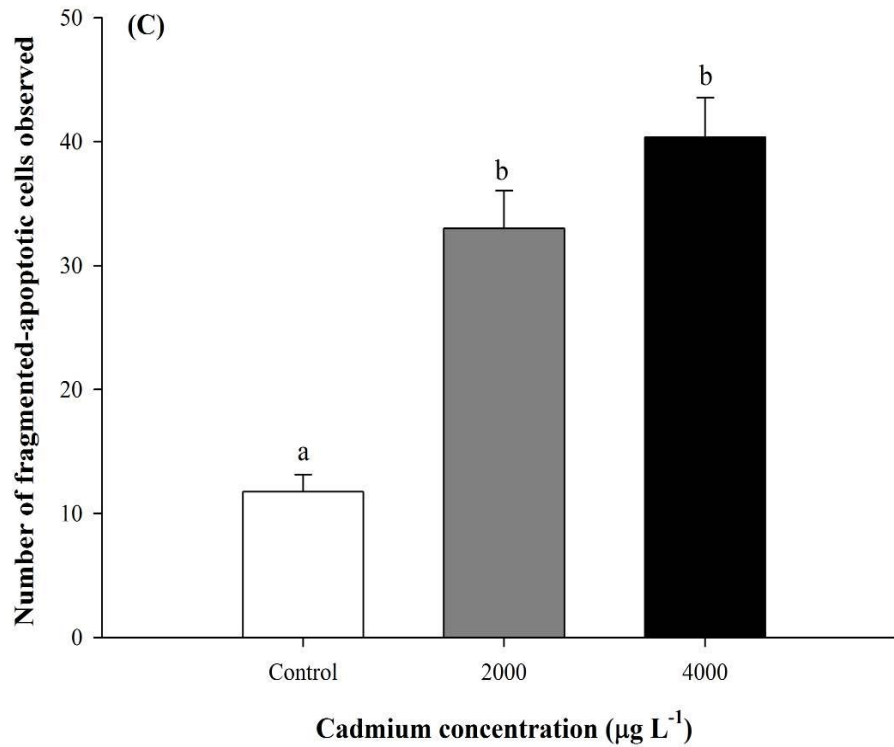
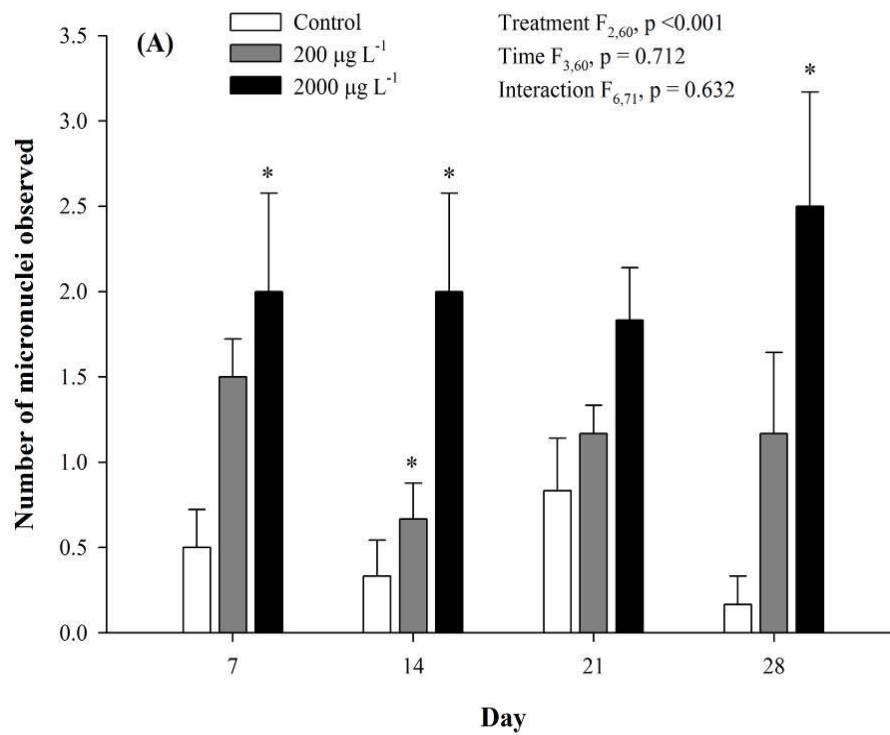
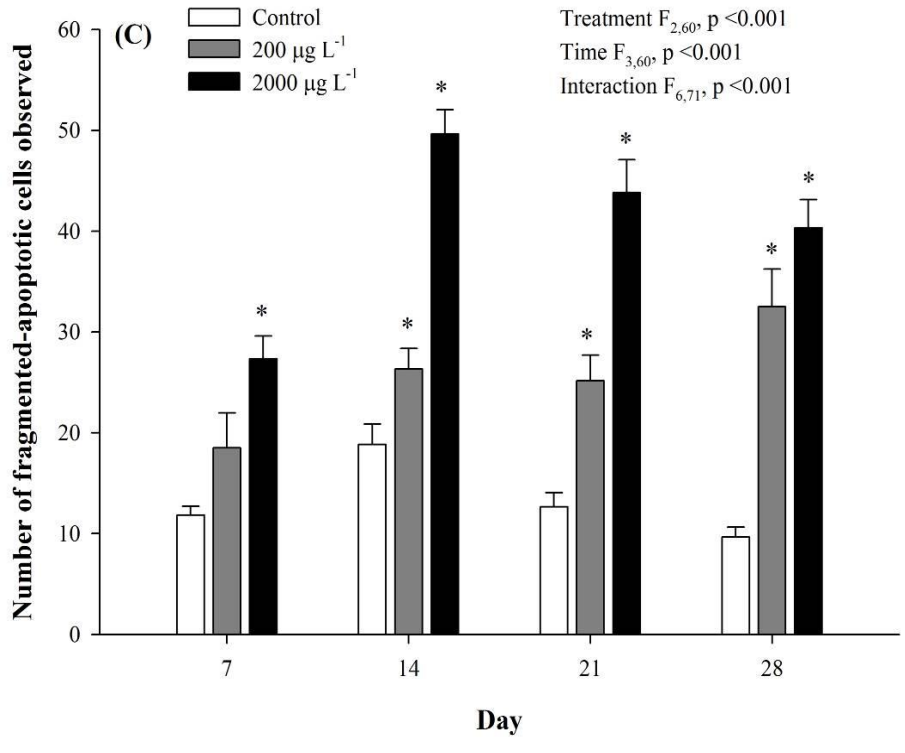
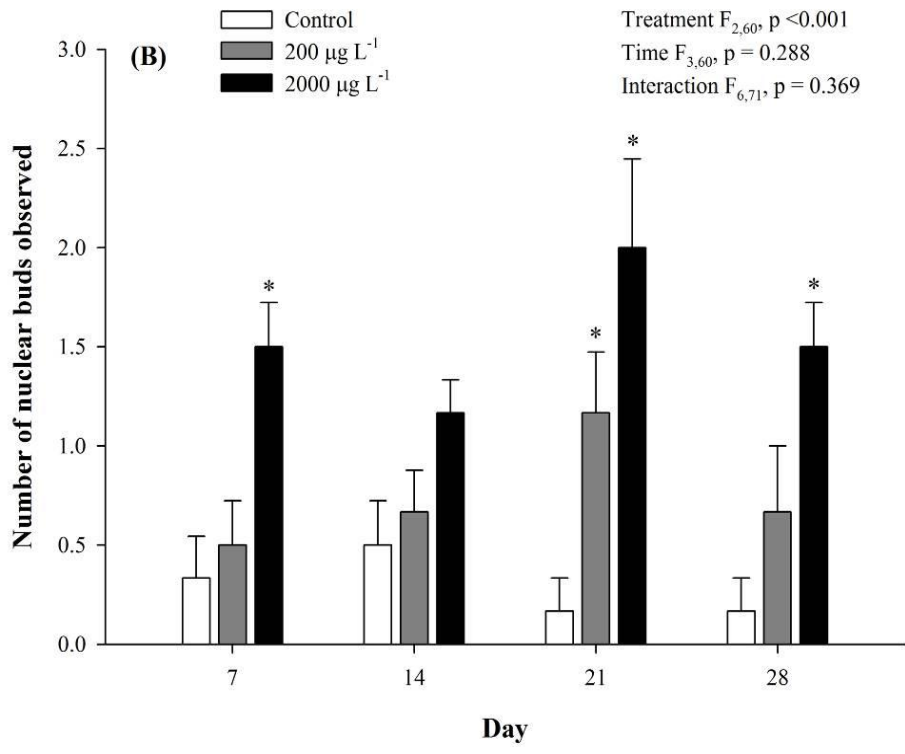


Figure 5.6 Number of micronuclei (A), nuclear buds (B), fragmented-apoptotic cells (C) and binuclei (D) observed in the gill cells of green-lipped mussels (*Perna canaliculus*) following acute (96 h) exposure to Cd (0 (control), 2000 and 4000 $\mu\text{g L}^{-1}$; $n = 8$). Values are presented

as mean \pm SEM. Plotted points sharing letters are not significantly different. All other details are identical to those in the Figure 5.4 legend, except that in panels A and B statistical significance ($p < 0.05$) was determined using Kruskal-Wallis ANOVA followed by a Tukey test and data in panel D were square-root transformed, prior to statistical analysis ($p = 0.050$).





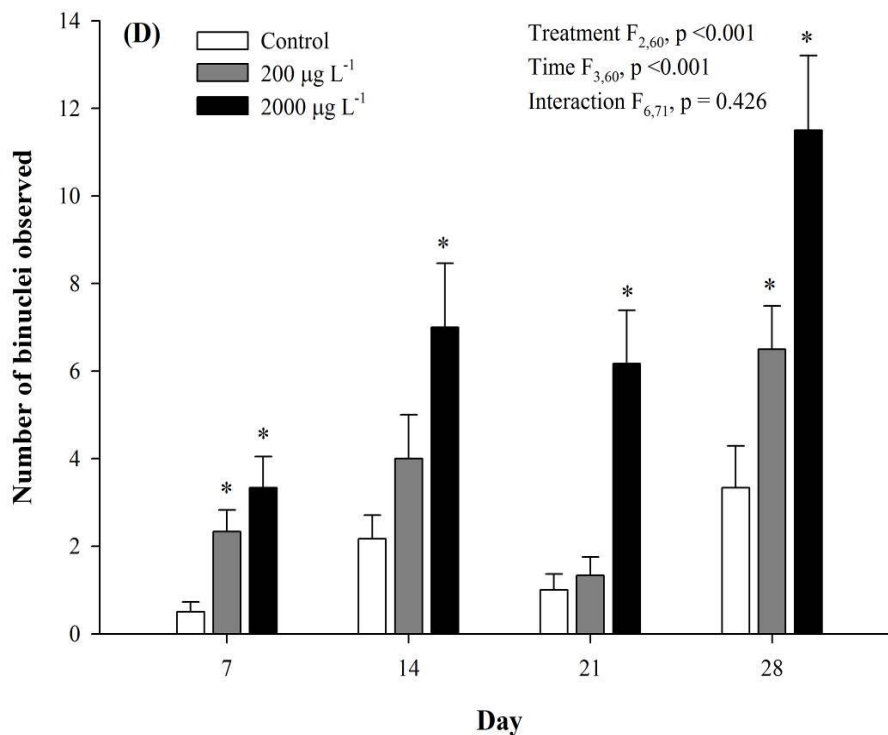


Figure 5.7 Number of micronuclei (A), nuclear buds (B), fragmented-apoptotic cells (C) and binuclei (D) observed in the gill cells of green-lipped mussels (*Perna canaliculus*) during subchronic (28 d) exposure to Cd (0 (control), 200 and 2000 µg L⁻¹, n = 6). Values are presented as mean ± SEM. All other details are identical to those in the Figure 5.5 legend, except that only the data in panels A and C were log-transformed, while data in panel D were square-root transformed, prior to statistical analysis.

With the exception of Day 21, subchronic exposure to Cd resulted in a significant increase in the numbers of micronuclei in 2000 µg Cd L⁻¹ while a significant increase was observed in the 200 µg Cd L⁻¹ mussels only on Day 14 of the exposure (Fig. 5.7A). Similarly, nuclear bud formation in 2000 µg L⁻¹ Cd-exposed mussels increased significantly during subchronic exposure (with the exception of Day 14) while an increase in this parameter was also observed in the 200 µg L⁻¹ Cd-exposed mussels, but only on Day 21 (Fig. 5.7B). Subchronic exposure to Cd resulted in a significant increase in the number of fragmented-apoptotic cells at both 200 and 2000 µg Cd L⁻¹ throughout the 28 d exposure (Fig. 5.7C; with the exception

of Day 7, at 200 $\mu\text{g Cd L}^{-1}$). A significant increase in the number of binuclei in the 2000 $\mu\text{g Cd L}^{-1}$ Cd-exposed mussels occurred throughout the 28 d exposure while the 200 $\mu\text{g Cd L}^{-1}$ Cd-exposed mussels showed a similar significant increase in binuclei only on Day 7 and 28 (Fig. 5.7D). Table 5.1 shows that all of these nuclear aberrations were strongly correlated with gill Cd accumulation (micronuclei, $R = 0.801$, $p = 0.0017$; nuclear buds, $R = 0.795$, $p = 0.0020$; fragmented-apoptotic cells, $R = 0.848$, $p = 0.0005$; binuclei, $R = 0.875$, $p = 0.0002$).

Table 5.1 Relationship between Cd accumulation in gill of mussels and cytogenotoxic biomarker response in gill cells after subchronic (28 d, 0-2000 $\mu\text{g Cd L}^{-1}$) exposure to waterborne Cd.

Cytogenotoxic Measure	Correlation coefficient (R)	Equation of the line of best fit	p value
Micronuclei	0.801	$y = 0.001x + 0.8029$	0.0017
Nuclear buds	0.795	$y = 0.0008x + 0.5318$	0.002
Fragmented-apoptotic cells	0.848	$y = 0.0192x + 0.7191$	0.0005
Binuclei	0.875	$y = 0.0048x + 2.1536$	0.0002

Significant values ($p < 0.05$) are indicated in bold

5.3.3 Comet assay

The percentage tail intensity of DNA in the haemocytes of green-lipped mussels increased significantly in Cd-exposed mussels during the 28 d subchronic exposure to Cd (Fig. 5.8). This effect was dose-dependent and independent of the time at which the response was measured.

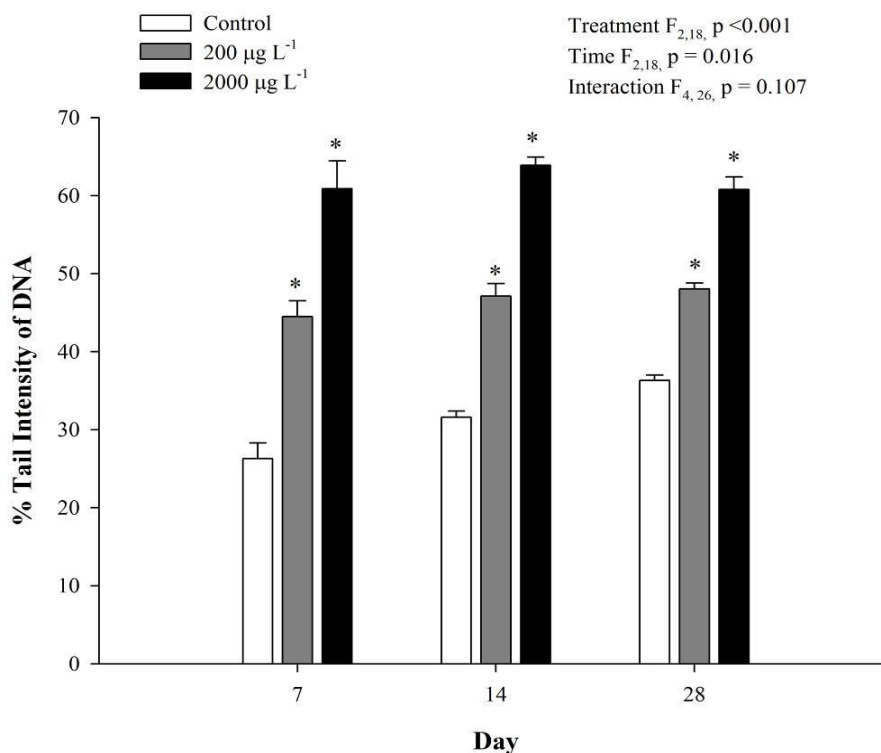


Figure 5.8 Percentage of DNA tail intensity of haemocytes in green-lipped mussel (*Perna canaliculus*) during subchronic (28 d) exposure to Cd (0 (control), 200 and 2000 $\mu\text{g L}^{-1}$, $n = 3$). Values are presented as mean \pm SEM. All other details are identical to those in the Figure 5.5 legend, except that only the data were not transformed prior to statistical analysis.

5.4 Discussion

5.4.1 Immunocytotoxic effects of cadmium

The classification of haemocytes presented in this study was based on the morphology and differential staining characteristics of these cells. Similar classifications, and indeed similar cell type identifications, have been reported in other mussel species (e.g. *Mytilus edulis*, Foll et al., 2010; *Perna viridis*, Wang et al., 2012). In general the haemocytes of mussels can be divided into those cells exhibiting intracellular granularity (granulocytes) and those that do not (agranulocytes or hyalinocytes). Granular cells can be further subdivided into basophils and eosinophils, on the basis of staining patterns. Granulocytes are considered to play

important roles in phagocytosis (Pipe, 1992), although the functional differentiation of the two cell types within this classification is not well understood. The roles of bivalve hyalinocytes are less well-defined but they are believed to be associated with the cytotoxic immune response (Soares-da-Silva et al., 2002), and are also important in the early stages of epithelial regeneration (Suzuki et al., 1991).

In the current study acute exposure to Cd resulted in an increase in the relative proportion of both granular cell types (Fig. 5.4). Conversely, subchronic Cd exposure resulted in a time- and concentration-dependent response in granular basophils (a decrease at 2000 $\mu\text{g Cd L}^{-1}$ at Day 7 and 28; an increase at 200 $\mu\text{g Cd L}^{-1}$ at Day 14), an elevation in granular eosinophils at both concentrations (on Day 14 and 21), and a dose-dependent relative increase in hyalinocytes observed only at 200 $\mu\text{g Cd L}^{-1}$ (with the exception of Day 21) (Fig. 5.5). A variety of responses in differential haemocyte count have been previously reported upon exposure to metal toxicants. For example, decreases in eosinophils and increases in basophils were noted after copper exposure in *Mytilus edulis* (Pipe et al., 1999). Conversely, Coles et al. (1995) reported no effect of Cd exposure (up to 400 $\mu\text{g L}^{-1}$) in the same species, while Cheng (1988) described an increase in hyalinocyte proportion following exposure of the oyster *Crassostrea virginica* to 1 mg L^{-1} Cd. Species differences, and particularly dose duration and concentration, are likely to explain these differences.

Changes in the relative proportions of haemocytes in bivalves can result from one of three mechanisms. Cell types that are more sensitive to Cd may be selectively eliminated from the circulating haemocyte population (Pipe et al., 1999); there may be an increase in haematopoiesis and/or cell differentiation resulting in a relative increase in a particular cell type (Cheng, 1988); and/or specific cell types may migrate to or from the tissue, thus altering their proportions in the sampled population (Pipe et al., 1999). Eosinophils appeared to be the

most resilient of the haemocytes to Cd effects, exhibiting increases in both acute and subchronic exposures at the highest concentrations examined. This may be a consequence of their role in phagocytosis. Phagocytic haemocytes of mussels utilise ROS as part of their defence against invading pathogens (Pipe and Coles, 1995). Consequently these cells are well protected against ROS damage (e.g. they exhibit elevated peroxidase activities; Soares-da-Silva et al., 2002). As Cd is known to generate oxidative stress (Pipe and Coles, 1995), it could be hypothesised that these cells have an inherent protective advantage upon Cd exposure compared to other cell types that lack these defences (e.g. hyalinocytes; Soares-da-Silva et al., 2002). Alternatively, it has been noted that Cd impacts the shape of granulocytes, making them rounder with less pseudopodia (Calisi et al., 2008). This would decrease their ability to aggregate in the tissue, causing an increase in their presence in the haemolymph (Auffret et al., 2002), an explanation that would also be consistent with the pattern observed in the current study.

As many researchers treat granulated cells as a single cell population in immunocytological studies (e.g. Calisi et al., 2008), the pattern observed for eosinophils would be expected to be replicated in the other granulated cell population, the basophils. However these two cell classifications showed a distinct pattern. Basophils decreased in relative numbers at Day 7 and 28 of subchronic Cd exposure. The mechanism behind this remains unexplored, but if these cells had a specific role in metal detoxification, then a decrease in circulating numbers might be expected as they transport accumulated Cd to tissues for storage and/or elimination. This has been previously hypothesised to occur in granulocyte populations of the oyster *Crassostrea virginica* upon Cd exposure (Cherkasov et al., 2007). It is, nevertheless, clear that the two granular cell types have a very different response to Cd exposure, and suggests that they should be treated as distinct populations in future studies. This finding is consistent with a study that identified unique epitopes in basophils and eosinophils, when these cells

were used as antigens to raise monoclonal antibodies (Dyrynda et al., 1997). The nature of these differences, and how they may confer different responses to Cd exposure, remains unknown.

The hyalinocytes showed a clear concentration-dependent pattern (Fig. 5.5C). The increase in relative hyalinocyte numbers at low concentrations could be a haematopoietic response (Cheng, 1988), while the return to basal levels at the higher concentration could represent a higher sensitivity to Cd exposure relative to granulocyte populations (e.g. Bolognesi and Hayashi, 2011). An alternative explanation is that hyalinocytes migrated to epithelial tissue such as the gill, to initiate repair induced by the higher level of Cd exposure (Suzuki et al., 1991). Although histological damage to gill was not assessed in the current study, dose-dependent epithelial damage to this tissue has been previously reported in mussels exposed to Cd (Sheir and Handy, 2010). The exact mechanism responsible for the observed changes in proportional cell populations in the current study clearly requires further investigation.

5.4.2 Cytogenotoxic effects of cadmium

In this study two assays were utilised to investigate the cytogenotoxic impacts of Cd exposure in green-lipped mussels. The comet assay detects single strand DNA breaks (Dixon et al., 2002; *Section 1.6.5*). This damage is relatively minor and often transient, and therefore the comet assay is considered to be more sensitive relative to the micronucleus test (Dixon et al., 2002). The micronucleus test evaluates significant perturbation to chromosomal structure, effects that can be observed only in actively-dividing cells. Consequently, such macrolesions are usually slower to develop and detect (Dixon et al., 2002). The results of the current study were generally supportive of this hypothesis. The comet assay showed impacts at the first tested day during subchronic exposure (Day 7), and at both tested exposure concentrations. Of the nuclear abnormalities examined via the micronucleus test, only the development of

binuclei showed a similar early onset in both 200 and 2000 $\mu\text{g Cd L}^{-1}$. However, significant increases in the other macrolesions (micronuclei, nuclear buds, fragmented apoptotic cells), were present only at the highest exposure concentration at Day 7. Consequently, although single stranded DNA breaks appeared to be a more sensitive endpoint of Cd exposure than gross nuclear abnormalities, the transition between the onset of these microlesions and the more deleterious macrolesions was not as clear as may have been predicted.

Differences in the cell types examined may explain this pattern. The comet assay was performed on haemocytes, whereas the micronuclei test was conducted on gill cells. It is generally considered that mussel gill cells are more sensitive to Cd exposure than haemocytes, likely owing to their direct exposure to the waterborne toxicant (e.g. Vincent-Hubert et al., 2011). Thus the presence of macrolesions in the gills might be expected to occur more rapidly than if these parameters had been measured in the haemocytes, leading to the patterns of genotoxicity onset observed. It is important to note, however, that haemocytes are considered the more reliable cells in which to monitor lesion effects, owing to their more homogeneous exposure to the toxicant, and their lower general variability in toxic response (Dixon et al., 2002).

The other notable finding derived from the comet assay results was the persistence of DNA strand breaks over the course of the 28 d subchronic exposure. This pattern was distinct from the usually reported transient effect (Dixon et al., 2002; Frenzilli et al., 2009). This suggests that Cd had a sustained cytogenotoxic impact that was persistent in spite of mechanisms that may act to lessen Cd toxicity (e.g. DNA repair, see below).

The effect of Cd on cytogenotoxic endpoints in mussels has been noted previously (e.g. Pruski and Dixon, 2002; Emmanouil et al., 2007; Vincent-Hubert et al., 2011), and these mutagenic impacts have been recommended for incorporation as biomarkers in mussel-based

environmental biomonitoring programmes for the detection of Cd and other stressors (Dixon et al., 2002). That the nuclear aberrations in branchial cells detected in this study correlated strongly with gill tissue Cd accumulation (Table 5.1), lends support to the idea that these endpoints are potentially effective indicators of Cd toxicity.

Although the literature is consistent in its description of Cd impacting cytogenotoxic markers, the mechanism by which this effect is generated is subject to debate. It is generally accepted that Cd is not directly mutagenic, in that it does not bind to DNA (Valverde et al., 2001). Instead it is believed that Cd exerts genotoxicity through the generation of ROS, by inhibiting DNA repair pathways, and/or by binding to cellular sulfhydryl groups that would be otherwise used to scavenge ROS (Pruski and Dixon, 2002; Emmanouil et al., 2007; Vincent-Hubert et al., 2011). Both the pro-oxidant effect, and the DNA repair inhibition, is believed to be generated by the ability of Cd to displace metal cofactors from cellular proteins. Metals such as Fe^{2+} and Cu^{2+} can directly generate ROS via the Fenton reaction, so the displacement of these cofactors from proteins by Cd is known to cause oxidative stress (Cuypers et al., 2010; see *Section 1.3.5.2*), and subsequent genotoxic damage. Similarly, key enzymes in DNA repair utilise Zn^{2+} as a cofactor, and the displacement of Zn^{2+} by Cd can lead to impairment of this process (Pruski and Dixon, 2002). The exact mechanism responsible for the genotoxic effects observed in the current study has not been investigated. However, in *Chapter 4* Cd exposure resulted in significant changes to branchial catalase activity. This suggests that a pro-oxidant effect is at least partially responsible for the observed cytogenotoxic changes described.

5.5 Conclusions

This study showed that Cd exposure in the green-lipped mussel had a significant impact on both immunocytological and cytogenotoxic biomarkers. The strong positive correlation

between tissue accumulation and nuclear aberrations detected by the micronucleus test, suggests that this assay is a useful tool to assess environmental Cd toxicity, a utility increased by the fact that this test is simple to perform and cost-effective. In combination with other biomarkers identified previously in the same species, such as clearance rate (*Chapter 3*) and MTLP (*Chapter 4*), a suite of endpoints have been established that respond to dose, duration and tissue metal accumulation levels. When applied in *P. canaliculus* exposed to metal pollutants found in the NZ environment these biomarkers could be used to develop an environmental risk assessment model that would be a valuable addition to biomonitoring programmes.

Aside from the utility of these cytogenotoxic endpoints in monitoring, the current study also indicates potentially important mechanisms of toxicity in this species. Alterations in the relative proportions of haemocytes suggest that exposure to Cd could weaken immune defence (Auffret et al., 2006), impacting disease resistance and survival capability of marine organisms (Pipe and Coles, 1995; Dyrinda et al., 1998). Similarly genotoxic effects could induce DNA damage leading to implications such as reduction in the population size. Furthermore, alterations in the DNA could ultimately induce changes in the genetic diversity affecting the entire ecosystem (Theodorakis, 2001).

6 Trace metal concentrations in sediment and tissues of green-lipped mussels from coastal regions of the South Island, New Zealand

6.1 Introduction

Coastal ecosystems are vulnerable to pollution from different sources. Among these pollutants, metal contaminants are of major concern because of their persistent and non-degradable nature (*Section 1.2*). Metals entering the coastal environment are present in the water column and/or associated with the sediments. The distribution and the fate of metals between environmental compartments are controlled by complex interactions between environmental variables. Changes in salinity, temperature, pH, oxygen content and DOM are known to affect the behaviour of metals in the coastal environment (e.g. Atkinson et al., 2007). These factors also influence metal uptake and accumulation in aquatic organisms.

Bivalves such as mussels are exposed to metals through different compartments of the environment namely the dissolved aquatic phase and the particulate phase in water, sediments and through their diet (e.g. Gagnon et al., 2006). One of the key characteristics that make mussels ideal bioindicators of the environment, is their ability to accumulate metals (see *Section 1.5.1*). Bioaccumulation can be defined as the net accumulation of a chemical into the tissues of an organism through all possible routes of uptake including water and food (Luoma and Rainbow, 2008). Metal uptake, accumulation, depuration in aquatic organisms is metal-, organ- and species-specific (McGeer et al., 2004).

Among the tissues, the gill is considered to play a significant role in metal uptake and accumulation due to its involvement in the filter-feeding mechanism (Marigoméz et al., 2002). Several mussel species use the digestive gland as the major organ for sequestration and detoxification of metals (Marigoméz et al., 2002). Apart from these two tissues, other organs such as the mantle and the foot are also involved in metal uptake and storage (Simkiss and Mason, 1983). Similar to the gill, both the foot and the mantle are also in direct contact with the external environment. However, few studies have reported organ-specific metal

accumulation levels in mussels (e.g. Yap et al., 2006). Within the different organs, metals are stored in granules, lysosomes and also bound to specialised proteins in detoxified form (Simkiss and Mason, 1983). The variations in distribution of metals between tissues can be related to their affinity towards metal-binding sites such as MT, and differences in the rate of metal uptake and elimination (Gundacker, 1999). Moreover, the physiological state of the mussels (e.g. spawning, feeding habits) and environmental factors such as salinity and temperature can also influence metal bioaccumulation in mussel tissues. Compared to whole body burden, metal concentration in different tissues provides information on metal uptake and accumulation (e.g. Yap et al., 2006). In a biomonitoring context, it would be valuable to understand the role of specific organs in metal accumulation to provide information on metal bioavailability (also see *Section 1.4*).

Marine sediments act as a reservoir for metals providing a time-integrated indication of metal contamination (e.g. Turner, 2000). Processes such as bioturbation, diffusion and resuspension are known to redistribute metals present in the sediments into the water column (Chapman et al., 1998). Although the measurement of total metal concentrations in sediments can provide information on metal contamination, it is the bioavailable fraction of metal (defined as the metal fraction that is available in free form for absorption by the organism) which has the potential to induce toxicity (Di Toro et al., 2005; *see Section 1.2*). The speciation of metals in sediments can be affected when metals form complexes with sulfides, oxides, carbonates or DOM (Chapman et al., 2003; Di Toro et al., 2005). Several environmental factors strongly influence metal complexation processes within sediments such as changes in redox reactions, benthic flux, presence of organic and detritus material (Di Toro et al., 1992; De Jonge et al., 2012).

In order to protect aquatic species from adverse metal toxic effects sediment quality guidelines (SQG) have been developed (McCauley et al., 2000). SQGs are derived from toxicity databases by environmental agencies that provide values that can be used to compare sediment metal concentrations from a particular site of interest (McCready et al., 2006). These guidelines are used to assess the degree of metal contamination and identify the risk associated to aquatic organisms (e.g. Christophoridis et al., 2009).

Metal concentrations in both mussel tissue and sediments are used worldwide as reliable indicators that reflect the health status of the coastal environment (e.g. Roméo et al., 2005). In NZ, the coastal environment is affected by several metal contaminants arising from different sources such as industrial activities (e.g. mining), agricultural runoff (e.g. dairy farms), sewage outfall and urban stormwater runoff (Burggraaf et al., 1997; Pilotto et al., 1998; Eagar, 1999). Other natural sources of metal input in NZ include volcanic and geothermal activity, earthquakes, soil erosion and sea upwelling (Croot and Hunter, 1998; Reyes et al., 2002; Brown and Simmons, 2003; Goldsmith et al., 2008). In NZ, there is limited information on sediment metal concentrations from coastal environments while there is no data on tissue metal concentrations of green-lipped mussels.

The objectives of this study were to collect green-lipped mussels, *P. canaliculus* and sediments from three coastal regions of the South Island in NZ including sites which differed in their contaminant histories to: (a) measure the metal concentrations in different mussel tissues from different sampling sites; and (b) to assess metal contamination levels in sediments using SQGs. In this study, the concentrations of Cu, Zn, Cd, As and Pb, were determined in the gill, digestive gland, mantle and foot of the green-lipped mussels collected from nine sites (three sites from each of the three regional locations) in coastal regions of NZ's South Island (Fig. 6.1). Sediment samples were also simultaneously

collected from each of those sampling locations and the levels of, Cu, Zn, As, Cd and Pb were determined. The information on metal concentrations collected from both mussel tissues and sediments can contribute towards understanding metal contamination in NZ and the use of green-lipped mussels as bioindicators of metal contamination in NZ.

6.2 Materials and methods

6.2.1 Description of sampling sites

Mussel and sediment samples were collected from three regions (West Coast, Canterbury and Nelson) in the South Island, NZ. The location, potential sources of pollution and physical properties of the nine sampling sites within these three regions are presented in Table 6.1 and briefly discussed below.

West Coast – Three sites, namely Carters Beach, Tauranga Bay and Ngakawau, were chosen as sampling sites within the West Coast region. Carters Beach is classified as a coastal recreational area and was chosen as the reference site (potentially least contaminated) for the West Coast region. Tauranga Bay receives discharges from local sewage and beach mining (WCRC, 2006). Coal mining is the primary source of contamination in Ngakawau (WCRC, 2006). The coastal area in the West Coast region has been reported to receive pollutants from adjoining farmlands, human sewage, stormwater discharge, industrial and commercial activities (WCRC, 2000).

Canterbury – Pigeon Bay, Lyttelton and Avon-Heathcote were chosen as the three sampling sites within the Canterbury region. Among these sites, Pigeon Bay was chosen as the reference site. Lyttelton is a major harbour close to Christchurch city and is characterised by shipping and port activity. Both Pigeon Bay and Lyttelton sites are of volcanic origin and

receive stormwater runoff (ECAN, 2007). Avon-Heathcote is an estuary where the Avon and the Heathcote rivers meet. Metal pollution has been a major problem in this site in the past with sewage discharge and storm water runoff being the two main contributors (ECAN, 2012). Recent earthquakes in 2011 have altered metal concentrations in the Canterbury region based on mussel tissue samples collected before and after the earthquakes.

Nelson – Adele Island, Tahuna Beach and Mapua were selected as sampling sites in the Nelson region. Adele Island is known for sightseeing and recreational activities (TDC, 2010). Adele Island was chosen as the reference site for the Nelson region. Tahuna Beach is a recreational area with road run-off, sewage discharge and boating activity being the primary contributors to metal pollution (TDC, 2010). Mapua is one of the most contaminated sites in New Zealand. It has a history of contamination from a pesticide manufacturing company that was closed down in 1988 (MfE, 2010).

6.2.2 *Mussel collection and maintenance*

Adult green-lipped mussels (n = 30 from each sampling site; 60-80 mm shell length) were collected from nine sampling sites (Fig. 6.1). A single reading of physical properties of the seawater such as temperature, salinity and pH at each sampling site was recorded using a calibrated hand-held meter (YSI 30 M, John Morris Scientific Ltd). Sampling was conducted during low tide, on shore from all the sites, with the exception of Pigeon Bay and Lyttelton that were accessed by boat. Sediment samples were not collected from these two sites. The mussels were collected by cutting the byssus threads off their substratum using a pair of scissors. After collection, the mussels were transferred into a polyethylene bag (90 x 60 cm) placed on ice-packs inside a cooler bin. Care was taken to cover the ice-packs with moist absorbent cloth to avoid direct contact with the mussels. The polyethylene bags containing

the mussels were half-filled with seawater from the collection site. The mouth of the bag was loosely tied with a nylon thread through which an aeration tube was inserted and connected to a battery-powered air-bubbler. This setup was transported to a 15°C controlled temperature room at the University of Canterbury within 24 - 48 h. On arrival at the facility, the mussels were maintained according to the protocol provided in *Section 2.1* and kept in fresh seawater (~20 L) that was collected in 30 L polypropylene containers from each collection site.

6.2.3 Sediment collection

Superficial oxic sediment samples (~0 to 2-5 cm depth; n = 3) from each site were collected in 50 ml acid-washed polypropylene tubes. All tubes used for sediment sample collection were acid-washed for 48 h in 2% HNO₃, thoroughly rinsed, dried and kept in clean polyethylene bags until use. These acid-washed tubes were used for storing sediments collected at each sampling site. The sediment samples were placed in clean polyethylene bags and transported to the laboratory inside the cooler bin along with the mussels. On reaching the laboratory, the sediment samples were stored at 4°C until processed.

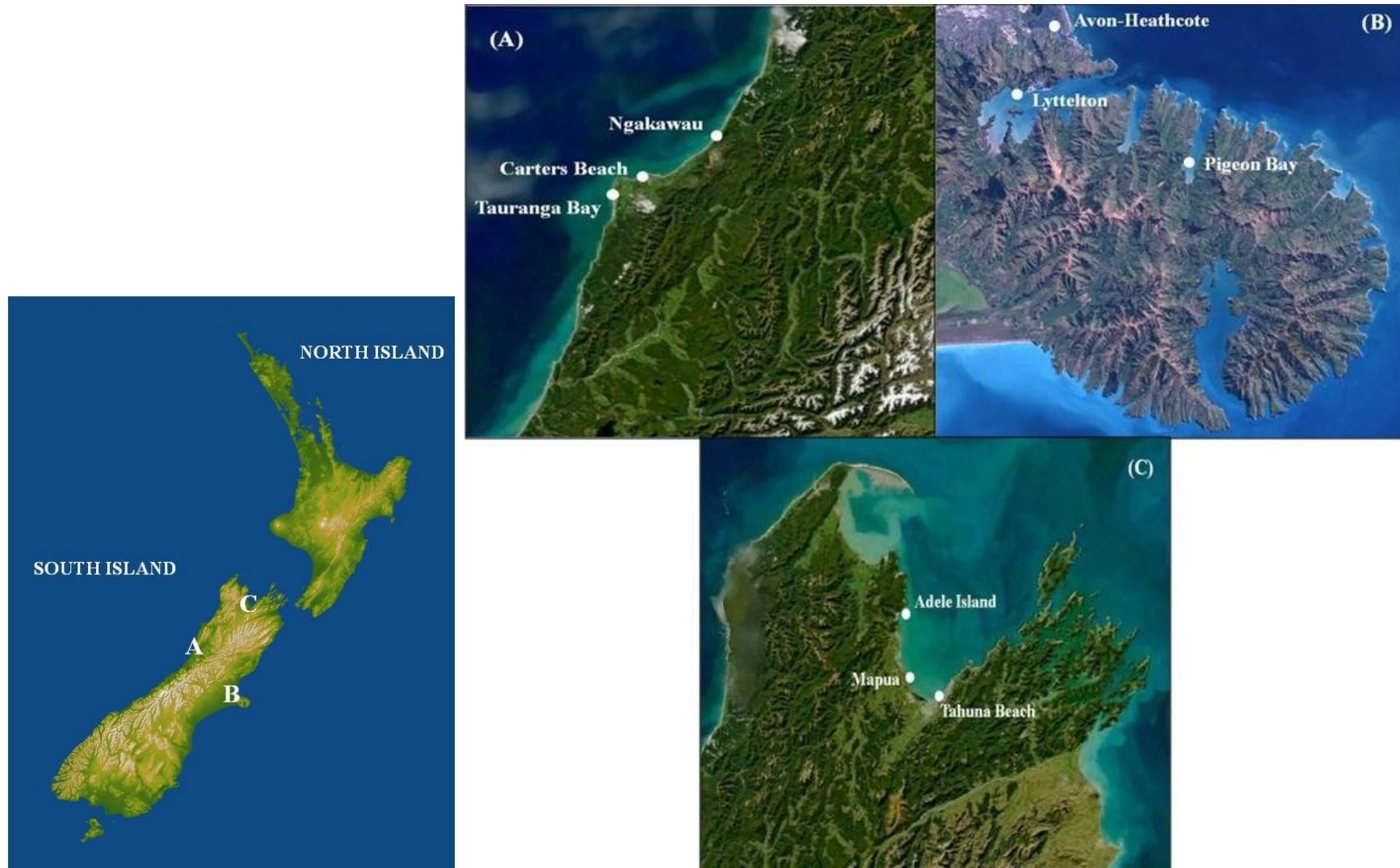


Figure 6.1 Location of sampling sites within each region in the South Island, New Zealand[†]. (A) West Coast (B) Canterbury (C) Nelson

[†]Satellite images reproduced from the source - NASA/JPL-Caltech

Table 6.1 Description of sampling sites and characteristics of seawater at the different sampling sites

Regions	Sampling sites	Sampling date	Location	Potential sources of pollution	Salinity (‰)	pH	Water Temperature (°C)
West Coast	Carters Beach	10 - 09 - 2011	S41° 43.779' E171° 35.243'	Industrial discharge, stormwater runoff	31.5	8.1	12.2
	Tauranga Bay	10 - 09 - 2011	S41° 46.533' E171° 27.250'	Sewage	32.2	7.8	12.2
	Ngakawau	11 - 09 - 2011	S41° 34.121' E171° 54.486'	Mining effluent	32.3	8.0	12.8
Canterbury	Pigeon Bay	03 - 10 - 2011	S43° 38.081' E172° 57.156'	Volcanism	32.0	7.6	10.4
	Lyttelton	03 - 10 - 2011	S43° 36.564' E172° 43.246'	Shipping activity, sewage, stormwater runoff	31.6	7.6	11.7
	Avon-Heathcote	04 - 10 - 2011	S43° 33.346' E172° 44.087'	Sewage, stormwater runoff	24.7	7.6	10.4
Nelson	Adele Island	27 - 09 - 2011	S40° 58.649' E173° 03.436'	Recreational activity	31.0	8.0	14.0
	Tahuna Beach	28 - 09 - 2011	S41° 16.549' E173° 15.238'	Sewage, stormwater runoff	32.6	7.6	15.8
	Mapua	27 - 09 - 2011	S41° 15.378' E173° 06.129'	Industrial discharge, stormwater runoff	29.7	7.8	15.1

Sampling date presented as dd/mm/yy

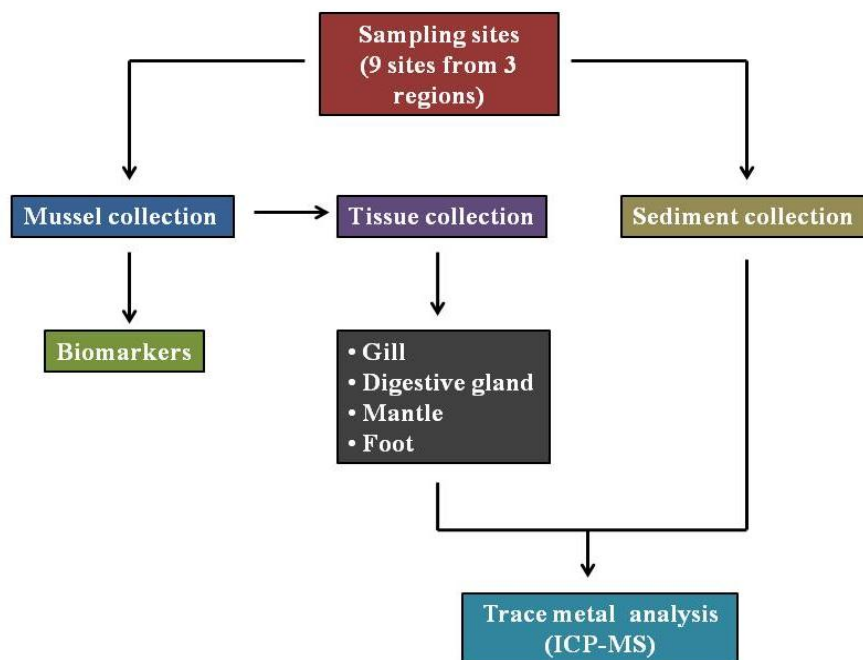


Figure 6.2 Flow chart illustration of field sampling and trace metal analysis

6.2.4 Trace metal analysis in mussel tissue and sediment samples

Mussels ($n = 6$) from each study site were dissected; gill, digestive gland, mantle and foot tissues were collected and stored at -80°C until analysis. The rest of the mussels ($n = 24$) were dissected and tissues stored at -80°C for future biomarker analysis. Tissue trace metal analysis was undertaken as described in *Section 2.8* using SRM 2976 (National Institute of Standards and Technology, US) mussel tissue as a certified reference material.

Prior to metal analysis, the sediments were transferred onto aluminium trays and dried in a heating oven at 60°C for 48 h. The samples were allowed to cool before transferring into clean polyethylene bags. A metal rod was used as a rolling pin to disaggregate the sediments in order to obtain uniform grain size prior to acid-digestion. Approximately 1 g of bulk sediment (not sieved and not washed) was weighed and acid digested in 5 ml 50% HNO_3

(Analar grade) and 10 ml of 20% HCl (Analar grade) made up to 20 ml at 90°C for 60 min. Once cooled overnight, the sediment samples were diluted using 2% Ultrapure HNO₃ for analysis. SRM 2702 (National Institute of Standards and Technology, US) was used as a certified reference material. The trace metal analysis conducted in sediment and mussel tissues in this field study is presented in Fig. 6.2.

Trace metal concentrations (Cu, Zn, As, Cd and Pb) in mussel tissue and in sediment samples were analysed using ICP-MS (Agilent-7500cx). The percentage recoveries for the different trace metals in the sediment and mussel tissue standard reference materials are reported in Table 6.2. Sediment Cd concentrations from all nine sampling sites were below the detection limit (0.1 µg g⁻¹).

Table 6.2 *Detection limits and recovery percentage of metals in standard reference material used for mussel tissue and sediment analysis*

Trace metal	Detection limit	Recovery (%)	
		Mussel tissue SRM 2976	Sediment SRM 2702
Cu	1	111 ± 0.51	86 ± 1.16
Zn	1	102 ± 12.68	93 ± 4.99
As	0.1	106 ± 1.21	84 ± 0.41
Cd	0.1	104 ± 0.06	108 ± 0.007
Pb	1	97 ± 0.10	108 ± 1.03

Detection limits expressed as µg g⁻¹.

Recovery levels expressed as % (mean ± SEM; n = 2 or 3).

6.2.5. Statistical analysis

Statistical analysis was conducted as described in Section 2.9. Variations in metal concentrations between sites and regions were analysed using one-way nested ANOVA followed by a post hoc LSD test (see Appendices 5-7). For concentrations of metals that fell

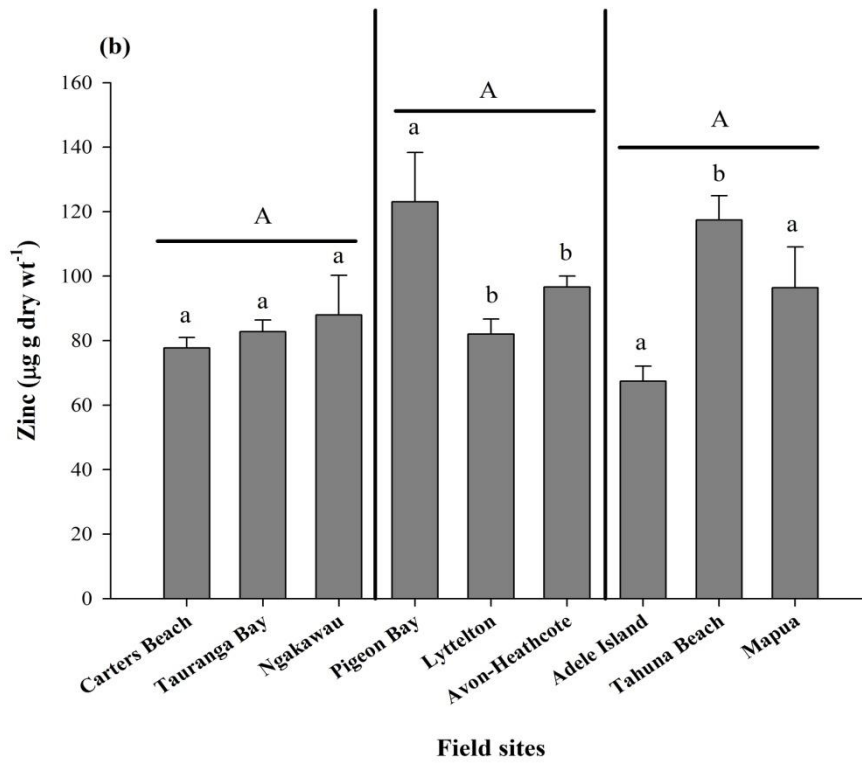
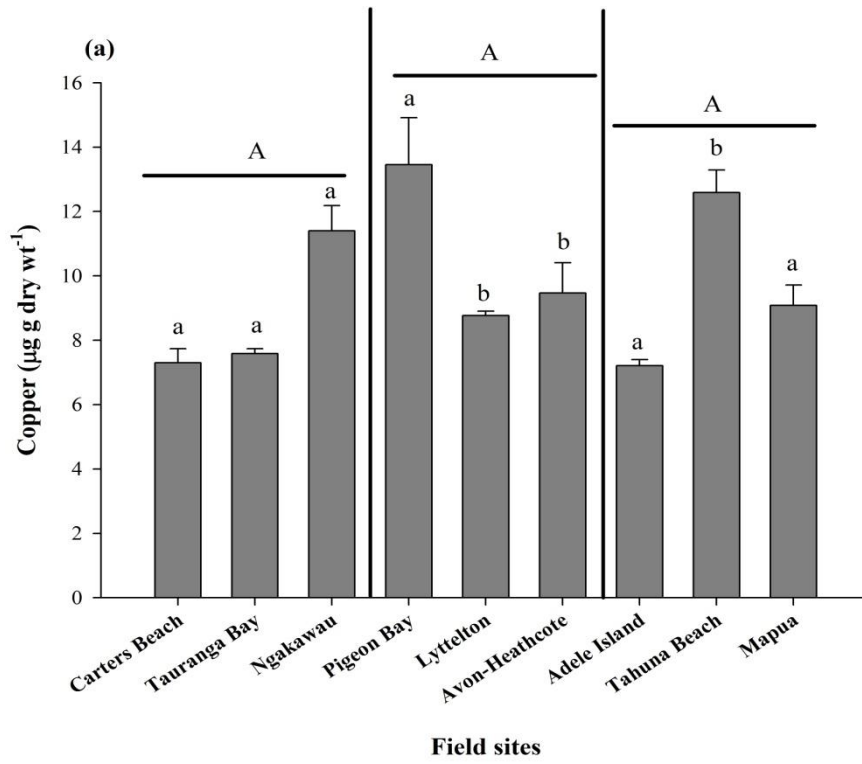
below the detection limit, a value representing half the detection limit concentration for that metal was used (e.g. for Cd, $0.05 \mu\text{g g}^{-1}$ was used based on $0.1 \mu\text{g g}^{-1}$ detection limit; Table 6.2).

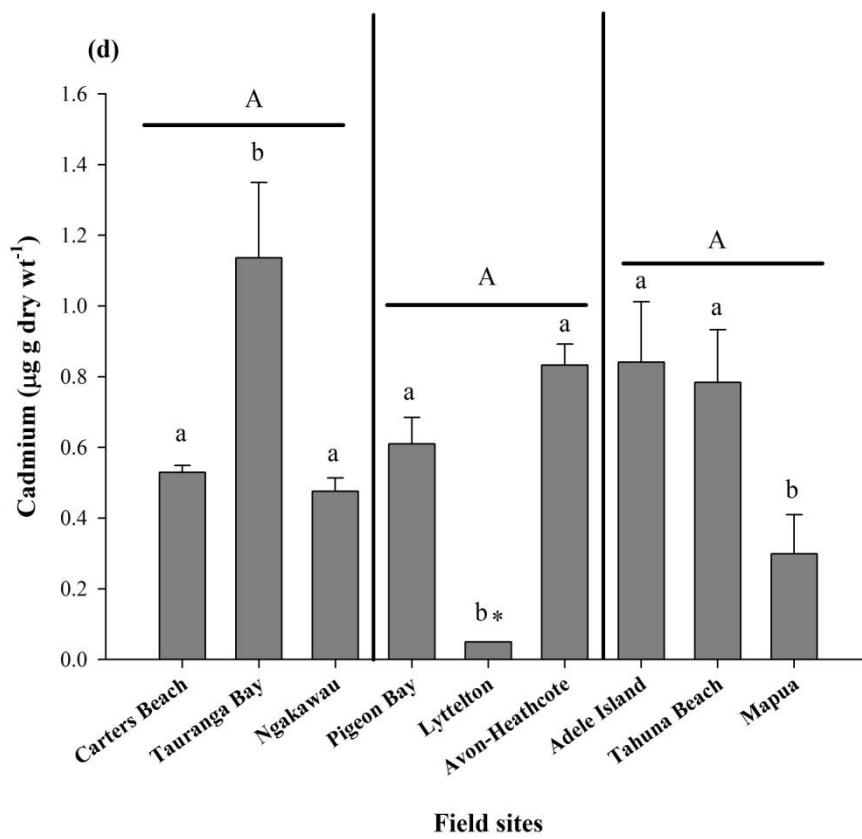
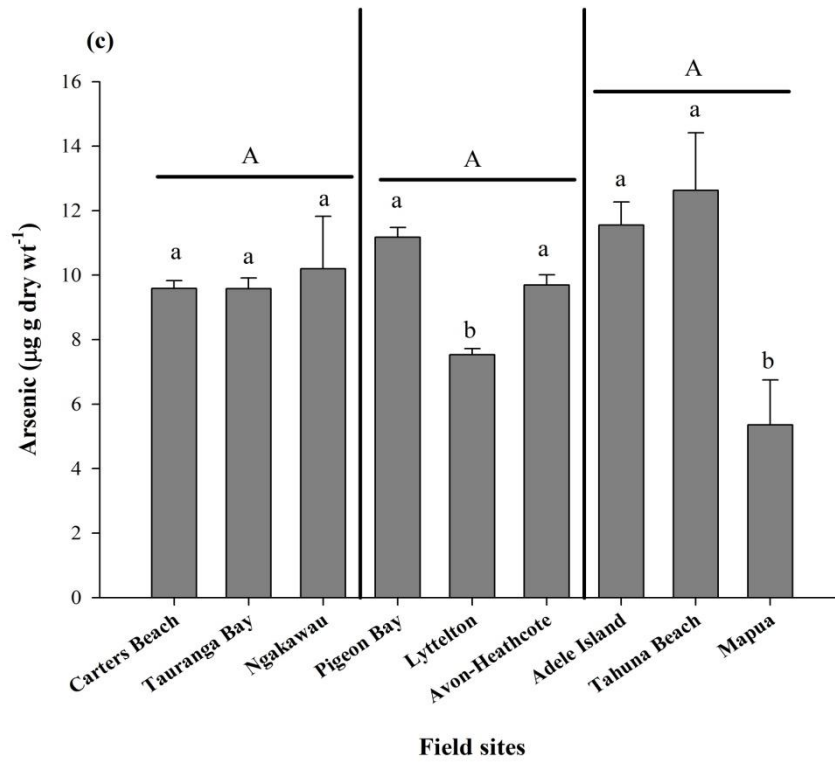
Correlation analyses between metal concentrations in each mussel tissue and sediment from different sampling sites were performed using Pearson correlation analysis to identify significant associations between corresponding sediment and tissue metal concentrations. Data were log-transformed for normalisation prior to correlation analyses (McGrath et al., 2004). Data are presented as mean \pm SEM. All statistical analyses were performed using SigmaStat (SYSTAT software 3.5).

6.3 Results

6.3.1 Metal concentrations in the gill

No significant regional differences were observed in Cu, Zn, As and Cd gill concentrations of mussels (Fig. 6.3a-d). The Canterbury mussels showed significantly higher concentrations of Pb in the gill than the West Coast and Nelson mussels (Fig. 6.3e).





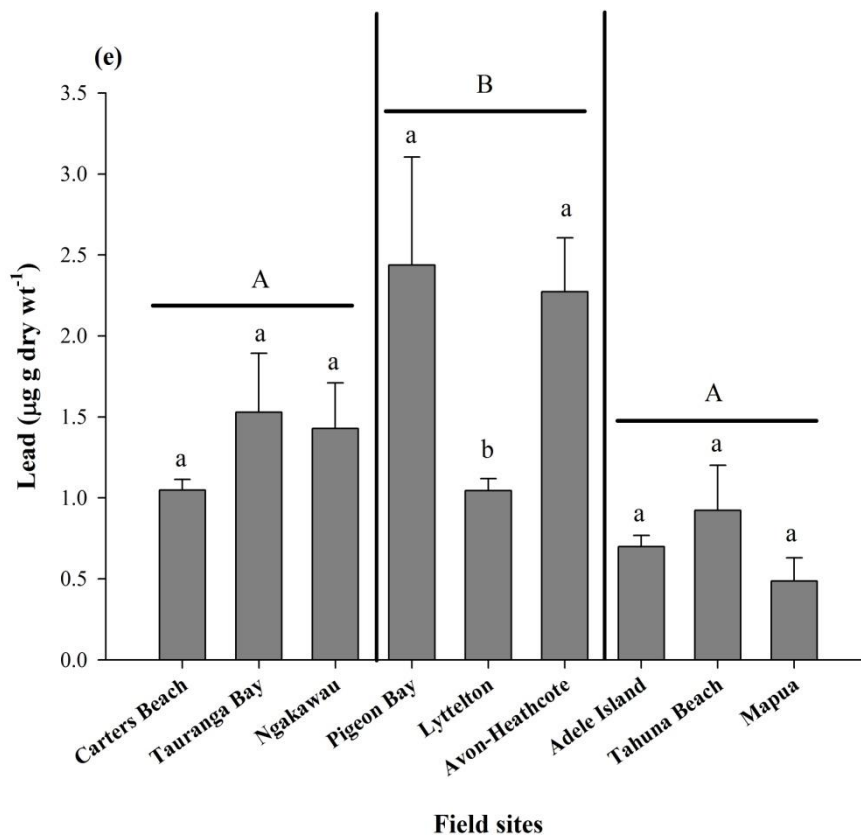
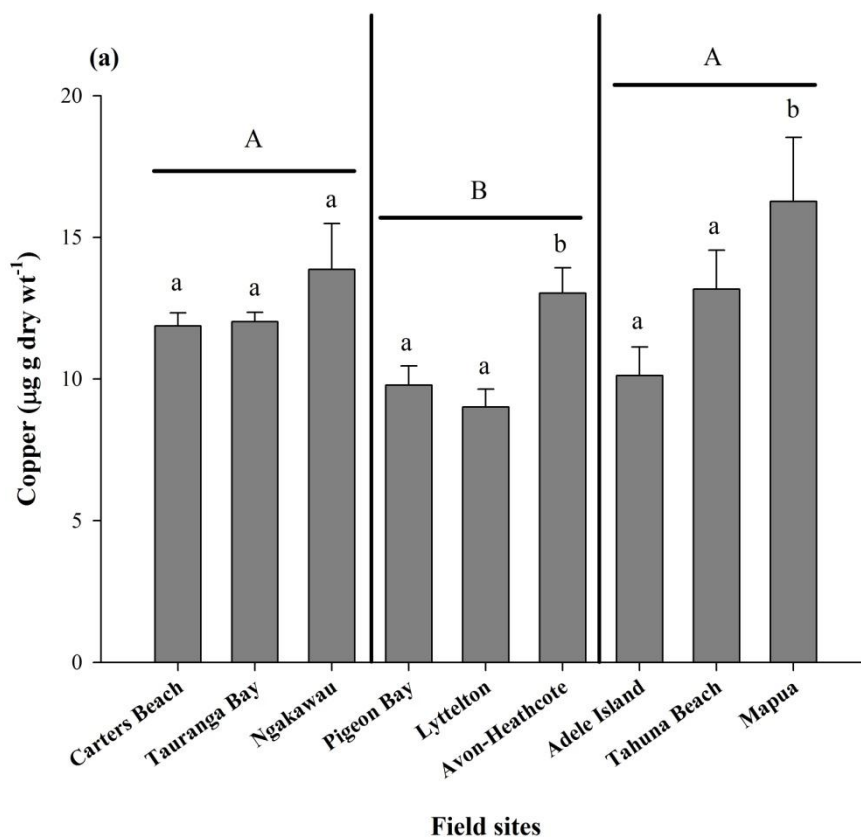
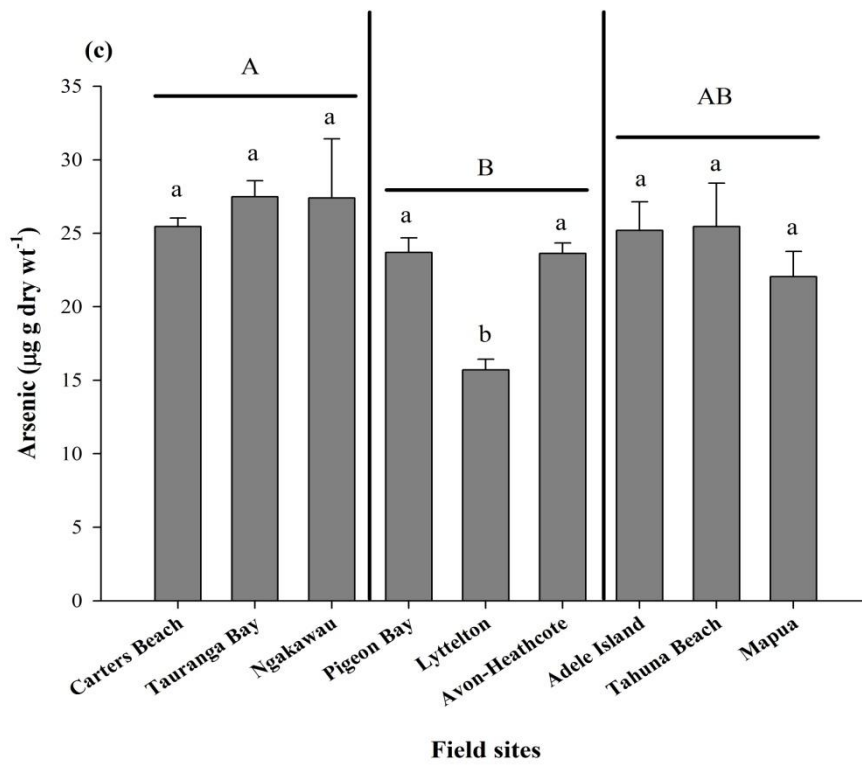
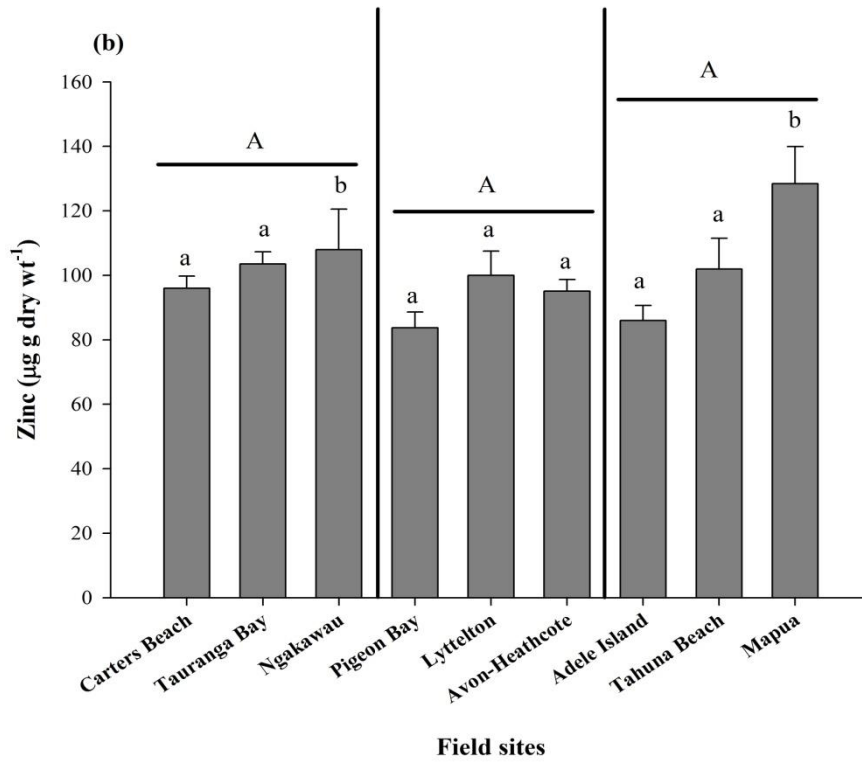


Figure 6.3a-e Concentration of trace metals (a) copper (b) zinc (c) arsenic (d) cadmium and (e) lead in the gill of green-lipped mussels collected from different sites in New Zealand. Statistical significance ($p < 0.05$) was determined using one-way nested ANOVA followed by post hoc LSD analysis to indicate differences between regions and differences between sites within regions. Data are presented as mean \pm SEM ($n = 5-6$). Plotted points sharing letters within a region are not statistically significant. Lowercase letters indicate differences between sites within a region, and uppercase letters indicate differences between regions. Asterisks indicate metal concentrations below detection limit and half the detection limit value was used to represent the metal concentration.

6.3.2 Metal concentrations in the digestive gland

The average metal concentrations in the digestive gland of green-lipped mussels collected from the different regions are presented in Figures 6.4a-e. The mussels from Canterbury had significantly lower concentrations of Cu than mussels from the other two regions (Fig. 6.4a). No significant regional differences were observed in Zn digestive gland accumulation levels (Fig. 6.4b). The Canterbury mussels also had significantly lower levels of As and Cd in the digestive gland compared to the West Coast mussels (Fig. 6.4c and d). The mussels from the Nelson region had significantly lower Pb concentrations in the digestive gland than the other two regions (Fig. 6.4e).





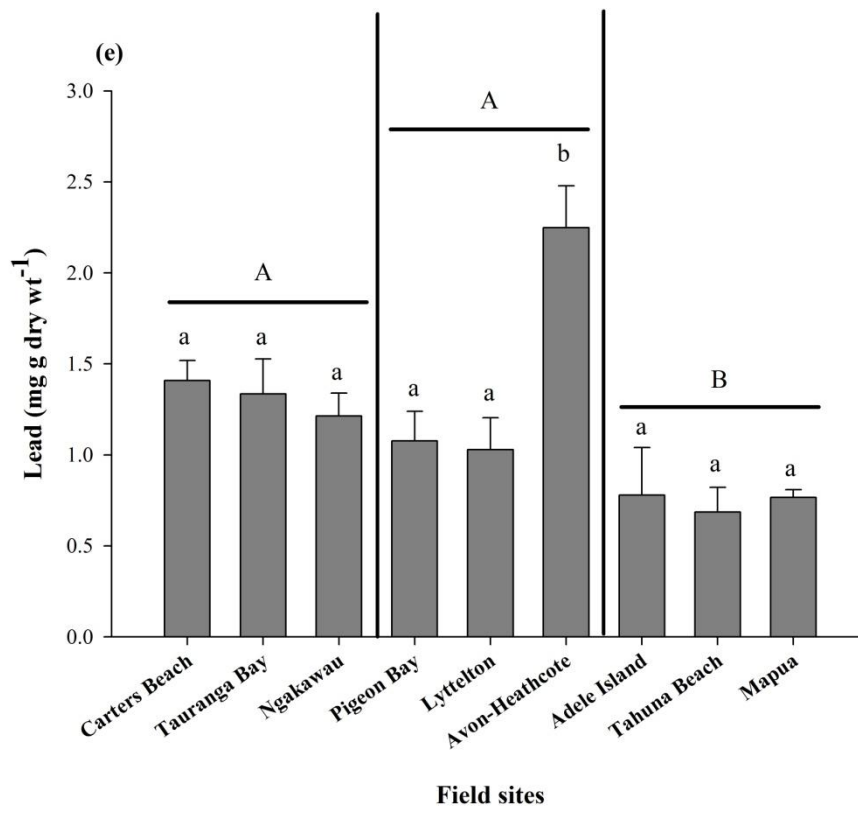
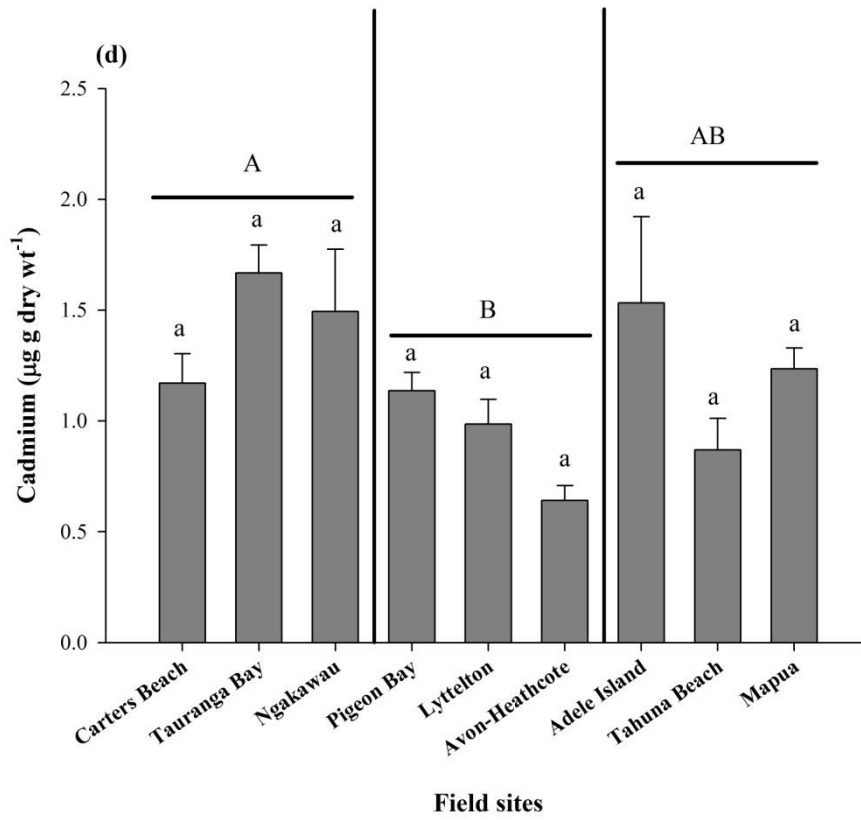
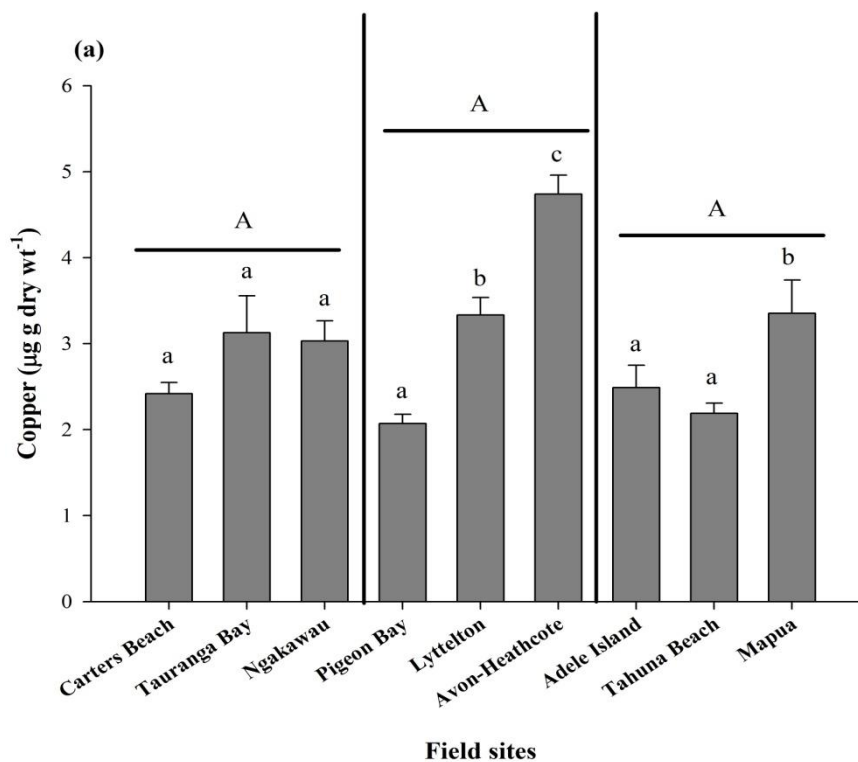
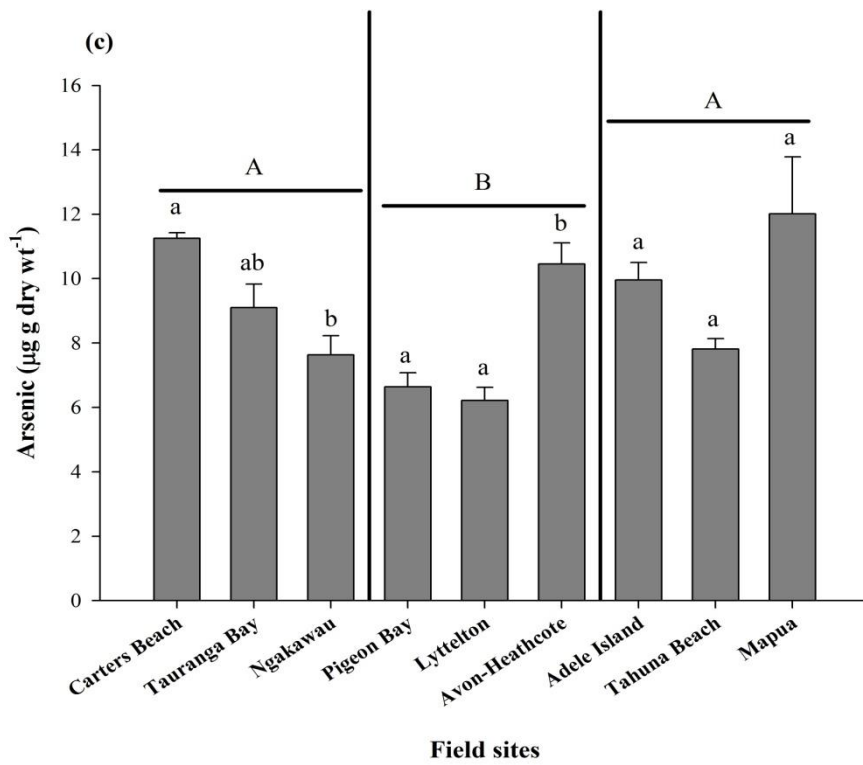
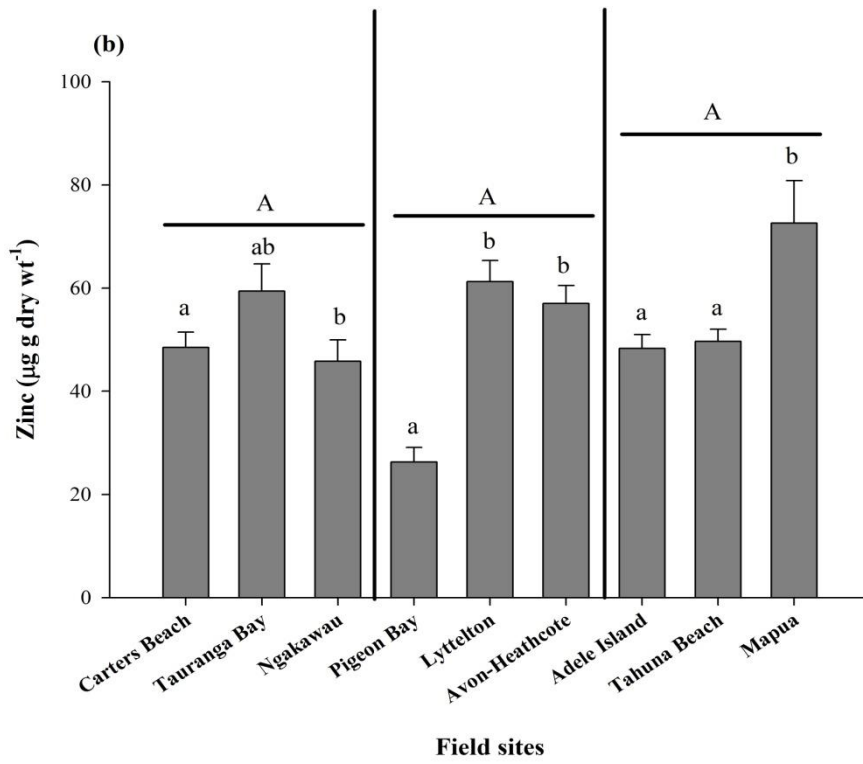


Figure 6.4a-e Concentration of trace metals (a) copper (b) zinc (c) arsenic (d) cadmium and (e) lead in the digestive gland of green-lipped mussels collected from different sites in New Zealand. Data are presented as mean \pm SEM ($n = 5-6$). See legend of Figure 6.3 for further details.

6.3.3 Metal concentrations in the mantle

There were no significant regional differences in Cu and Zn concentrations in the mantle of mussels (Fig. 6.5a and b). The concentrations of As and Cd were significantly lower in mantle of mussels from Canterbury region (Fig. 6.5c and d), while Pb concentrations were significantly higher in Canterbury mussels than the other two regions (Fig. 6.5e).





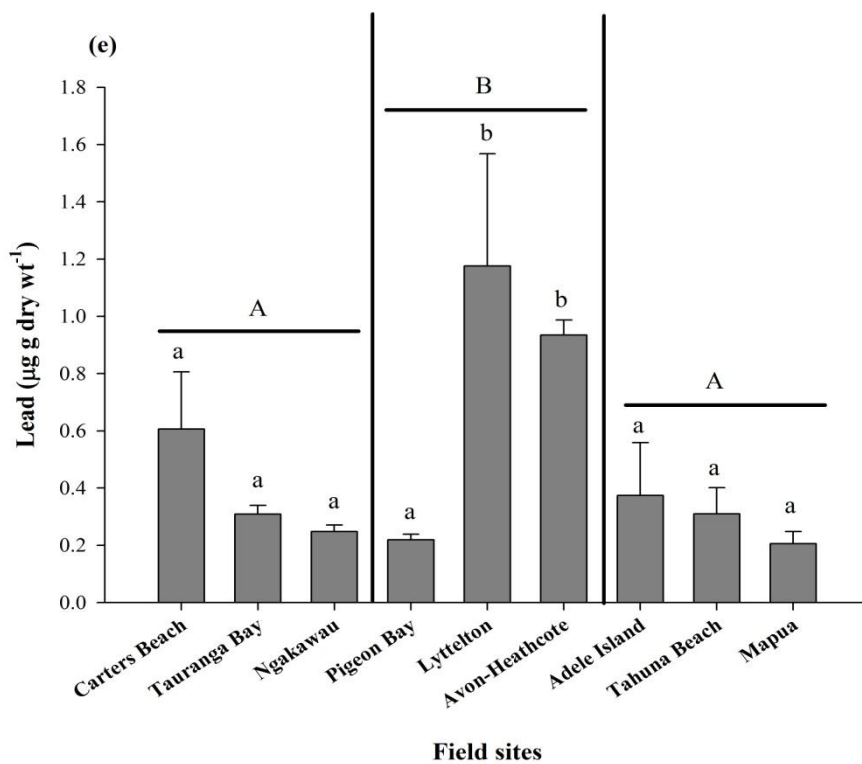
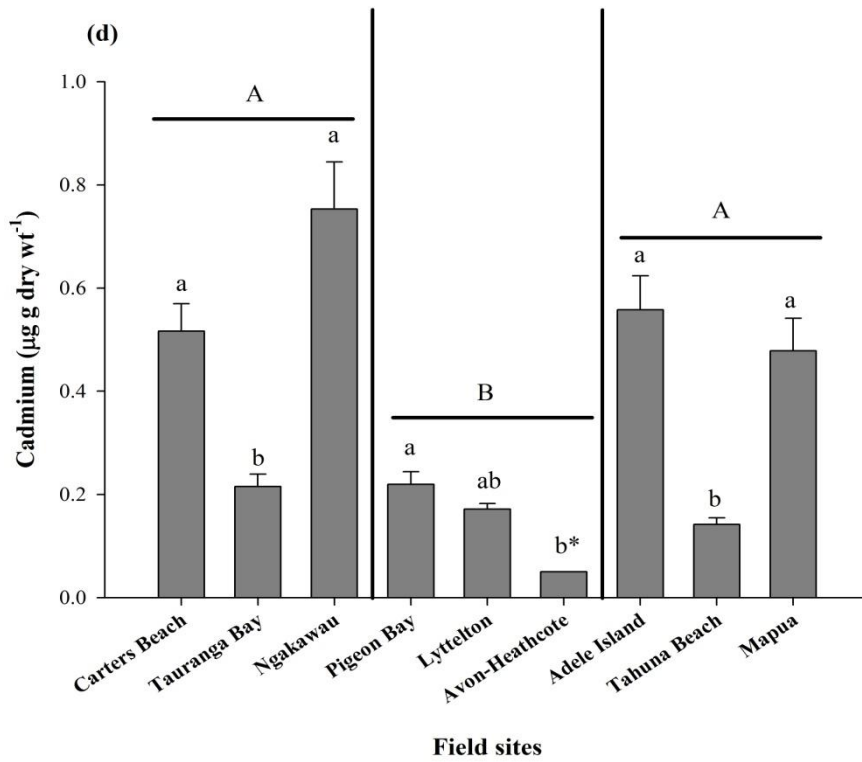
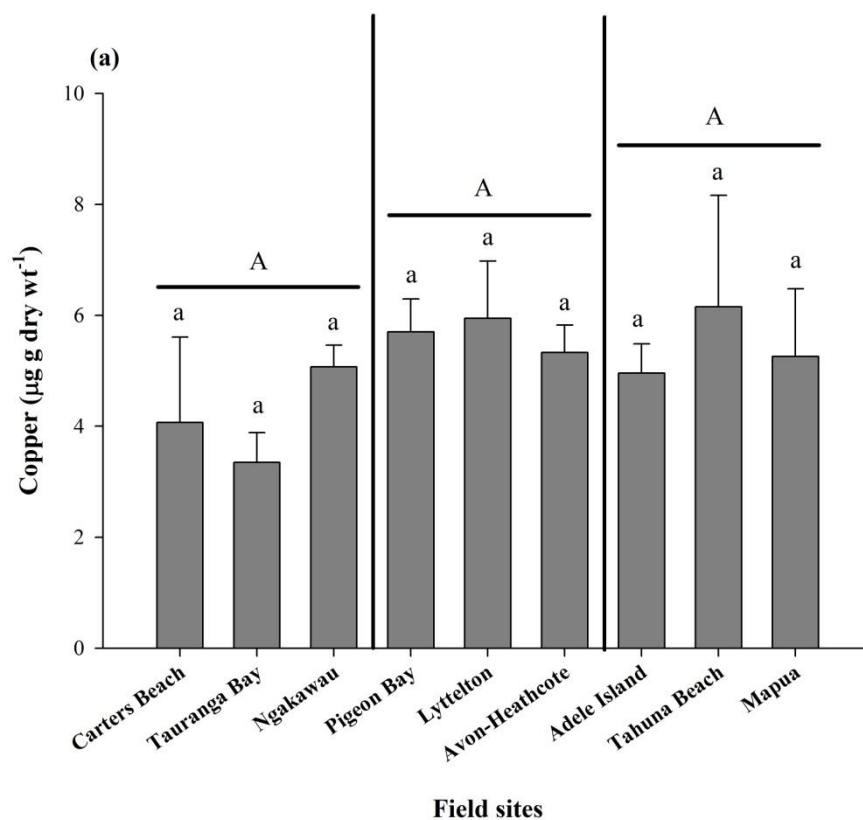
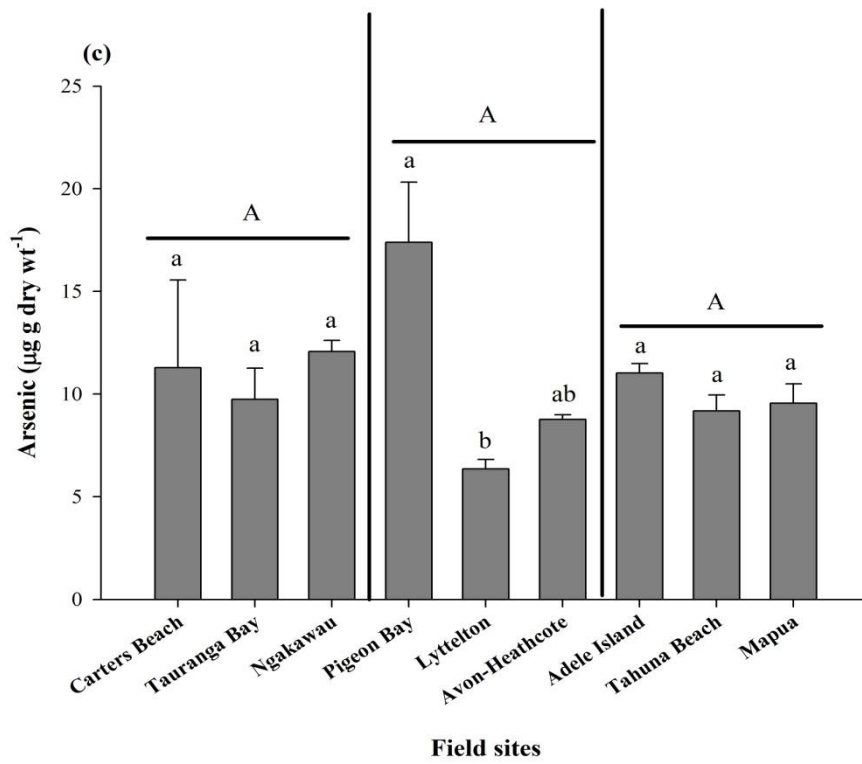
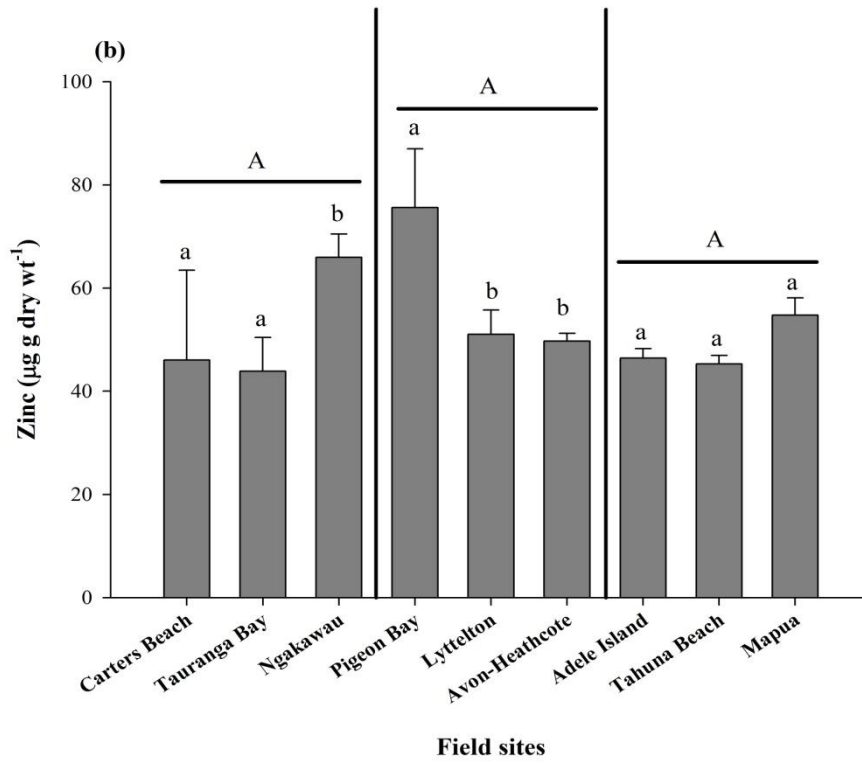


Figure 6.5a-e Concentration of trace metals (a) copper (b) zinc (c) arsenic (d) cadmium and (e) lead in the mantle of green-lipped mussels collected from different sites in New Zealand. Data are presented as mean \pm SEM ($n = 5-6$). See legend of Figure 6.3 for further details.

6.3.4 Metal concentrations in the foot

The concentration of metals measured in the foot of mussels is presented in Figures 6.6a-e. No regional differences were observed in the concentrations of Cu, Zn, As and Pb measured in the foot of mussels (Fig. 6.6a-c and e). The Nelson mussels had significantly higher Cd concentrations in the foot than the mussels from the other two regions (Fig. 6.6d).





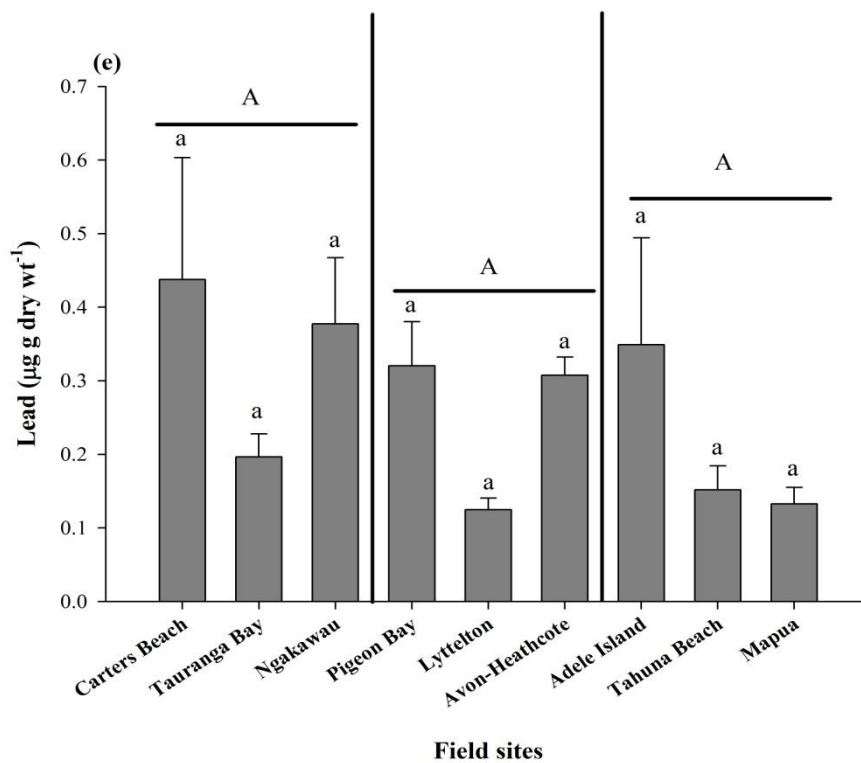
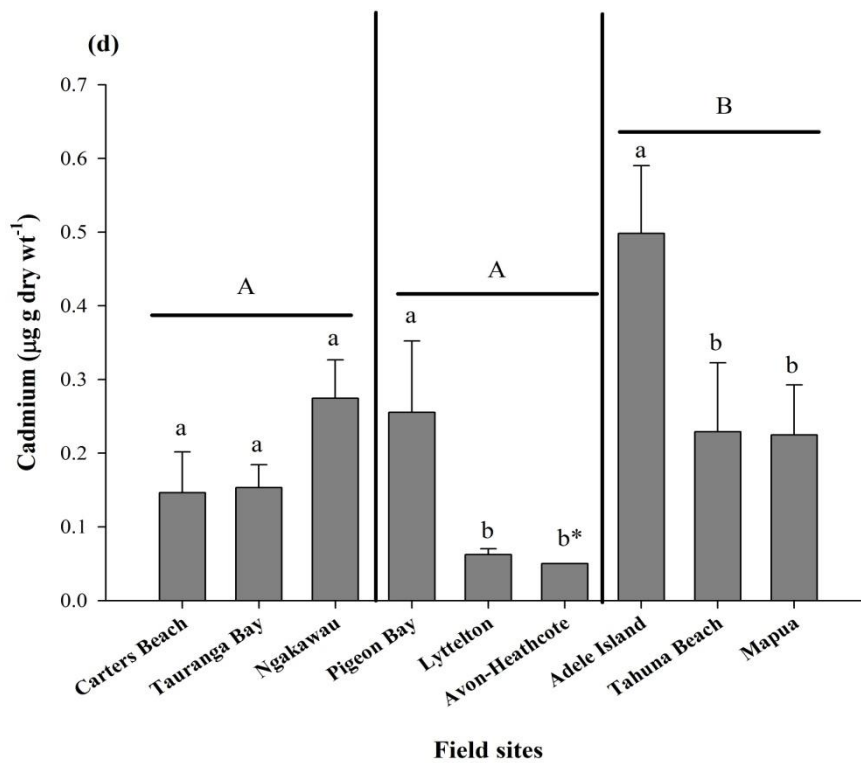
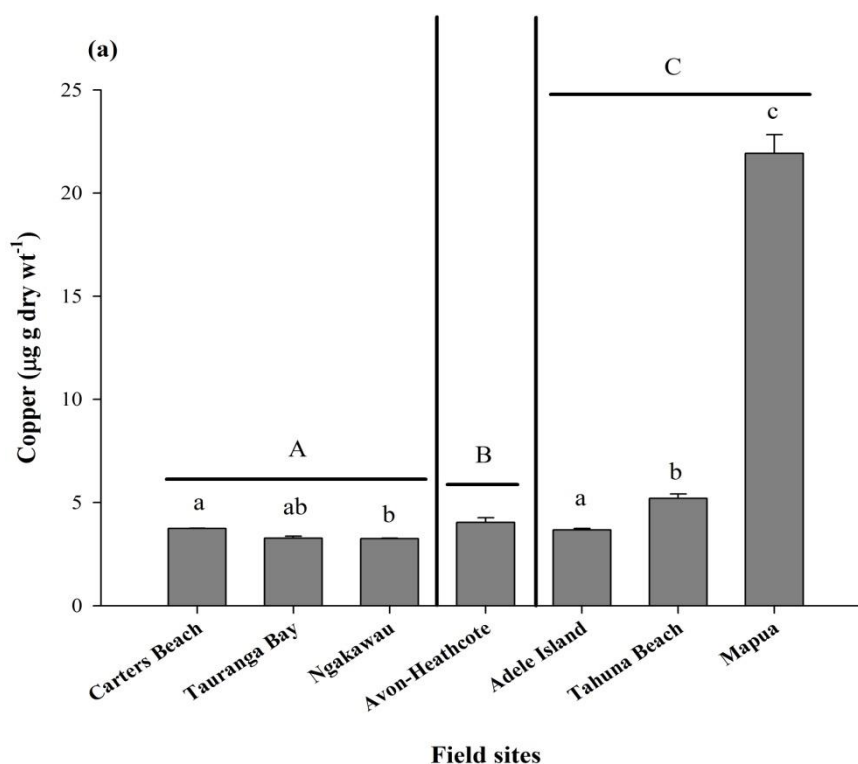
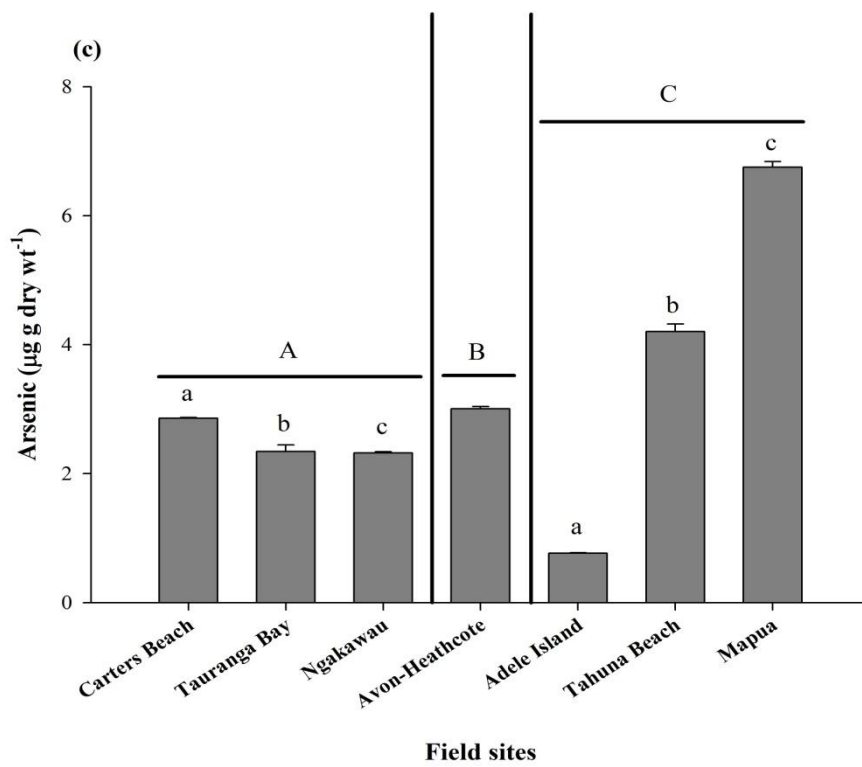
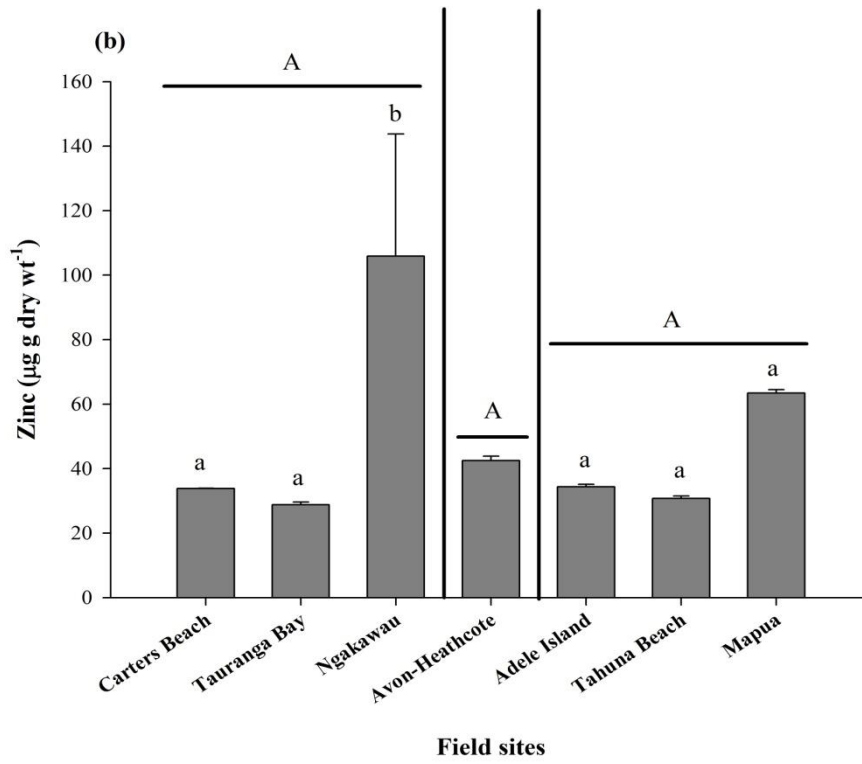


Figure 6.6a-e Concentration of trace metals (a) copper (b) zinc (c) arsenic (d) cadmium and (e) lead in the foot of green-lipped mussels collected from different sites in New Zealand. Data are presented as mean \pm SEM ($n = 5-6$). See legend of Figure 6.3 for further details.

6.3.5 Metal concentrations in the sediment

The total concentrations of Cu, Zn, As and Pb measured in the sediment samples collected from different sites are presented in Figures 6.7a-d (also see Table 6.8). The Cu concentrations in the sediment showed significant regional differences between the West Coast, Canterbury and Nelson regions ranging between 3.2 - 22 $\mu\text{g g}^{-1}$ (Fig. 6.7a). The highest concentration of 22 $\mu\text{g Cu g}^{-1}$ sediment was measured at Mapua (Nelson). Sediment Zn concentrations showed no significant regional differences with values ranging between 29 and 106 $\mu\text{g g}^{-1}$ (Fig. 6.7b). The concentrations of As ranged between 0.8 to 6.8 $\mu\text{g g}^{-1}$ with significant differences between the West Coast, Canterbury and Nelson regions (Fig. 6.7c). Concentrations of Pb varied between 3.4 - 12.3 $\mu\text{g g}^{-1}$ among the sampling sites, with significantly higher Pb concentrations in Nelson region compared to Canterbury and West Coast regions (Fig. 6.7d). The Cd concentrations were below the detection limits for all nine sampling sites (0.1 $\mu\text{g g}^{-1}$; Table 6.2).





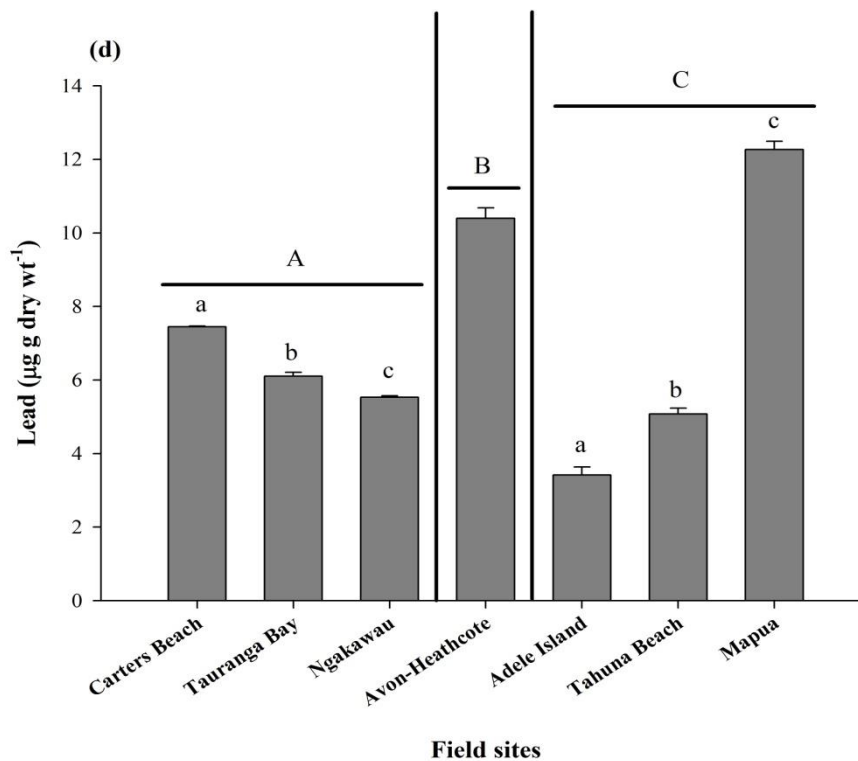


Figure 6.7a-d Concentration of trace metals (a) copper (b) zinc (c) arsenic and (d) lead in the sediments collected from different sites in New Zealand. Statistical significance ($p < 0.05$) was determined using one-way nested ANOVA followed by post hoc LSD analysis to indicate differences between regions and differences between sites within regions. Data are presented as mean \pm SEM ($n = 3$). Plotted points sharing letters are not statistically significant. Lowercase letters indicate differences between sites within a region, and uppercase letters indicate differences between regions. Pigeon Bay and Lyttelton sediment samples were not available and hence metal concentrations in these two sites were not determined. Cadmium concentrations were below detection limits for all sampling sites.

Correlation analyses between metal concentrations in the tissues of green-lipped mussels and sediment samples are presented in Table 6.4. A significant positive correlation was observed between Zn concentrations in the foot of mussels and Zn in the sediment ($R = 0.997$, $p <$

0.0001; Table 6.3, see *Appendix 4*). There were no other significant correlations between tissue and metal concentrations.

Table 6.3 *Correlations between metals in different mussel tissues and sediment across all collection sites*

Metal	Tissue	Correlation coefficient (R)	Equation of the line of best fit	p value
Cu	Gill	0.2920	$y = -0.5144x + 1.1872$	0.5252
	Digestive gland	0.7299	$y = 3.373x - 3.0432$	0.0626
	Mantle	0.1420	$y = 0.3728x + 0.5142$	0.7614
	Foot	0.3538	$y = 1.1972x - 0.1262$	0.4363
Zn	Gill	0.2105	$y = -0.3724x + 2.3603$	0.6506
	Digestive gland	0.5305	$y = 2.0006x - 2.3800$	0.2206
	Mantle	0.0138	$y = 0.0403x + 1.5688$	0.9766
	Foot	0.9974	$y = 3.2825x - 3.9337$	<0.0001
As	Gill	0.5937	$y = -1.4435x + 1.8422$	0.1599
	Digestive gland	0.4752	$y = -4.0648x + 6.1239$	0.2812
	Mantle	0.1837	$y = 0.7059x - 0.2647$	0.6933
	Foot	0.4896	$y = -2.7477x + 3.1973$	0.2648
Cd	Gill	0.4406	$y = -0.455x + 0.0207$	0.3224
	Digestive gland	0.5294	$y = -0.0716x + 0.0344$	0.2218
	Mantle	0.4796	$y = 0.0313x + 0.0403$	0.2761
	Foot	0.3927	$y = 0.0253x + 0.0429$	0.3836
Pb	Gill	0.1209	$y = 0.0929x + 0.8168$	0.7962
	Digestive gland	0.4015	$y = 0.4113x + 0.8018$	0.3720
	Mantle	0.1753	$y = 0.1452x + 0.8827$	0.7070
	Foot	0.3023	$y = -0.2791x + 0.6552$	0.5099

Significant values ($p < 0.05$) are indicated in bold. Data were \log_{10} transformed prior to regression analyses.

6.4 Discussion

The current study showed that the metal concentrations in the sediments and mussel tissues differed significantly between the three regions; in addition, significant differences also existed between reference sites and potentially contaminated sites within a region. The

mussels collected from sites with elevated metal concentrations in sediments had high concentrations of metals in their tissues. These results indicate the ability of green-lipped mussels to bioconcentrate metals in their tissues and the potential use of *P. canaliculus* as a bioindicator of coastal metal pollution in NZ.

6.4.1 Metal concentrations in mussel tissues

Based on the average of metal accumulation levels analysed in the tissues, results from the current study indicate that the digestive gland is the major metal accumulation organ followed by the gill, mantle and the foot. However, there are no previous studies on metal concentrations in the different tissues of *P. canaliculus* that would allow direct comparison of the results from this study. There are studies of metal tissue distribution of other bivalves from around the world (Table 6.4). For example, Bustamante and Miramand (2004) reported similar metal accumulation patterns in the tissues of different scallop species. The findings from the present study are also consistent with previous studies reported in other bivalves presented in Table 6.4.

Mussels accumulate metals through various routes and then redistribute them amongst different tissues (Widdows and Donkin, 1992). Often an increase in metal accumulation in the gill would suggest uptake of metals through facilitated diffusion from the dissolved phase as the gill is in direct contact with the external environment (Cooper et al., 2010). In addition, as the major organ involved in filter-feeding mechanism, the gill can also be associated with dietary metal uptake. The digestive gland is considered to be the organ involved in storage of metals associated with food, organic matter and sediment (Marigomé et al., 2002). This could explain the elevated metal concentrations measured in the digestive gland and the gill of mussels in the current study. The foot and mantle accumulated lower concentrations of metals than the gill and the digestive gland, which is in agreement with studies on other

bivalves (Table 6.4). Both the foot and the mantle are possibly involved in uptake and transfer of metals or used as transient storage organs (e.g. Yap et al., 2006). In the present study, it is likely that the low metal accumulation levels of the foot and the mantle is due to the functional role played by these tissues.

The differences in metal concentrations between the four tissues observed in the present study could be related to a combination of factors. Yap et al. (2010) hypothesised that the differences in metal accumulation between tissues can be explained on the basis of biochemical pathways (e.g. MT) and the similarities in chemical characteristics of the metals. In addition, different metal uptake pathways, the biological functions of individual metals and the role played by each tissue in storage and excretion can also affect metal distribution between tissues (Wang and Fisher, 1999; Blackmore and Wang, 2003a). In the current study, metal concentrations in all four mussel tissues were as follows, $Zn > As \geq Cu > Cd \geq Pb$.

6.4.1.1 Essential metals

Metals such as Cu and Zn are essential as they have key biological functions such as being enzyme co-factors vital for the normal functioning of aquatic organisms (see *Section 1.2*). Although both Cu and Zn are essential metals, Cu and Zn follow different bioaccumulation patterns in bivalves (e.g. Fukunaga and Anderson, 2011). It is known that bivalves can regulate essential metal ions such as Cu and Zn in their tissues (Bervoets et al., 2004). This includes organism-specific strategies and pathways for regulation of uptake, accumulation and elimination of Cu and Zn in the tissues (Vijver et al., 2004). Although essential for various physiological and cellular processes, excessive concentrations of Cu and Zn can be toxic to the organisms. The excess concentrations of these metals are stored in the detoxified form to prevent interaction with sensitive cell organelles. Metal sequestration studies in zebra mussels showed that Cu was predominantly associated with MT and heat shock proteins

while most Zn was found to be associated with cell organelles and to a lesser extent with detoxification proteins (Voets et al., 2009). However, the strategies involved in regulation of Cu and Zn differ between species (e.g. Ju et al., 2011).

Copper – The results of the current study showed that compared to the other regions the Canterbury mussels had significantly lower concentrations of Cu in their digestive gland. Cu concentrations measured in the gill, mantle and foot did not show significant regional differences. The tissue Cu concentrations measured in the present study ranged between 3.34 - 16.3 $\mu\text{g g}^{-1}$ dw were comparable to Cu bioaccumulation levels reported in the mussel, *Perna viridis* from a polluted site in Malaysia (Yap et al., 2006; Edward et al., 2009; Table 6.9). In the current study, Cu concentrations were similar in both the gill and digestive gland of mussels. Similar pattern of Cu accumulation in the range of $\sim 4 - 6 \mu\text{g g}^{-1}$ dw has been reported in the gill and digestive gland of mussel, *Mytilus galloprovincialis* (Sakellari et al., 2013). Cu is stored in the cytoplasmic granules of the gill in the mussel, *Mytilus galloprovincialis* (Soto et al., 1996) while the lysosomes in the digestive gland of mussels are known for detoxification of Cu (Viarengo et al., 1985). The similar concentrations of Cu measured in the gill and the digestive gland indicates that *P. canaliculus* can regulate internal Cu concentrations to maintain homeostasis.

Zinc – In the present study, Zn concentrations analysed in the four tissues ranged between 26.3 - 128.4 $\mu\text{g g}^{-1}$ dw and did not show any significant regional differences. Zn accumulation in the gill and the digestive gland of mussels were similar and followed the same pattern as Cu. Results of Zn concentrations in the four tissues from the current study were comparable with concentrations of 47.6 - 92.8 $\mu\text{g g}^{-1}$ dw reported in the mussel *Perna viridis* from a polluted site in Malaysia (Edward et al., 2009; Table 6.9). Zn concentrations reported in other bivalves were similar to the range of concentrations reported in the four

tissues for green-lipped mussels in the present study (see Table 6.9). In general, bivalves accumulate more than required concentrations of Zn in the gill, the mantle and the digestive gland (Eisler, 2010). The elevated Zn accumulation levels are due to the involvement of Zn in biological functions as a co-factor for several enzymes (e.g. carbonic anhydrase, alkaline phosphatase). In the tissues of the mussel *Perna viridis*, Zn was stored in the form of metal-insoluble inclusions (Blackmore and Wang, 2002). Similar to Cu, the Zn distribution in the mussel tissues suggests homeostatic regulation of Zn probably through specific accumulation and detoxification strategies.

6.4.1.2 Non-essential metals

Metals such as As, Cd and Pb have no known biological functions and are highly toxic to aquatic organisms (Wood et al., 2011). Mussels possess inbuilt mechanisms that aid in storage and detoxification of non-essential metals (Rainbow, 2002). For example, metal binding proteins such as MT, and storage of metals within lysosomal granules can limit toxic metals from binding to metal-sensitive fractions of the cell (Marigómez et al., 2002). The concentrations of As in digestive gland of mussels along with Cd and Pb concentrations from the current study suggest sequestration of these metals for detoxification. The toxic metals are bound to metal-rich granules, which can be eliminated (Rainbow and Smith, 2010). Though Cd, Pb and As are non-essential the uptake, storage, transport and elimination pathways for these metals may vary amongst species (e.g. Wallace et al., 2003).

Arsenic – The concentrations of As in the digestive gland of West Coast mussels were significantly higher than the Canterbury mussels while mantle concentrations in Canterbury mussels were significantly lower than the other two regions. No regional differences were found between the gill and foot concentrations. Overall, As concentrations observed in green-lipped mussels showed higher accumulation in the digestive gland of 15.7 - 27.5 $\mu\text{g g}^{-1}$ dw

compared to the other tissues (Table 6.4). Whaley-Martin et al (2012) also reported high As accumulation in the digestive gland of blue mussels, *Mytilus edulis*. These authors hypothesised that As enters mussels via the gills by association with sediment or food and is eventually absorbed in the digestive gland.

Marine sediments and seawater contain inorganic As in toxic chemical forms such as As (III) and As (V) (e.g. Fattorini et al., 2006; Mamindy-Pajany et al., 2013). Arsenobetaine and arsenosugars are the organic forms of As found in algae, and can accumulate in mussels via the diet. In mussels (e.g. *Mytilus galloprovincialis*), As is present mainly in the digestive gland in organic and less toxic inorganic forms; (Argese et al., 2005).

Cadmium – Cd accumulation in the digestive gland and mantle was significantly higher in West Coast mussels compared to the Canterbury region. The concentrations of Cd measured in the mussels from the current study ranged between 0.05 - 1.7 $\mu\text{g g}^{-1}$ dw (Table 6.9). The high Cd concentrations in the West Coast mussels could be due to the presence of Cd in mining effluents and acid-mine drainage. The presence of metal-rich rocks could also leach different metals including Cd into the environment (e.g. Brown et al., 2005).

Cd accumulation was higher in the digestive gland than the other tissues. This is in agreement with previous results reported earlier in the thesis where mussels showed significantly higher accumulation levels in the digestive gland than the gill (*Section 3.3.1*). The digestive gland acts as the major site for Cd sequestration in bivalves via detoxification by MT production (e.g. Ngo et al., 2011; *Chapter 4*). In addition, the slow rate of Cd excretion in the digestive gland of bivalves has also been well documented (e.g. Hervé -Fernández et al., 2010). In the current study, although Cd concentrations in the sediment were below detection limits for all the sites, Cd concentrations in the mussel tissues were similar to the values recorded in other mussel species from contaminated sites (Table 6.4). Similar to the current study, other studies

have also reported Cd bioaccumulation in mussels even though sediment Cd concentrations were not detectable (e.g. Giarratano and Amin, 2010; Duarte et al., 2011). It is known that Cd concentration in biota is of a higher magnitude than the Cd levels present in the sediment and seawater (e.g. Apeti et al., 2009). This is primarily due to the linear uptake of Cd from the dissolved aquatic phase and low Cd elimination rate in bivalves (e.g. Fisher et al., 1996). The low excretion rate can be explained by the presence of detoxification mechanisms such as MT (Viarengo and Nott, 1993). The Cd results from this study are in agreement with this concept.

Lead – Accumulation of Pb was significantly higher in the gill and mantle of mussels from the Canterbury region compared to the other two regions whereas Nelson mussels had significantly lower Pb concentrations in the digestive gland compared to the other two regions. No regional differences were noted in Pb concentrations in the foot of mussels. The relatively high Pb concentrations in Canterbury mussels could be related to the increased urbanisation, industrial pollution and sewage discharge in this region (e.g. stormwater runoff in Avon-Heathcote).

In the current study, the concentrations of Pb were similar in mussel gill and digestive gland ranging between 0.4 - 2.43 and 0.6 - 2.24 $\mu\text{g g}^{-1}$ dw, while the mantle and foot values were between 0.13 - 1.18 $\mu\text{g g}^{-1}$ dw (Table 6.4). Differences in tissue Pb concentrations have been previously reported in other bivalve species. Accumulation of Pb in the gills was the highest in the mussel, *Perna viridis* exposed to Pb followed by the viscera, mantle and foot (Yap et al., 2004b; Table 6.4). The digestive gland of the scallop *Pecten maximus*, accumulated higher Pb concentrations than the gill, mantle and foot (Saavedra et al., 2008; Table 6.4). Compared to Pb accumulation levels in other bivalves, the relatively low Pb accumulation levels in mussels from this study could be related to low bioavailability (e.g. Fisher et al.,

1996). Metals such as Pb have been reported to accumulate in the mitochondria of gills and lysosomes present in the digestive gland of mussels, *Mytilus edulis* (e.g. Einsporn and Koehler, 2008). Exposure of scallops to Pb showed that Pb was associated with the insoluble subcellular fractions and accumulated in the digestive gland (Metian et al., 2009).

Table 6.4 Metal concentrations in soft tissue of different mussel species expressed as $\mu\text{g g}^{-1}$ dry weight.

Mussel species	Tissues	Metals					References
		Cu	Zn	Cd	As	Pb	
<i>Perna canaliculus</i> (New Zealand)	Gill	7.21 - 13.46	67.40 - 123.05	0.30 - 1.14	5.35 - 12.63	0.49 - 2.44	This study
	Digestive gland	9.01 - 16.27	83.76 - 128.47	0.64 - 1.67	15.71 - 27.48	0.69 - 2.25	
	Mantle	2.07 - 4.74	26.30 - 72.58	0.05 - 0.75	6.22 - 12.01	0.21 - 1.18	
	Foot	3.34 - 6.15	43.89 - 75.60	0.05 - 0.50	6.36 - 17.40	0.13 - 0.44	
<i>Perna viridis</i> (Malaysia)	Gill	11.0	89.8	1.53	-	28.6	Edward et al., 2009
	Remainder	12.3	92.8	0.925	-	6.47	
	Mantle	12.0	47.6	1.29	-	23.1	
	Foot	8.77	49.3	1.2	-	35.2	
<i>Perna viridis</i> (Malaysia)	Gill	11.7	78.9	1.76	-	2.82	Edward et al., 2009
	Remainder	13.6	61.3	0.94	-	9.08	
	Mantle	6.69	48.7	1.23	-	1.34	
	Foot	5.94	51.6	1.76	-	0.673	
<i>Perna viridis</i> (Malaysia)	Gill	6.07 - 14.70	61.70 - 167.00	0.93 - 2.38	-	5.90 - 24.90	Yap et al., 2006
	Remainder	5.52 - 15.70	62.70 - 145.00	0.85 - 2.87	-	6.98 - 26.20	
	Mantle	4.99 - 12.50	39.10 - 108.00	0.78 - 3.31	-	5.50 - 38.90	
	Foot	6.39 - 10.00	40.70 - 89.70	0.93 - 1.94	-	2.68 - 22.90	
<i>Pecten maximus</i> (Iberian Peninsula)	Gill	7.6	213.0	6.1	16.0	1.7	Saavedra et al., 2008
	Digestive gland	28.0	210.0	120.0	15.0	4.8	
	Mantle	4.5	95.0	3.6	9.9	0.91	
	Foot	6.5	75.0	5.6	8.3	0.95	
<i>Mytilus edulis chilensis</i> (Beagle Channel)	Gill	6.49 - 11.98	83.97 - 270.63	0.38 - 2.11	-	BDL - 15.57	Giarratano et al., 2010
	Digestive gland	12.07 - 25.76	63.17 - 102.54	0.30 - 3.25	-	3.37 - 9.17	

BDL - Below Detection Limit

6.4.2 Metal concentrations in the sediments

Overall, in the current study, analysis of the metal content in sediment samples showed significant regional and site differences in Cu, As and Pb concentrations. Of the metals analysed in the sediments, the average total metal concentrations indicate Zn (29 - 106 $\mu\text{g g}^{-1}$ dw) as the most abundant metal followed by Cu (3.2 - 22 $\mu\text{g g}^{-1}$ dw), Pb (3.4 - 12 $\mu\text{g g}^{-1}$ dw) and As (0.76 - 6.8 $\mu\text{g g}^{-1}$ dw). However, no regional differences were observed in Zn sediment concentrations while Cd levels were consistently below detection limits at all sampling sites. The results of the sediment metal concentrations were compared with SQGs (Table 6.5 and 6.6). The use of SQGs help to assess the level of ecological risk associated with the metal contamination measured in sediments from different sampling sites (e.g. Simpson et al., 2005; see *Section 1.3.3*).

Sediment trace metal concentrations from the different sampling sites were compared to the ISQG values for metal concentrations issued for Australia and New Zealand (ANZECC & ARMANC, 2000). The metal concentrations from all sampling sites were well below the ISQG-low trigger values (ANZECC & ARMANC, 2000; Table 6.5). Comparison of sediment metal concentrations from the current study with ISQG indicated that the possibility of any biological impacts on organisms was highly unlikely at the metal concentrations reported in the current study.

Sediment metal concentrations from the current study were also compared to US EPA SQGs, to classify the level of metal pollution between sites (Table 6.6). The results indicated moderate Zn contamination in the West Coast region and moderate As contamination in Canterbury and Nelson regions (Table 6.6).

Table 6.5 Australian New Zealand interim sediment quality guideline (ISQG) values issued by ANZECC & ARMICANZ (2000).

Metals	ISQG-low	ISQG-high	Sediment (this study)
Arsenic	20	70	0.8 - 6.8
Copper	65	270	3.3 - 21.9
Lead	50	220	3.4 - 12.3
Zinc	200	410	28.8- 105.9

Values expressed as mg kg^{-1} dry weight

Cadmium concentrations were below detection limits

Table 6.6 US EPA sediment quality guidelines (SQG) and average sediment metal concentrations measured in three regions in this study.

Metals	US EPA SQG			This study		
	No pollution	Moderate pollution	Heavy pollution	West Coast	Canterbury [†]	Nelson
Arsenic	<3	3-8	>8	2.3 - 2.9	3.0*	0.8 - 6.8*
Cadmium	-	-	>6	<0.1	<0.1	<0.1
Copper	<25	25-50	>50	3.3 - 3.7	4.0	3.7 - 21.9
Lead	<40	40-60	>60	5.5 - 7.4	10.4	3.4 - 12.3
Zinc	<90	90-200	>200	28.8 - 105.9*	42.5	30.8 - 63.5

Values expressed as mg kg^{-1} dry weight

*Moderate pollution

US EPA SQG values (Pedersen et al., 1998)

[†]Canterbury region is represented by Avon-Heathcote, sediment samples were not available for Pigeon Bay and Lyttelton.

A well documented natural source of As in NZ is the presence of gold and coal mines along with As-enriched geothermal sites (e.g. Mroczek, 2005; ECAN, 2012). Additionally, the long-term use of As in pesticides, in biocides for sheep, cattle and pig dips and leaching of As from treated timber are the other common sources of As input throughout NZ (e.g. ARC,

2002; Bourne, 2003). Sediments from Mapua in the Nelson region had the highest concentration of As (Table 6.7). Pesticide manufacturing industry at Mapua was operational during 1938 to 1988 and is likely to be the major source of As at this site (MfE, 2010). The overall average concentration of As reported in sediments in the current study was lower in comparison to the concentrations reported in other countries as shown in Table 6.7. The As concentrations from the current study was also found to be lower than the As concentrations reported at other sites within NZ; 12 to 72 $\mu\text{g g}^{-1}$ As measured at Richmond (Nelson region) and 19 - 61 $\mu\text{g g}^{-1}$ As at Waikato (Rumsby, 2009; TDC, 2010; Table 6.7).

Apart from elevated As concentrations, the Nelson region also had significantly higher concentrations of Cu and Pb compared to the other regions (Fig. 6.7 a and d). A combination of different natural and anthropogenic sources of metal input could be responsible for metal contamination within this region. The leaching of metals from ultramafic rocks in the Tasman district, use of pesticides and fertilisers in orchards, sewage disposal and stormwater runoff could contribute to the elevated sediment metal concentrations observed in the Nelson region (Brown and Peake, 2006; TDC, 2012). Sources of As, Cu and Pb include insecticide, fungicide and paint formulations used in NZ (ARC, 2002).

With the exception of Mapua, the concentrations of Cu (3.3 - 5.21 $\mu\text{g g}^{-1}$ dw) and Pb (3.4 - 10.4 $\mu\text{g g}^{-1}$ dw) reported in the current study were comparable to 6.98 $\mu\text{g g}^{-1}$ dw of Cu and 5.48 $\mu\text{g g}^{-1}$ dw of Pb in the sediments at a low contaminated site in Argentina (Marinho et al., 2013; Table 6.7). The concentrations of both Cu and Pb concentrations in sediments from the current study were lower than those reported in other sites within NZ and in other countries (Table 6.7).

No regional differences were observed in Zn sediment concentrations. Comparison with US EPA SQG values indicate that Ngakawau in the West Coast region is affected by moderate

Zn contamination (Table 6.6). Mining activity is the most likely cause of Zn contamination in the West Coast region. The leaching of metals from pyrite rocks due to acid mine drainage is an important source of Zn and other metals such as As and Pb (McLaren et al., 1998; WCRC, 2006; Haffert and Craw, 2008). Other sources of Zn in NZ include weathering of galvanised iron roofing and sewage sludge inputs and stormwater runoff (Kingett Mitchell Ltd., 2003; Brown and Peake, 2006). This includes metal input from discharge of treated sewage water from Christchurch into the Avon-Heathcote estuary from 1958 to 2010 (McMurtrie and Kennedy, 2012). Several stormwater runoff pipes also open directly into the estuary discharging metals from local industrial and urban areas (ECAN, 2012). The sediment metal concentrations reported for Avon-Heathcote estuary in the current study were within a similar range to the values reported previously for the estuary by ECAN (2011a) (Table 6.8). The metal concentrations reported by ECAN were measured from samples collected at Avon-Heathcote in March-April, 2011 (Table 6.8). Avon-Heathcote estuary was affected by the February 2011 earthquakes with liquefaction resulting in new sediment inputs and discharge of untreated sewage into the estuary (ECAN, 2011b). Environmental factors such as salinity and temperature can also affect the behaviour of metals present in the sediments. For example, the low salinity values measured at Avon-Heathcote Estuary could have affected metal partitioning in the sediments at this site. In general, estuaries are prone to fluctuations in salinity that inevitably causes changes in metal bioavailability and consequently, toxicity in organisms (e.g. Chapman and Wang, 2001; see *Section 6.1*). Among the five metals analysed in the sediments, Cd concentrations were below detection limits. However, previous reports of Cd concentrations at sites within NZ were within the range of $< 0.01 - 0.22 \mu\text{g g}^{-1}$ dw sediment.

Overall the metal concentrations from the current study were similar to average values of $< 0.63 - 6.98 \mu\text{g g}^{-1}$ Cu, $7.9 - 46.5 \mu\text{g g}^{-1}$ Zn, and $5.48 \mu\text{g g}^{-1}$ Pb reported at uncontaminated to

low contamination sites at San Jorge Gulf in Argentina (Marinho et al., 2013; Table 6.7). Certain sites from other countries were tabulated as uncontaminated or low contamination sites based on the findings and conclusions reached from the study in the given region (Table 6.7). Comparison of metal concentrations from the current study with other study sites within NZ and at different locations around the world also suggests that metal contamination at the sampling sites used in the present study were relatively low (Table 6.7).

6.4.3 Possible effects of environmental factors

Differences in salinity and temperature could have had an effect on the metal uptake and accumulation in mussels from the different sampling sites. For example, low salinity can increase metal uptake and accumulation in mussels (e.g. *Perna viridis*, Blackmore and Wang, 2003b). In this study, the salinity measured at Avon-Heathcote was 24.7 ‰, lower than the other sampling sites which ranged between 30-33 ‰ (Table 6.1). The Avon-Heathcote estuary is known to have salinity values as low as 8-12 ‰ (Marsden, 2004). Compared to the other sampling sites, the low salinity levels at Avon-Heathcote estuary (Canterbury) could have influenced the higher metal accumulation in those mussels.

High temperatures can increase metal uptake in mussels, although the effects of temperature are reported to be metal-specific (e.g. Mubiana and Blust, 2007). The water temperature measured at the different sampling sites was between 10.4°C - 16°C (Table 6.1). The average water temperature measured in the Nelson region was 15°C, which could have increased metal uptake in mussels compared to the other sites. The present study indicates that variations in metal accumulation in mussels could be influenced by differences in environmental factors between sampling sites.

The variations in metal accumulation patterns in the four tissues observed in this study can also be related to variability in uptake and accumulation rate of different metals (Rainbow,

2002; Luoma and Rainbow, 2005). Moreover, pre-exposure to metals could influence changes in uptake and accumulation rates of different metals (Wang and Rainbow, 2005). The difference in metal accumulation in mussels between regions could also be related to variations in food resources (phytoplankton). Overall, the factors influencing metal accumulation in mussels discussed above have similar effects on other mussel species used as bioindicators around the world (see *Section 1.5.1* and Table 1.5).

ISQG values are derived using an empirical approach. The values are derived from a database of matched sediment chemistries and biological effects to assess the likelihood of any adverse biological impacts at a given sediment concentration. The empirical approach does not include bioavailability as a factor for assessment of impacts (Burgess et al., 2013). Metals are distributed in the acid-soluble, exchangeable, oxidisable and/or residual phases of the sediments (e.g. Morillo et al., 2002). Moreover, the effects of sediment characteristics such as grain size, presence of organic matter and its association with metal concentrations can influence metal bioavailability. These factors could explain the lack of correlation between metal concentrations in the mussels and sediments, with the exception of the significant correlation observed between Zn concentration in the mussel foot and sediment (Table 6.3). Understanding metal partitioning in sediments can provide information on the bioavailability of metals and associated toxic effects in aquatic organisms. Further studies are required to investigate the distribution and fate of metals in sediments for better interpretation of the results from the current study.

Table 6.7 Sediment metal concentrations reported in New Zealand and in other countries

Location	Sampling site	Cu	Zn	As	Cd	Pb	References
Current study (New Zealand)	Carters Beach	3.75	33.85	2.86	<0.1	7.45	This study
	Tauranga Bay	3.27	28.8	2.34	<0.1	6.1	"
	Ngakawau	3.25	105.89	2.32	<0.1	5.53	"
	Avon-Heathcote	4.04	42.46	3	<0.1	10.4	"
	Adele Island	3.67	34.41	0.76	<0.1	3.42	"
	Tahuna Beach	5.21	30.76	4.2	<0.1	5.08	"
	Mapua	21.93	63.48	6.75	<0.1	12.27	"
Other sites (New Zealand)	Port Waikato (Waikato)	1.4 - 28.0	23.0 - 92.0	19.0 - 61.0	<0.01 - 0.05	1.5 - 17.0	Rumsby, 2009
	Lyttelton (Canterbury)	7.4 - 9.7	50.0 - 60.0	-	<0.1	17.0 - 18.0	Sneddon and Bailey, 2009
	Avon-Heathcote (Canterbury)	3.5 - 11.8	34.0 - 92.0	-	0.025 - 0.157	7.5 - 23.0	ECAN, 2011a
	Richmond (Nelson)	24.0 - 120.0	120.0 - 730.0	12.0 - 72.0	<0.1 - 0.22	15.0 - 58.0	TDC, 2010
	Tamaki Estuary (Auckland)	21 - 47.0	138 - 272	-	0.11 - 1.0	51 - 122	Abraham and Parker, 2008

Metal concentrations are expressed as $\mu\text{g g}^{-1}$ dry weight

(Continued on next page)

Location	Sampling site	Cu	Zn	As	Cd	Pb	References
Other countries (Contaminated)	Australia (Sydney Estuary)	137.0 - 432.0	538.0 - 1569.0	-	-	220.0 - 694.0	Birch et al., 2013
	Spain (Galicia coast)	4.5 - 1205.0	29.6 - 826.7	6.5 - 25.0	-	32.5 - 177.5	Bellas et al., 2008
	Italy (Taranto Gulf)	42.4 - 52.3	86.8 - 129.0	-	-	44.7 - 74.8	Buccolieri et al., 2006
	China (Bohai Bay)	7.9 - 46.7	34.0 - 123.0	-	0.05 - 0.19	18.8 - 39.1	Gao and Li, 2012
	Croatia (Reijka Harbour)	30.6 - 429	69.8 - 1260	9.50 - 37.7	0.14 - 4.66	23.6 - 637	Cukrov et al., 2011
	Turkey (Izmit Bay)	24.5 - 102.4	440 - 1900	13.5 - 28.2	2.5 - 9.5	55.2 - 172	Pekey, 2006
	Korea (Masan Bay)	13.45 - 90.69	79.98 - 378.73	-	0.10 - 7.47	13.03 - 82.16	Hyun et al., 2007
	Malaysia (Peninsular Malaysia)	1.63 - 150.81	23.70 - 609.20	0.11 - 311.84	1.06	7.97 - 93.11	Zulkifli et al., 2010
	Argentina (Ushuaia Bays)	18.76 - 33.32	63.82 - 96.22	-	0.74 - 2.14	21.59 - 35.81	Comoligo et al., 2011
Uncontaminated/ low contamination	Turkey (Gulf of Saros)	19	73	-	-	22	Sarı and Çağatay, 2001
	USA/Mexico (Baja California)	12.8	68.3	-	0.17	13.6	Villaescusa-Celaya et al., 2000
	UK (Medway Estuary)	42	138	14	-	67	Spencer, 2002
	Argentina (San Jorge Gulf)	<0.63 - 6.98	7.9 - 46.5	-	<0.25	5.48	Marinho et al., 2013

Metal concentrations are expressed as $\mu\text{g g}^{-1}$ dry weight

6.5 Conclusions

This is the first study conducted to assess metal concentrations in separate tissues of green-lipped mussels and in the environment (i.e. sediment). This study has shown both regional and site-specific variation in the metal concentrations in mussel tissues and sediments. For example, the significantly high Cu and Zn concentrations in Mapua and Ngakawau sediments were reflected in the digestive gland of mussels from these two sites (Fig. 6.4a-b and 6.7a-b). Similarly, high concentrations of Pb in Avon-Heathcote sediment coincided with the elevated Pb concentrations in the digestive gland of the Avon-Heathcote mussels (Fig. 6.4d and 6.7d). The findings from the current study indicate that the green-lipped mussels are ideal bioindicators that reflect metal contamination levels in the environment. *P. canaliculus* is a suitable endemic species that can be used for monitoring metal pollution in NZ. Additionally, based on the four tissues analysed, the digestive gland and gill could provide a more accurate understanding of metal accumulation in mussels than whole-body measurements.

In general, sediment metal concentrations reported in this study reflected low metal contamination levels. Despite metal input from potentially different natural and anthropogenic sources, the comparison of sediment metal concentrations with ISQGs indicates that there is no ecological risk associated to the environment. The results from the current study suggest that metal accumulation in mussels and in sediments may be influenced by the existence of a complex interaction between several biotic and environmental variables. Further studies are required to fully understand the complex metal interactions between the environment and the mussels, in the NZ setting.

7 Field-to-laboratory transport protocol impacts subsequent physiological biomarker response in the marine mussel, *Perna canaliculus*[†]

[†]**Chandurvelan, R.,** Marsden, I.D., Gaw, S., Glover, C.N., 2013. Field-to-laboratory transport protocol impacts subsequent physiological biomarker response in the marine mussel, *Perna canaliculus*. **Comparative Biochemistry and Physiology**, 164A: 84-90.

7.1 Introduction

Biomonitoring programmes using mussels largely rely on field-collected data. However, experimental manipulation of mussels under laboratory conditions plays a key role in providing meaningful information to environmental settings (Akaishi et al., 2007). Such experiments involve transferring animals from the field to the laboratory, a process which could range in time from a few hours to a few days. Furthermore, mussel transplantation between natural environments is common for environmental biomonitoring and similar ecological assessments (e.g. Honkoop et al., 2003).

Collection and transport of mussels from the field involves multiple steps that might induce a stress response in the mussels, and which could have impact on the subsequent biomarker analyses. These steps include: (a) sampling procedure – cutting byssus threads of mussels off rocky substrates; (b) transportation medium – air or water; (c) duration of transport; (d) physiochemical properties maintained during transport – temperature, salinity, pH; (e) cleaning or removal of epibionts (barnacles, chitons, seaweeds) off mussel shells; (f) duration of maintenance in laboratory conditions before experimentation. Hence any data obtained from transported mussels will reflect the cumulative effect of stress responses related to these artificial stressors, in addition to those that relate to the ecosystem of origin. This could confound interpretation of environmental monitoring data.

There are few guidelines available for transportation of mussels and these differ in their recommendations. Widdows and Staff (2006) suggest that mussels should be transported air-exposed in insulated containers maintained at $\sim 5^{\circ}\text{C}$. Another common procedure involves the transportation of mussels between sites in either artificial seawater (Hicks and McMahon, 2002) or in aerated water sourced from the collection site (Viarengo et al., 1982). The quality of the seawater used and the duration between collection and experimentation are considered

crucial factors in limiting the effects of transportation stress (Widdows and Staff, 2006). It has also been reported that the duration of recovery from stress depends on the species tested (Widdows, 2009). Among the numerous biomarkers that have been applied to assess stress responses in mussels, physiological measurements and SFG are considered to have the greatest utility (*Section 1.6.2*).

The primary objective of this study was to investigate the transport-related stress in *P. canaliculus* by measuring physiological changes during a simulated transport period and after recovery. This study would help elucidate the effects of transportation stress in *P. canaliculus*. As green-lipped mussels are a valuable aquaculture species (Jeffs et al., 1999) and a potential bioindicator species it is essential that minimal stress is induced through the harvesting and transport of the mussels. This will ensure that responses adequately reflect exposure to environmental stressors, and/or the mussel maintains optimal health for further processing and/or live sale to consumers.

7.2 Materials and methods

7.2.1 Collection and maintenance

Adult green-lipped mussels, *P. canaliculus* (70-80 mm; 38 ± 7 g) were collected from Lyttelton Harbour, Canterbury, New Zealand ($43^{\circ} 36.564'S$, $172^{\circ} 43.246'E$). The mussels were removed from wharf pilings during low tide by using scissors to cut their byssus threads, with care taken to ensure there was no damage to the foot of the mussel. Conditions at the collection site were as follows: salinity 32 ‰, pH 7.5, air temperature $13.4^{\circ}C$, and water temperature $12.7^{\circ}C$. Mussels were transported to the aquarium facility at the University of Canterbury within 1 h of collection in a clean 30 L polypropylene container filled with seawater from the collection site, with constant aeration provided by battery-powered air-bubblers. Once in the aquarium mussels were cleaned, by gently scraping epibionts off the

shells, and were maintained for 4 days in seawater from the collection site prior to initiation of the ‘transport’ experiment.

7.2.2 Experimental design

This study comprised of two phases - a simulated “transport” period lasting for 24 h and a “recovery” period lasting for 48 h. Mussels were divided into four groups (n = 6 each). The first group of mussels (site seawater; SSW) were placed in individual 5 L polypropylene tanks exposed to 4 L of seawater taken from the collection site ($32 \text{ ‰} \pm 0.2$) at 15°C with gentle aeration. In the second group mussels were held under identical conditions, except in artificial seawater (ASW). The ASW used was prepared according to the manufacturer’s instructions (Reef Crystals, Instant Ocean). The third group (EMS 15°C) were placed without any water (i.e. emersed) in individual polypropylene containers covered with moist absorbent cloth and maintained at 15°C . The fourth group was also emersed (EMS 5°C) with each mussel placed in an insulated container with an ice-pack covered in moist paper towels, to avoid direct contact of mussels with ice. The lid of the each container was covered with moist absorbent cloth. The temperature inside the container was $5 \pm 0.5^{\circ}\text{C}$.

The mussels were maintained in these treatments for 24 h, which constituted the “transport” period. This time period was considered a worst-case scenario for transport of *Perna* within a NZ setting. Physiological biomarkers were measured on mussels from all four treatments at the end of this phase. Mussels were then transferred to aerated natural seawater used in the aquarium facility at the University of Canterbury. This seawater was collected from the same location as the mussels. Each treatment was maintained identically, but separately, at 15°C with gentle aeration, and allowed to recover from their respective transport method for 48 h. This period of recovery was chosen on the basis of a preliminary study. Physiological

biomarkers were then measured on mussels from the four treatments at the end of the recovery period.

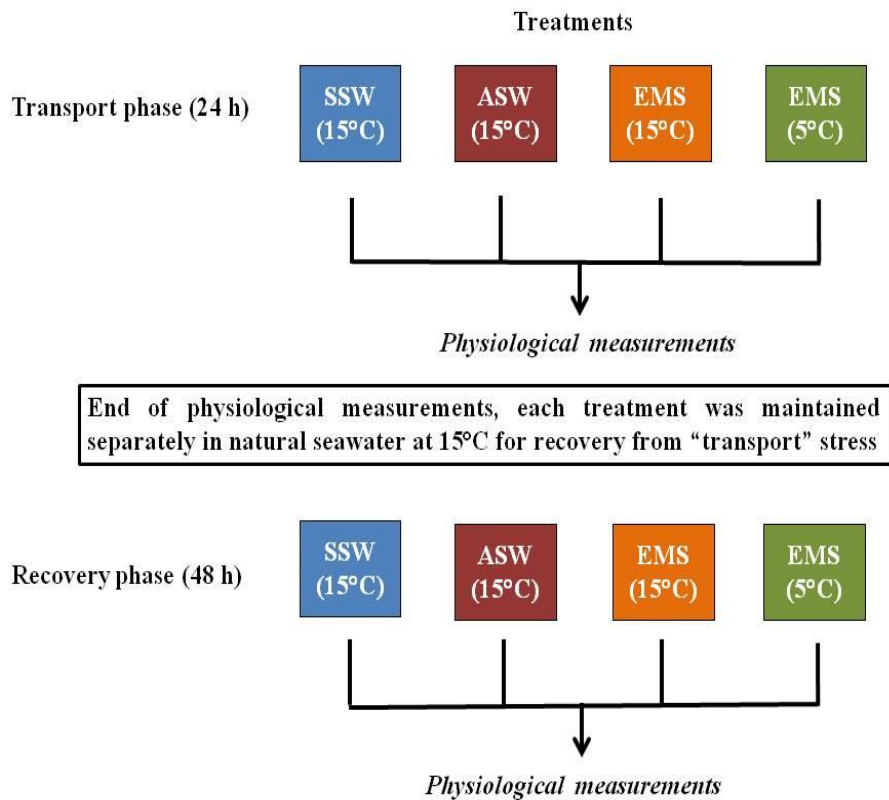


Figure 7.1 Flow chart illustration of the experimental design of the field-to-laboratory transportation protocols used in this study. SSW – Site seawater; ASW – Artificial seawater; EMS – Emerged at 15°C and 5°C.

7.2.3 Physiological biomarkers

All physiological measurements were conducted on individual mussels. The respiration rate, clearance rate, excretion rate and absorption efficiency of the mussels from each treatment

was conducted according to the physiological biomarker measurements detailed in *Section 2.3*.

At the end of the “recovery period” physiological measurements, mussels were sacrificed and soft tissues were collected and dried at 60°C for 24 h to obtain dry weight measurements. All physiological measurements of individual mussels were then converted to corresponding dry weight in grams. The SFG was then calculated from the physiological measurements using the equation described in *Section 2.3.6*.

7.2.4 Statistical analysis

All experimental data were analysed using the statistical software STATISTICA 7.0 (Statsoft). Data were tested according to the methods detailed in *Section 2.9*. Data that passed these tests were then analysed by two-way repeated measures ANOVA with transport condition (SSW, ASW, EMS 5°C, EMS 15°C) and time (i.e. immediately after the transport phase or after a further 48 h of ‘recovery’) as the two factors, followed by post hoc LSD test. The level of significance of the results was set at $p < 0.05$. Data are presented as mean \pm SEM.

7.3 Results

Clearance rate (Fig. 7.2) showed a significant effect of transport condition, time (i.e. transport phase versus recovery phase; $p < 0.05$) and a significant interaction between these two factors ($p < 0.05$). The EMS 5°C transport condition displayed a significant decline in clearance rate relative to SSW after both the transport and recovery period. At the end of the 48 h recovery period this inhibition in clearance rate was significantly lower than all the other groups. Following a 48 h recovery in natural seawater the ASW and EMS 15°C groups showed significant elevations in clearance rate relative to those observed in these same groups after the transport phase of the study.

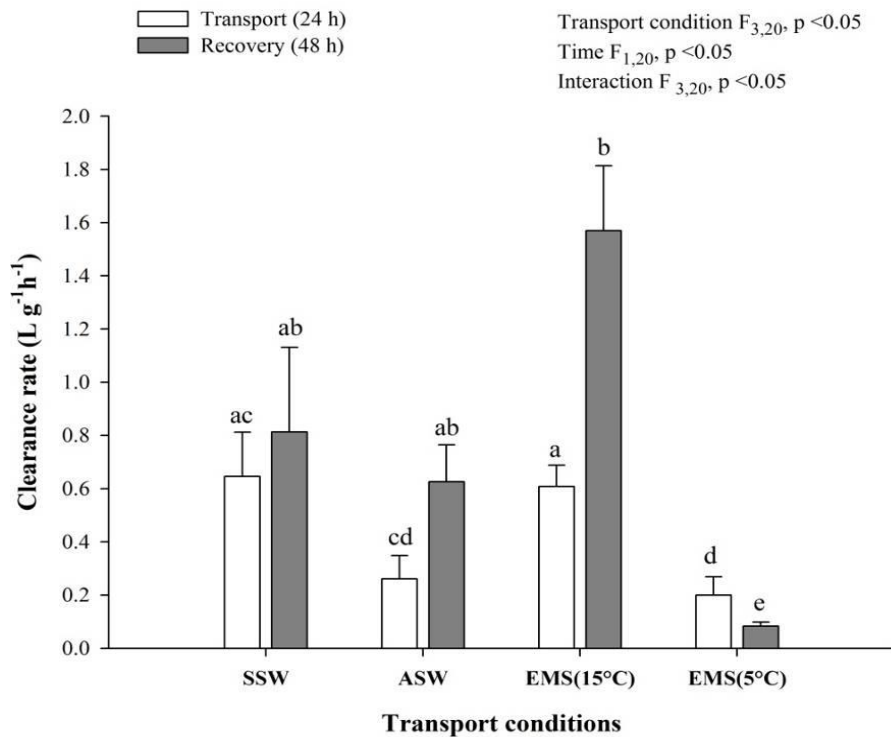


Figure 7.2 Clearance rate (mean \pm SEM) of *Perna canaliculus* ($n = 6$) after initial 24 h transport treatment (holding mussels in site seawater (SSW), in artificial seawater (ASW) or emersed (EMS) at 15°C and 5°C), and a subsequent 48 h recovery in natural seawater. Level of significance was set at $p < 0.05$; Plotted points sharing letters are not significantly different as determined by two-way repeated measures ANOVA, followed by post hoc LSD test. Statistical analyses were performed on log-transformed data.

Absorption efficiency (Fig. 7.3) was significantly impacted by transport condition ($p < 0.05$), however no significant effects of time ($p = 0.873$), or interaction between these two factors ($p = 0.650$) were observed. Mussels in the EMS 5°C group had an absorption efficiency that was significantly elevated when compared to the ASW mussels after the initial 24 h transport period, but no other significant changes were observed.

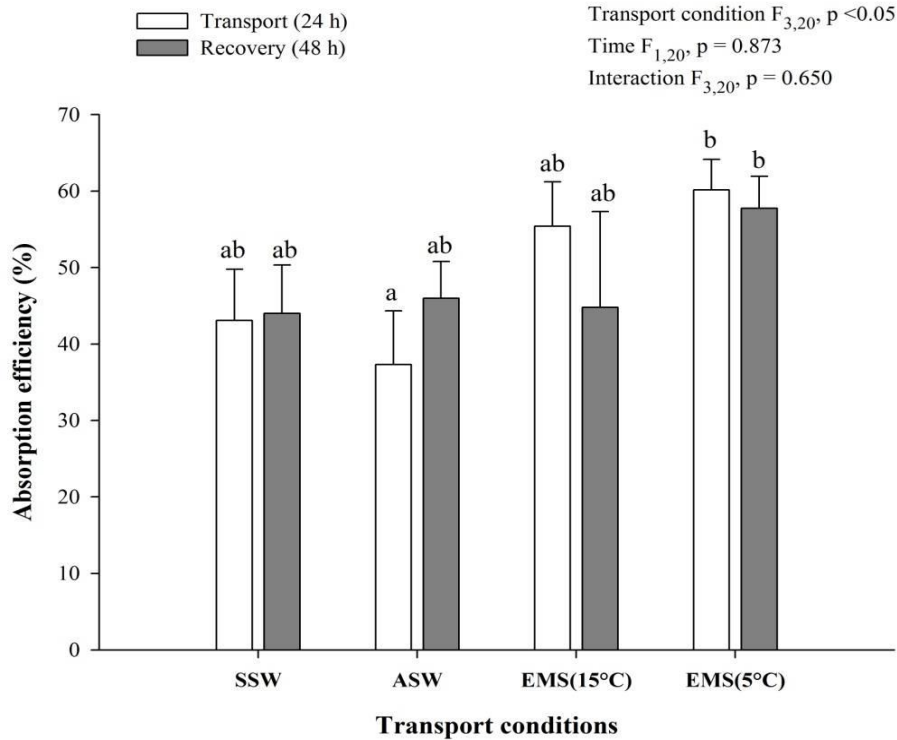


Figure 7.3 Absorption efficiency (mean \pm SEM) of *Perna canaliculus* ($n = 6$) after initial 24 h transport treatment (holding mussels in site seawater (SSW), emersed (EMS), or in artificial seawater (ASW)), and a subsequent 48 h recovery in natural seawater. Level of significance was set at $p < 0.05$; Plotted points sharing letters are not significantly different as determined by two-way repeated measures ANOVA, followed by post hoc LSD test.

Significant effects of transport condition, time, and transport protocol \times time ($p < 0.05$) were observed on excretion rate (Fig. 7.4). Mussels in the ASW transport condition displayed a significant increase in excretion rate relative to all other groups at the end of 24 h transport period, although this effect dissipated after the recovery phase. A significant decline in recovery excretion rate was observed in EMS 15°C mussels relative to the SSW group and to the transport phase of the EMS 15°C group.

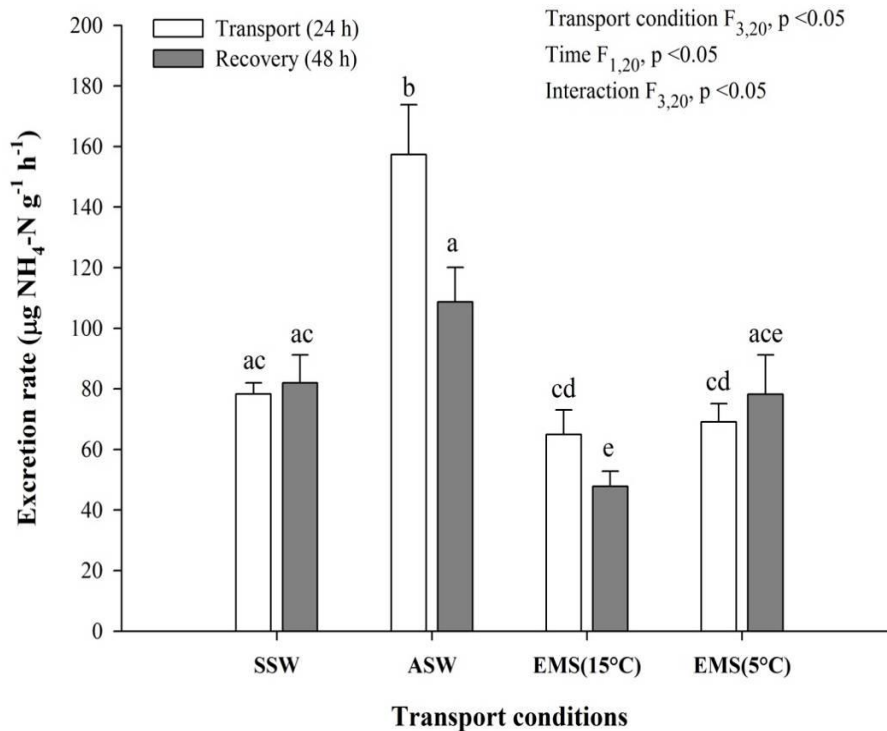


Figure 7.4 Excretion rate (mean \pm SEM) of *Perna canaliculus* ($n = 6$) after initial 24 h transport treatment (holding mussels in site seawater (SSW), emersed (EMS), or in artificial seawater (ASW)), and a subsequent 48 h recovery in natural seawater. Level of significance was set at $p < 0.05$; Plotted points sharing letters are not significantly different as determined by two-way repeated measures ANOVA, followed by post hoc LSD test. Statistical analyses were performed on log-transformed data.

Respiration rate (Fig. 7.5) was significantly impacted by transport condition, time, and interaction between these factors ($p < 0.05$). After the transport phase ASW mussels had a significantly elevated respiration rate relative to all other groups, while EMS 15°C mussels displayed lower respiration rates than the SSW group. After recovery the ASW group respiration rate was much reduced, and was not significantly different from any other recovery group. The respiration rate of the EMS 5°C group was significantly less than that of the SSW group following recovery.

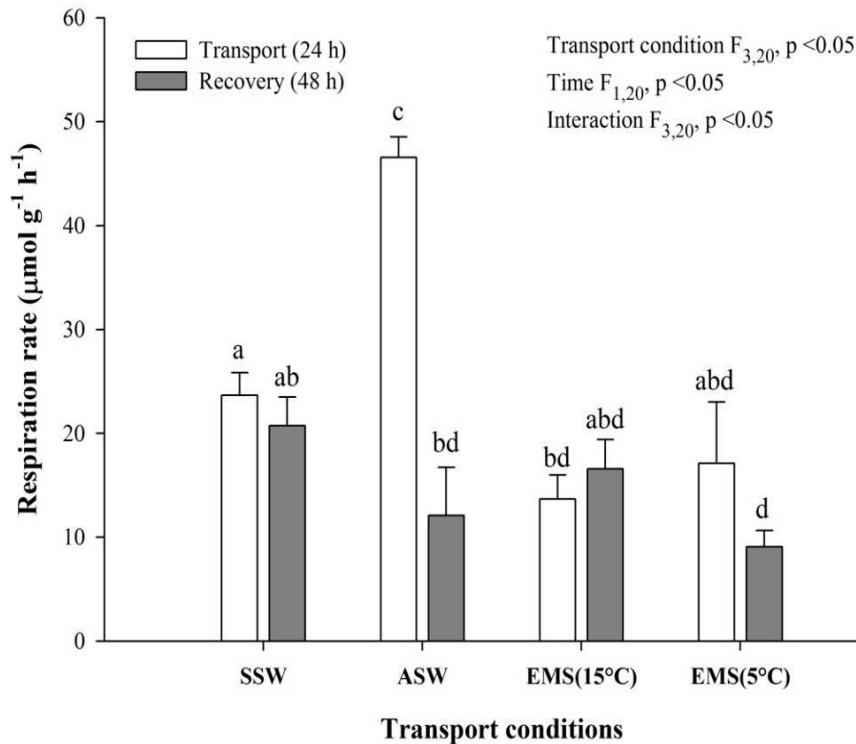


Figure 7.5 Respiration rate (mean \pm SEM) of *Perna canaliculus* ($n = 6$) after initial 24 h transport treatment (holding mussels in site seawater (SSW), emersed (EMS), or in artificial seawater (ASW)), and a subsequent 48 h recovery in natural seawater. Level of significance was set at $p < 0.05$; Plotted points sharing letters are not significantly different as determined by two-way repeated measures ANOVA, followed by post hoc LSD test.

Significant effects of transport condition and time were documented for SFG ($p < 0.05$; Fig. 7.6). No significant effects of the interaction between these factors were observed, however ($p = 0.089$). Following the 24 h transport phase of the study, only mussels in the EMS 15°C group exhibited positive SFG values, although this effect was not significantly different from the SSW group (Fig. 7.6). Conversely ASW mussels displayed a large negative SFG, which was significantly different from all other treatments. After recovery the EMS 15°C scope-for-growth was significantly elevated with respect to the same group at 24 h and both the SSW and the EMS 5°C post-recovery groups.

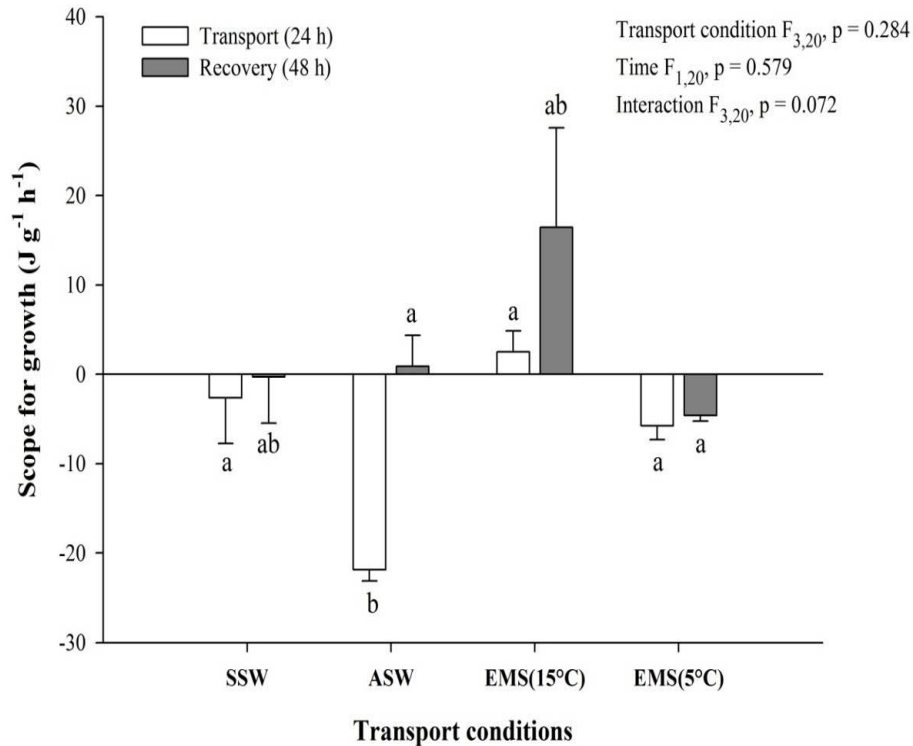


Figure 7.6 Scope for growth (mean \pm SEM) of *Perna canaliculus* ($n = 6$) after initial 24 h transport treatment (holding mussels in site seawater (SSW), emersed (EMS), or in artificial seawater (ASW)), and a subsequent 48 h recovery in natural seawater. Plotted points sharing letters are not significantly different as determined by two-way repeated measures ANOVA, followed by post hoc LSD test.

7.4 Discussion

This is the first study that describes transport-related stress in mussels using physiological biomarkers. The results indicate that different transportation methods induced varied levels of impact on the physiology of mussels. Menezes et al. (2006) and Lorenzon et al. (2007) suggested that handling and transport-related stress in experimental organisms can greatly enhance the chances of physiological changes that would impact assessment of pollutant-related stress, a hypothesis confirmed for mussels in the present study.

7.4.1 *Physiological biomarkers*

The results of the current study showed that emerging mussels at 5°C for transport (the EMS 5°C group) impaired clearance rates. The clearance rate did not recover following a 48 h period, indicating that transport emersed at 5°C is a significant stressor to mussels. This counters the claim that transport under such conditions is preferred (Widdows and Staff, 2006), at least for *P. canaliculus*. This depression in clearance rate was not observed in the EMS 15°C group, indicating that it is the temperature component of this treatment that is likely the source of stress to the mussels.

The low temperature could have induced the well documented behavioural response of shell valve closure in mussels (Jørgensen et al., 1990). Studies by Loayza-Muro and Elias-Letts (2007) found that at 5°C freshwater mussels showed a significant decline in filtration that was directly related to shell valve closure, a finding also supported by other researchers (e.g. Clarke and Griffiths, 1990). The most important factor appears not to be the temperature itself, but the fact that the exposure temperature varies significantly from that in the ambient environment (Clarke and Griffiths, 1990; Bartsch et al., 2000). The mussels in the current chapter were collected at an ambient air temperature of ~13°C, and thus the 5°C emersion treatment represents a significant temperature shock. Interestingly, these mussels failed to recover clearance rate even 48 h after being returned to 15°C, indicating a significant long-term impairment of filtration associated with this temperature shock effect. This likely stems from the so-called “chill coma” effect that has been previously described for mussels of the *Mytilus* genus. It is thought that cold shock stimulates nitric oxide production, resulting in inhibition of mitochondrial respiration, and an inhibited ability of the mussel to open the shell valves (Jansen et al., 2007).

An alternative explanation for decreased clearance rates is that there is a physical impairment of the feeding apparatus (i.e. damage to cilia and gill filaments; Jones et al., 1992). If this was the case then it would be expected that the decrease in clearance rate would be accompanied by a decrease in absorption efficiency, which reflects how effectively an organism utilises its nutritional source. In the present study, there were no major impacts of transport protocol on this parameter, suggesting that the decrease in clearance rate was not a consequence of a direct physical impairment of the feeding or digestive apparatus, but was a response related to valve closure. Previous studies support the present findings, in that absorption efficiency is documented to be relatively independent of the presence of organismal stress, and is known to be significantly influenced only by the quantity and quality of the food available to the mussel (Honkoop et al., 2003).

Ammonia excretion and oxygen consumption can be utilised as measures of energy balance in mussels. The ASW treatment exhibited significantly elevated respiration and excretion rates after a simulated 24 h transport protocol, demonstrating that ASW appears to induce significant stress in mussels. Lee et al. (2007) studied the effects of ASW on hard clams and found that an imbalance of magnesium and potassium salts in ASW could potentially affect the cellular, physiological and behavioural functions of organisms. This was hypothesised to be due to the role of magnesium as an enzyme co-factor, and the role of potassium in cellular homeostasis. In particular, changes in these salt concentrations could induce changes in cellular osmoregulation (Castille and Lawrence, 1981; Garton and Berg, 1989). As osmoconformers, mussels cope with changes in intracellular salinity by utilising protein/amino acid catabolism to regulate cell volume (Livingstone et al., 1979; Bishop et al., 1983), which would increase nitrogenous waste.

The significant increase in oxygen consumption of mussels in ASW may have a similar aetiology as the excretion rate. An increase in respiration in the ASW group may reflect the increased costs associated with alterations in energetically-expensive osmoregulatory pathways. This is a hypothesis that remains to be tested. It has also been suggested that the organic matter composition of ASW may have directly or indirectly (e.g. via influencing metal bioavailability; Arnold et al., 2007) have negative effects on marine organisms, and which could also result in perturbation of basic physiological functions in the animal, increasing the costs of homeostasis, and leading to an increase in respiration rate. It is, however, worth noting that both excretion and respiration rates recovered quickly in ASW mussels. This can be explained by the fact that bivalves tend to quickly resume their normal physiological functions once they are transferred to more favourable conditions (Akberali and Trueman, 1985).

SFG primarily depends on the feeding and oxygen consumption rates of bivalves (Sobral and Widdows, 1997). These two parameters comprise the major sources of energy gain and energy loss of an organism. The maintained low clearance rates observed in the EMS 5°C mussels is the obvious explanation for the maintained negative SFG values in this group. Conversely, high energy loss in the form of oxygen consumption and ammonia production contributed towards the high negative SFG value observed in ASW mussels. However, since both these measures showed recovery after 48 h, a significant recovery to a positive SFG was also observed. Negative SFG is likely to reflect an impaired ability to perform ecologically important processes related to growth and reproduction (Navarro et al., 2006). This implies that mussels subjected to the EMS 5°C transport condition may suffer significant long-term impairments.

Positive SFG values represent conditions where mussels have sufficient excess energy to contribute towards growth and reproduction (Tsangaris et al., 2010). The EMS 15°C mussels displayed positive SFG values after the 24 h transport period and a significantly higher positive SFG at the end of 48 h recovery period. These results reflect that EMS 15°C transport condition caused least stress to the mussels and was the optimal transport condition tested in this study.

7.4.2 Transportation treatments

The results of this research indicate that transporting *P. canaliculus* emersed at air temperatures close to the ambient temperatures at the point of collection minimises transport stress. This finding could reflect the intertidal nature of these mussels. As *P. canaliculus* is regularly exposed to air, it might be expected that it is well-adapted to the aerial transport manipulation used in the present study. This species is equally adept at respiring in water or air (Marsden and Weatherhead, 1998), demonstrating its capacity to withstand emersion.

It is interesting to note that several previous studies support water transport conditions for bivalves. Chen et al. (2001) noted that water transportation was less stressful compared to air transportation after analysing glycogen levels in five different freshwater mussel species. Similar conclusions were obtained in transportation studies on the freshwater mussel, *Lamellidens corrianus* where glucose concentrations measured in different tissues showed significantly higher concentrations in mussels transported by air than water, indicating higher stress (Yusufzai et al., 2010). Maguire and colleagues (1999) utilised adenylic energetic charge as a stress indicator in juvenile scallops, *Pecten maximus* and found that dry transport was not a conducive form of transportation. These latter two species are less likely to be aerially exposed than *P. canaliculus* supporting the idea that intertidal habitat may have an important role in determining optimal transport conditions.

In support of the findings of the present investigation, however, are a number of studies that favour transport of marine fauna in an emersed environment. For example, Ocaño-Higuera and colleagues (2011) suggested that emersion was most favourable for shipment of adult Giant Lion's Paw scallop. A similar finding has been recorded for crabs transported in "chilled air" versus water, with the former protocol causing fewer physiological perturbations (Robson et al., 2007; Lorenzon et al., 2008). There are several studies conducted in bivalve species that consider out-of-water transport or emersion conditions as a better transport method as long as factors such as temperature, relative humidity and duration of transport are taken into account (Wells and Baldwin, 1995; Christophersen et al., 2008). These results were based on the reduced stress levels, lower mortality rates and smaller relative costs associated with shipment in air.

7.4.3 Aquaculture and ecological implications

P. canaliculus is of significant economic importance to New Zealand. In 2010, green-lipped mussels comprised 72% of aquaculture exports, earning \$202.5 million internationally (New Zealand Aquaculture Farm Facts, 2010). Mussels are an inexpensive source of protein and other essential minerals and hence add high nutritional value to the human diet (Vareltzis, 1996). In recent times there has been an increased demand for fresh or raw mussels. However mussel flesh quality has been found to deteriorate depending on processing, handling and storage conditions (Caglak et al., 2008). Several factors such as temperature and oxygen are known to cause a marked change in the odour, colour, texture, and flavour of mussel meat (Brooks and Harvie, 1981; Warwick, 1984; Pastoriza et al., 2004). As stress has a significant impact on seafood quality (Bjornevik and Solbakken, 2010), it is crucial to maintain optimal conditions during and after mussel harvesting, to ensure that customer satisfaction is not compromised.

From an ecological perspective, it has been previously shown that translocation of mussels between field sites induces significant stress, due to handling and transportation (Cope and Waller, 1995). In this study, almost all mussels that were not transported by emersion at ambient temperature displayed negative SFG values, indicating a stress effect that persisted beyond the 24 h of transport and the 48 h recovery period.

7.5 Conclusions

The transport of mussels emersed at 5°C or in artificial seawater had a negative impact on physiological parameters of mussels. This study recommends the transport of mussels under emersion at ambient temperatures. This would ensure that the mussels are subjected to minimal stress during transportation. The 15°C emersion temperature used in this study was successful for transport of this particular *Perna* species, although this outcome may differ for other mussel species. This study also indicates that the water transportation method used in *Chapter 6*, could have possible negative impact on the mussels. If biomarker responses were measured in the field-collected mussels used in *Chapter 6*, the possibility of significant effects of transportation-related stress in those mussels cannot be ruled out. When the effects of transport stress are minimised by using transportation protocols recommended in the the current chapter, the physiological status of the mussel will more accurately reflect environmental field conditions. This will be of particular importance in scenarios where mussels are being used to assess ecosystem health. Transportation related stress cannot be avoided but can certainly be minimised by implementing appropriate protocols.

8 General Discussion

8.1 Summary of findings

The objectives of this thesis were to (a) determine Cd-induced toxic mechanisms in mussels (b) identify multiple biomarkers that respond to Cd exposure in mussels and (c) assess the potential utility of employing green-lipped mussels as a bioindicator species for metal contamination in coastal habitats in NZ. This was the first study to assess Cd toxicity effects in *P. canaliculus*. The toxicological effects of Cd in green-lipped mussels at different doses and durations of exposure were investigated by conducting a mechanistic study. This consisted of acute (96 h; at 2000 and 4000 $\mu\text{g Cd L}^{-1}$) and subchronic (28 d; at 200 and 2000 $\mu\text{g Cd L}^{-1}$) Cd treatments. This thesis has shown that *P. canaliculus* is relatively tolerant to Cd, characterised by a 96 h LC_{50} value of 8160 $\mu\text{g L}^{-1}$ (Section 3.3.1). This tolerance to Cd could be explained on the basis of the ability of green-lipped mussels to induce detoxification and defence mechanisms against Cd toxicity effects (Chapters 3-5).

Exposure to Cd induced a cascade of cellular, genotoxic, immunocytotoxic and physiological effects in green-lipped mussels (Chapters 3-5). During both acute and subchronic exposure to Cd, clearance rate of mussels declined, with a concomitant increase in excretion rate (Chapter 3). Although not measured in this thesis, shell valve closure was hypothesised to be responsible for the decline in feeding and oxygen consumption levels in Cd-exposed mussels (Chapter 3) (e.g. Tran et al., 2001). In addition, a significant decline in glycogen levels was also observed in Cd-exposed mussels (Chapter 4). The utilisation of reserve energy resources is an indication of Cd-related stress which was also responsible for the high protein catabolism and low O:N ratios of mussels (Chapter 3). This decline in feeding and in glycogen levels, along with increase in protein catabolism and nitrogen excretion, likely reflected an energy imbalance, indicating low energy gain and high energy loss. This scenario

would result in very little or no energy available for growth of the mussels characterised by a negative SFG induced by exposure to Cd (*Chapter 3*).

The energy imbalance could also be attributed to the use of energy towards detoxification and repair mechanisms. For instance, Cd induced MTLP production for detoxification and storage of Cd in non-toxic forms (*Chapter 4*). The defence mechanism also included the production of catalase, an enzyme that is part of the anti-oxidant defence system. Although significant increases in MTLP and catalase activity were observed, exposure to Cd induced lipid peroxidation in the digestive gland of mussels at subchronic concentrations (*Chapter 4*). This could be attributed to the production of ROS, another important effect of Cd toxicity. The induction of ROS and increase in oxidative stress can affect cell membranes causing lipid peroxidation, and also lead to DNA damage (*Section 1.3.5.2*). The effects of ROS production could explain the significant DNA damage observed in the haemocytes of mussels and the formation of different macrolesions in the gill cells (*Chapter 5*). The occurrence of damage at the cellular level suggested that detoxification mechanisms were not sufficient enough to protect the mussels against oxidative stress and DNA damage (*Chapter 4-5*).

Overall, the effects of Cd on biomarker responses were tissue-specific, dose- and time-dependent, with duration of exposure being the predominant effect (*Chapter 4*). The acute toxicity responses indicated that during short-term exposure to high Cd concentrations, the detoxification and defence mechanisms of mussels were overwhelmed and inhibited by the level of Cd exposure. Consequently, the mussels were unable to induce detoxification and antioxidant defence mechanisms during acute exposure. This inadequacy to induce protective mechanisms could also be partly due to the absence of energy resources to drive these processes (*Section 4.4.2*). On the other hand, subchronic exposure to Cd (long-term exposure to low Cd doses) induced MTLP and catalase activity that could provide protection against

toxic Cd effects. However, induction of these mechanisms was not sufficient to protect against Cd-induced physiological impairments, oxidative stress, damage to the gill cells, and DNA damage in the haemocytes.

The findings from the laboratory studies clearly delineated the differences in physiological, biochemical and cellular toxicity responses based on duration of exposure and concentrations of Cd (*Chapters 3-5*). The relative differences in sensitivity to Cd exposure among aquatic organisms could be related to the species-specific ability of organisms to distribute Cd at the subcellular level and regulate detoxification mechanisms (see *Section 1.3.4*; Wang and Rainbow, 2006).

Metal concentrations in different mussel tissues were measured to assess the utility of green-lipped mussels as bioindicators for coastal metal pollution in NZ (*Chapter 6*). There were no significant correlations between metal concentrations in the sediment and different mussel tissues, with the exception of Zn accumulation in the foot (*Section 6.3*). The field study indicated differences between contaminated sites within each region as well as regional differences, in tissue and sediment metal concentrations. Specific sites with the highest concentration of metals in the sediments reflected elevated metal concentrations in the tissues (e.g. Cu in Mapua). These results suggested the utility of the green-lipped mussel as a coastal bioindicator for metal contamination in NZ.

Collection and transportation of mussels are part of both field and laboratory studies. In this thesis, *Chapter 7* included a field study that simulated the investigation of the possible effects of transportation related stress in mussels. Physiological biomarker responses were used to indicate the level of stress involved in transportation of mussels. Among the four treatments, emersion at 15°C was found to be the best method of transportation from the field to the laboratory. Green-lipped mussels are intertidal organisms exposed to tidal fluctuations.

Compared to the other treatments used, a positive SFG during emersion at 15°C reflected the well-being of the organism and also highlighted the feasibility of transport in air (*Chapter 7*). The findings in *Chapter 7* also highlighted that the green-lipped mussels were affected by short-term changes in temperature (during air transport) and possible differences in ion concentrations of the artificial seawater used during water transport, relative to natural seawater.

8.2 Implications of the findings

There are numerous studies on Cd toxicity effects in aquatic invertebrates. In this thesis, the mechanistic study on Cd toxicity under both acute and subchronic concentrations revealed that toxic effects in mussels were both dose and duration dependent and differed compared to other species such as *P. viridis*. The differences in Cd toxicity between species can be attributed to the site of action, bioaccumulation, detoxification and elimination mechanisms of specific organisms. It has been established that toxicity occurs when the rate of metal uptake exceeds the combined rate of metal elimination and detoxification (Rainbow, 2007). This thesis has provided both acute and subchronic toxicity data on responses to Cd that could be of use in future ecological risk assessment and regulation. The mechanistic differences between acute and subchronic Cd toxicity shown in this thesis can be extrapolated to understand the effects of mussels exposed to sources such as mining effluents, industrial discharges, sewage outfalls and stormwater runoff (e.g. Brix et al., 2010). Moreover, a risk-based approach can be developed to investigate species vulnerability, water quality and help prioritise remediation measures at affected contaminated sites (e.g. Beiras and Albentosa, 2004).

From an ecotoxicological perspective, delineating the mechanisms of Cd toxicity during different durations of exposure in green-lipped mussels is a valuable addition towards hazard

identification of metals (e.g. McGeer et al., 2003). Environmental risk assessments are based on exposure and effects in organisms. However, risk assessments are often incomplete due to limited availability of data on metal toxicity effects for a species of interest (Fairbrother et al., 2007). A Biotic Ligand Model (BLM) approach can be developed which uses metal accumulation at a sensitive binding site of the organism to predict toxicity (Paquin et al., 2000). Use of a BLM can help environmental regulators and decision makers to rapidly assess risk associated with toxic metals by using simple and cost-effective scientific tools (e.g. Cu toxicity using *Mytilus* species, Arnold et al., 2005).

In this thesis, the feeding rate of mussels was reduced significantly, irrespective of changes in duration and concentration of Cd exposure. This characteristic made clearance rate one of the key biomarker endpoints of Cd exposure and effect. A decline in feeding will most likely have an effect on the growth of the organism. This was observed in the thesis with the negative SFG of green-lipped mussels as shown in *Chapter 3*. Exposure to Cd is known to reduce the probability of long-term survival of aquatic organisms (e.g. Hansen et al., 2002). For example, Ward and Robinson (2005) reported that exposure to Cd reduced fitness, increased sensitivity of cladocerans to other metals, and impacted their survival. This thesis has also demonstrated that the tolerance to Cd in green-lipped mussels came at a cost (*Section 3.4.3*), which indicates implications of ecological relevance such as reduced fitness and possible disease susceptibility. Additionally, there are reports indicating that metal absorption rate in bivalves is related to the filtration/clearance rate (e.g. Baines et al., 2006). Reduced clearance rate and associated decline in metal uptake can be considered to be a detoxification process (e.g. Pan and Wang, 2012). This indicates the further use of clearance rate as a biomarker of both Cd exposure and effect in mussels.

One of the findings from the laboratory studies was the measurement of strong biomarker responses in the digestive gland of mussels. For example, MTLP concentration, catalase and alkaline phosphatase activities in the digestive gland were higher than the gill (*Chapter 4*). The digestive gland is the major organ involved in metabolism, detoxification and storage of metals (Marigómez et al., 2002). The implication of this finding is the possible utility of the digestive gland for biomarker measurements in *P. canaliculus* for future biomonitoring studies in NZ. Additionally, this also indicates that biomarkers validated and used in other mussel species could have similar utility in *P. canaliculus*.

The field study in this thesis was the first attempt to assess metal contamination levels in NZ using green-lipped mussels as a bioindicator species (*Chapter 6*). The results indicate that green-lipped mussels can be used as “early-warning” signals for coastal metal pollution in NZ. This study has provided baseline concentrations of metals in the sediments collected from different sampling sites that indicate level of metal contamination. Based on these results, environmental management agencies in NZ could develop risk assessment and water quality guidelines for metals. Mussel tissue accumulation levels can also be monitored from different coastal areas to assess human health risk associated with consumption of green-lipped mussels from contaminated sites.

8.3 Methodological considerations

Detoxification mechanisms and Cd sequestration allow Cd to be stored in a non-toxic form. It is important to determine the level of bioreactive fraction of Cd in tissues to link the active internal Cd concentration to the toxic response (e.g. Wallace et al., 2003). In this thesis, the determination of metabolically active Cd in mussel tissues using centrifugation process and subcellular tissue partitioning studies could have provided a better understanding of toxicity mechanisms. The metabolically active Cd and binding of Cd to metal sensitive cell organelles

is responsible for induction of toxic responses. Tolerance to Cd can be related to storing Cd in the detoxified form (e.g. bound to MT, Viarengo and Nott, 1993). Additionally, the redistribution of Cd to metal-sensitive and inactive fractions of the cell could also act as a protective mechanism conferring tolerance to the organism (e.g. Ng and Wang, 2005b). Subcellular distribution of Cd in the tissues could provide information on toxicity and tolerance (Vijver et al., 2004). Moreover, the distribution of metals by subcellular partitioning is known to be both metal- and organism- specific (Wang and Rainbow, 2006). Hence, the compartmentalisation of Cd in different cell components can also be used as an indicator of toxicity (e.g. Pan and Wang, 2008).

During Cd exposure treatments the mussels were fed a daily diet of measured algal feed by introducing algae into the Cd exposure waters (*Section 2.3.1*). Consequently there is strong likelihood that some of the accumulation and toxic effects relate to dietary exposure (see *Section 8.4*). However, no attempt was made to isolate the pathways of exposure, as this was not a specific goal of this study. Under natural conditions, the response of mussels to Cd in the seawater would also potentially contaminate the food source, so the findings of the study still hold for an environmental setting. It is, however, worth noting that one of the responses of mussels to Cd exposure is to greatly reduce clearance rate, indicating that the dietary component of Cd exposure would become less important as waterborne concentrations increase (see *Section 3.4.2*).

Physiological biomarkers were used in this thesis to assess Cd toxicity effects in mussels. The standardised protocol by Widdows and Staff (2006) suggested the measurement of oxygen consumption in mussels after completion of clearance rate measurements. Accordingly, physiological biomarkers in mussels were measured starting with the clearance rate, followed by excretion rate and then the respiration rate. The order in which the physiological

measurements were conducted could have impacted the results to a certain extent. For example, the impacts could be related to the effects of specific dynamic action (SDA). SDA refers to an increase in the oxygen consumption after feeding (Secor, 2009). In *Chapter 3*, mussels were provided with ~2 h interval between feeding and respiration measurements. A recent study by Lurman et al. (2013) on energetics of *P. canaliculus* found that SDA effects are highly controlled in this species. This implies that when under stress *P. canaliculus* has the ability to stop both feeding and digestion, instead utilising oxygen for other vital biological functions. They also reported that there was no correlation between feeding and subsequent metabolic rate (Lurman et al., 2013). However in this thesis when the physiological biomarkers were measured during Cd exposures, preliminary experiments could have been conducted to determine the effects of feeding response and its impacts on oxygen consumption in *P. canaliculus*, to rule out possible effects of SDA.

In *Chapter 5* as part of the immunocytotoxic biomarker response in mussels, the differential haemocyte cell count was measured. The total haemocyte cell count or number of circulating haemocytes in the green-lipped mussel was not measured immediately after collection. This was due to limiting factors such as time and lack of equipment (e.g. automated cell counter, flow cytometer). Viable haemocytes are required for measuring total haemocyte cell counts. The lack of total cell count information in this study is a minor limitation to the interpretation of the differential cell count results. Bivalves are found to have elevated total haemocyte cell count when exposed to contaminants such as metals (e.g. Brousseau et al., 2000). Total haemocyte count is an indication of the number of circulating cells in the haemolymph at a particular time point. Generally when exposed to metals, an increase in total haemocytes in mussels has been known to reflect haematopoiesis (e.g. Coles et al., 1995). On the contrary, a decrease in total haemocyte number is indicative of migration towards the tissues (e.g. Coles et al., 1995). In this thesis, the differential haemocyte proportions in mussels during Cd

exposure, showed significant fluctuations at any given time point. The additional information on total circulating haemocyte numbers would have aided in interpreting the specific functions of each haemocyte type based on the distribution of haemocytes between the haemolymph and the tissues (*Section 5.4.1*).

The comet assay is conducted using freshly collected samples of mussel haemocytes or gill cells (e.g. Pavlica et al., 2001; Vincent-Hubert et al., 2011). However, the comet assay used to measure DNA damage in *Chapter 5* was conducted using haemocyte samples that had undergone a freeze-thaw cycle. Preliminary experiments were conducted to validate the method of using freeze-thawed haemocyte samples for comet assay. The validation procedure involved exposure of both fresh and thawed haemocytes to different concentrations of H₂O₂ prior to testing the comet assay. No significant differences were observed in DNA damage between the freshly collected and freeze-thawed haemocyte samples (unpublished data). Laboratory studies used to measure DNA damage in mussel haemocytes in *Chapter 5* followed a validated protocol along with the use of a time-matched control series for the Cd treatments (*Section 5.3.3*). However, the same protocol that was used under controlled laboratory conditions may not be suitable for haemocytes collected from mussels in field studies. Mussels are exposed to a mixture of metal contaminants along with organic pollutants under field conditions. So the application of the laboratory protocol may not be adequate to delineate the differences in damage induced by several pollutants and haemocyte storage method. The application of a standardised protocol that takes into account factors such as the storage buffers, the duration of storage, and thawing period should be considered to avoid confounding results. Future studies could also consider the use of cryopreservation methods for storage of mussel haemocyte samples (e.g. Kwok et al., 2013).

8.4 Future studies

The relative importance of both aqueous and dietary exposure in metal uptake kinetics has been highlighted in the mussel, *Perna viridis* (Chong and Wang, 2001). However, apart from the waterborne toxicity to Cd reported in this thesis, there are no metal toxicity studies in green-lipped mussels. As a future directive, Cd kinetics and dietary route of exposure should also be explored in *P. canaliculus* (e.g. Shi and Wang, 2005). Studies on Cd uptake, accumulation, retention, elimination and depuration mechanisms of green-lipped mussels would allow comparison between Cd exposure routes (e.g. Blackmore and Wang, 2002). For example, Ng and Wang (2005b) showed that subcellular distribution of Cd varied between waterborne and dietborne exposures (see *Section 8.3*). Hence it would be interesting to investigate the effects of dietary metal exposure to further understand the effects of exposure route, distribution and kinetics of Cd toxicity in *P. canaliculus*. The accumulation and elimination of Cd through both the dietary and aqueous exposure routes can be useful in biodynamic modelling (e.g. Luoma and Rainbow, 2005). Biodynamic modelling can be used to predict metal accumulation differences among species.

The laboratory study on waterborne Cd toxicity was a mechanistic study that helped identify biomarkers of Cd exposure and the possible mechanisms of Cd toxicity. From an ecotoxicological perspective, study of metal toxicity in early life stages of mussels such as embryo or larva can prove to be useful. The sensitivity of the larval stage to contaminants is a key factor that determines the survival of a species in a contaminated environment. The use of early life stages of mussels holds both toxicological and ecological relevance (e.g. Beiras and Albentosa, 2004). Studies on metal toxicity effects in early life stage forms of *P. canaliculus* can provide metal toxicity data that can help develop a BLM. The development of tools such as BLM's can help predict Cd toxicity in aquatic species. This would help in the

derivation of environmental regulation and protection criteria for aquatic species in NZ. For example, larvae of mussels belonging to the *Mytilus* genera are used in the development of saltwater quality criteria for Cu toxicity (US EPA, 2003). The development of acute and chronic water quality criteria based on toxicity tests on the embryo-larval life stages of mussels, along with modifying water chemistry parameters such as salinity and DOM has proved to be informative in predicting metal toxicity characteristics in the environment (e.g. *Mytilus trossolus*; Nadella et al., 2009).

Measurement of metal concentrations in mussels as part of coastal biomonitoring in NZ could benefit from using the shell and the byssus threads of *P. canaliculus*. These two mussel tissues are known to accumulate high concentrations of metals. Studies on green-lipped mussels, *Perna viridis* indicated that the byssus threads and mussel shells accumulate metals such as Cd, Pb and Zn (Yap et al., 2003a; 2003b). For example, Yap et al. (2003a) found that Zn concentrations in the byssus threads were higher than Zn concentrations in the soft tissue of mussels and can be used as a biomonitoring tool for Zn pollution. Both the byssus and shell of mussels are constantly exposed to the external environment and are used for excretion of metals (e.g. Yap et al., 2003a; 2003b). This approach should be considered as part of future studies in this species.

Apart from metal contaminants, industrial and agricultural activities in NZ are also a source of organic pollutants (e.g. pesticides, fertilisers, antifouling paints; Taylor et al., 1997). The long-term use of insecticides, fertilisers, sheep-dips containing organochlorine (e.g. dieldrin, lindane and dichlorodiphenyltrichloroethane (DDT)) and organophosphate (e.g. diazinon) pesticides is a cause of concern in NZ (Buckland et al., 1998). In addition, other organic contaminants such as pentachlorophenol and dioxins are major constituents of pesticides used in horticultural and agricultural lands in different regions of NZ (Buckland et al., 1998). For

example, organochlorine pesticides such as dieldrin, chlordanes, and other contaminants such as PCBs were present at elevated concentrations in several fish species such as eel, trout and flounder collected from the South Canterbury region in NZ (Stewart et al., 2011). The concentrations of these organic pollutants posed a potential health risk to humans even at low consumption rate (Stewart et al., 2011). Previous studies have shown that the green-lipped mussel, *Perna viridis* can accumulate elevated concentrations of organic pollutants in their tissues that could have potential impacts on seafood consumers (e.g. Liu and Kueh, 2005; So et al., 2005). Further studies on bioaccumulation levels of organic pollutants in the green-lipped mussel, *P. canaliculus* could help understand the cumulative effects of environmental contaminants.

Green-lipped mussels were collected from different sampling sites in NZ and the metal accumulation levels were measured (*Chapter 6*). In addition, the biomarker responses of the mussels were measured, however the findings have not been included in this thesis. The sampling sites used in the South Island, NZ for mussel collection discussed in *Chapter 6* have varied exposure histories to different contaminants. These differences in environmental factors and history of exposure between sampling sites can induce different metal accumulation levels in bivalves that would make direct comparisons difficult (e.g. Mersch et al., 1996). Caging and transplantation experiments using green-lipped mussels would be helpful to develop monitoring strategies for coastal biomonitoring in NZ that could overcome such differences within a contaminated site. Previous studies in other mussels such as *Mytilus edulis* and *Mytilus galloprovincialis* used in transplantation and field translocation studies also showed biomarker responses to a mixture of contaminants (e.g. Regoli et al., 2004; Rank et al., 2007). A major advantage of using this approach is that fluctuations in factors such as season, salinity, temperature and pH within a sampling site can be minimised, as both indigenous and transplanted mussels will be exposed to similar environmental conditions

within a given sampling site. Moreover, any adaptive mechanisms exhibited by indigenous mussels in case of pre-exposure to contaminants at a specific sampling site could bias the outcome of biomarker responses (e.g. Riedel et al., 2002). For example, compared to the other sites investigated, the Avon-Heathcote (Canterbury) is an estuary where regular fluctuations in salinity occur. Mapua in the Nelson region has a known history of pesticide contamination that could have induced an adaptive response in mussels from that site. This disadvantage can be overcome by measuring biomarker responses in both caged and indigenous mussels termed as the “active biomonitoring” method (see *Section 1.4*). This approach allows parallel measurements of metal concentrations and biomarker responses in both caged as well as indigenous mussels within a sampling site that can provide a time-integrated assessment of chronic pollution levels (e.g. Nigro et al., 2006; Marigómez et al., 2013). Active biomonitoring is more useful than the passive method used in *Chapter 6*, in establishing the pollution status within a particular sampling site (see *Section 1.4*). Biomarker responses have been measured in mussels from the nine sampling sites discussed in *Chapter 6*. The findings on biomarker responses (unpublished data) suggest that future studies should consider caging and transplantation strategies using *P. canaliculus* for developing a suitable bioindicator to monitor coastal pollution in NZ.

8.5 Conclusions

This is the first study to assess the effects of metal toxicity in *P. canaliculus*. The major findings of this thesis include the mechanistic understanding of acute and subchronic Cd toxicity effects in the green-lipped mussel, *P. canaliculus*. The effects of Cd toxicity in mussels were dependent on the concentration and duration of exposure to Cd. The field study identified and investigated the utility of employing green-lipped mussels as a bioindicator of coastal metal contamination in NZ. This research has opened new avenues for exciting

research in metal ecotoxicology that would protect aquatic species in NZ. Potential future studies have been discussed that will help in realising the full potential of this endemic coastal species in environmental monitoring through the use of biomarkers.

9 References

- Abel, P.D., 1996. Water Pollution Biology, second ed. Taylor and Francis, London.
- Abraham, G., Parker, R., 2008. Assessment of heavy metal enrichment factors and the degree of contamination in marine sediments from Tamaki Estuary, Auckland, New Zealand. *Environmental Monitoring and Assessment* 136, 227-238.
- Aebi, H.E., 1984. Catalase, in: Bergmeyer, H.U. (Ed.), *Methods in Enzymatic Analysis*. Verlag Chemie, Basel, pp. 273-286.
- Ahsanullah, M., 1976. Acute toxicity of cadmium and zinc to seven invertebrate species from Western port, Victoria. *Australian Journal of Marine and Freshwater Research* 27, 187-196.
- Akaishi, F.M., St-Jean, S.D., Bishay, F., Clarke, J., Rabitto, I.S., Ribeiro, C.A.O., 2007. Immunological responses, histopathological finding and disease resistance of blue mussel (*Mytilus edulis*) exposed to treated and untreated municipal wastewater. *Aquatic Toxicology* 82, 1-14.
- Akberali, H.B., Trueman, E.R., 1985. Effects of environmental stress on marine bivalve molluscs. *Advances in Marine Biology* 22, 101-198.
- Aldridge, D.W., Payne, B.S., Miller, A.C., 1995. Oxygen consumption, nitrogenous excretion, and filtration rates of *Dreissena polymorpha* at acclimation temperatures between 20 and 32°C. *Canadian Journal of Fisheries and Aquatic Sciences* 52, 1761-1767.
- Alfven, T., Elinder, C.G., Carlsson, M.D., Grubb, A., Hellström, L., Persson, B., Petersson, C., Spång, G., Schütz, A., Järup, L., 2000. Low-level cadmium exposure and osteoporosis. *Journal of Bone and Mineral Research* 15, 1579-1586.
- Allen, H.E., 1993. The significance of trace metal speciation for water, sediment and soil quality criteria and standards. *Science of the Total Environment* 134, 23-45.
- Allen, Y., Calow, P., Baird, D.J., 1995. A mechanistic model of contaminant-induced feeding inhibition in *Daphnia magna*. *Environmental Toxicology and Chemistry* 14, 1625-1630.
- Almeida, J.A., Diniz, Y.S., Marques, S.F.G., Faine, L.A., Ribas, B.O., Burneiko, R.C., Novelli, E.L.B., 2002. The use of the oxidative stress responses as biomarkers in Nile

- tilapia (*Oreochromis niloticus*) exposed to in vivo Cd contamination. *Environment International* 27, 673-679.
- Almeida, J.A., Novelli, E.L.B., Silva, M.D.P., Júnior, R.A., 2001. Environmental cadmium exposure and metabolic responses of the Nile tilapia, *Oreochromis niloticus*. *Environmental Pollution* 114, 169-175.
- Al-Subiai, S.N., Moody, A.J., Mustafa, S.A., Jha, A.N., 2011. A multiple biomarker approach to investigate the effects of copper on the marine bivalve mollusc, *Mytilus edulis*. *Ecotoxicology and Environmental Safety* 74, 1913-1920.
- Amiard, J.-C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. *Aquatic Toxicology* 76, 160-202.
- Anandraj, A., Marshall, D.J., Gregory, M.A., McClurg, T.P., 2002. Metal accumulation, filtration and O₂ uptake rates in the mussel *Perna perna* (Mollusca: Bivalvia) exposed to Hg²⁺, Cu²⁺ and Zn²⁺. *Comparative Biochemistry and Physiology* 132C, 355-363.
- Anderlini, V.C., 1992. The effect of sewage on trace metal concentrations and scope for growth in *Mytilus edulis aoteanus* and *Perna canaliculus* from Wellington Harbour, New Zealand. *Science of the Total Environment* 125, 263-287.
- Ansaldo, M., Nahabedian, D.E., Holmes-Brown, E., Agote, M., Ansay, C.V., Guerrero, N.R.V., Wider, E.A., 2006. Potential use of glycogen level as biomarker of chemical stress in *Biomphalaria glabrata*. *Toxicology* 224, 119-127.
- ANZECC & ARMCANZ (2000) Australian and New Zealand Guidelines for Fresh and Marine Water Quality. Volume 1, Section 3.5: Sediment Quality Guidelines. Australian and New Zealand Environment and Conservation Council, and Agriculture and Resource Management Council of Australia and New Zealand. Canberra, Australia.
- Apeti, D.A., Lauenstein, G.G., Riedel, G.F., 2009. Cadmium distribution in coastal sediments and mollusks of the US. *Marine Pollution Bulletin* 58, 1016-1024.
- ARC (Auckland Regional Council), 2002. Pesticide Residues in Horticultural Soils in the Auckland Region, Working Report No. 96, Auckland Regional Council, February 2002.

- Argese, E., Bettiol, C., Rigo, C., Bertini, S., Colombari, S., Ghetti, P.F., 2005. Distribution of arsenic compounds in *Mytilus galloprovincialis* of the Venice lagoon (Italy) Science of The Total Environment 348, 267-277.
- Arnold, W.R., Cotsifas, J.S., Winter, A.R., Klinck, J.S., Smith, D.S., Playle, R.C., 2007. Effects of using synthetic sea salts when measuring and modeling copper toxicity in saltwater toxicity tests. Environmental Toxicology and Chemistry 26, 935-943.
- Arnold, W.R., Santore, R.C., Cotsifas, J.S., 2005. Predicting copper toxicity in estuarine and marine waters using the Biotic Ligand Model. Marine Pollution Bulletin 50, 1634-1640.
- Atkinson, C.A., Jolley, D.F., Simpson, S.L., 2007. Effect of overlying water pH, dissolved oxygen, salinity and sediment disturbances on metal release and sequestration from metal contaminated marine sediments. Chemosphere 69, 1428-1437.
- ATSDR (Agency for Toxic Substances and Disease Registry). 2012. Toxicological profile for cadmium. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service. <http://www.atsdr.cdc.gov/toxprofiles/tp.html>.
- Aubry, A., Elliott, M., 2006. The use of environmental integrative indicators to assess seabed disturbance in estuaries and coasts: Application to the Humber Estuary, UK. Marine Pollution Bulletin 53, 175-185.
- Auffret, M., Mujdzic, N., Corporeau, C., Moraga, D., 2002. Xenobiotic-induced immunomodulation in the European flat oyster, *Ostrea edulis*. Marine Environmental Research 54, 585-589.
- Auffret, M., Rousseau, S., Boutet, I., Tanguy, A., Baron, J., Moraga, D., Duchemin, M., 2006. A multiparametric approach for monitoring immunotoxic responses in mussels from contaminated sites in Western Mediterranean. Ecotoxicology and Environmental Safety 63, 393-405.
- Baines, S. B., Fisher, N. S., Kinney, E. L., 2006. Effects of temperature on uptake of aqueous metals by blue mussels *Mytilus edulis* from Arctic and temperate waters. Marine Ecology Progress Series 308, 117-128.

- Baker, C.F., Montgomery, J.C., 2001. Sensory deficit induced by cadmium in banded kokopu, *Galaxias fasciatus*, juveniles. *Environmental Biology of Fishes* 62, 455-464.
- Baldisserotto, B., Chowdhury, M.J., Wood, C.M., 2005. Effects of dietary calcium and cadmium on cadmium accumulation, calcium and cadmium uptake from the water, and their interactions in juvenile rainbow trout. *Aquatic Toxicology* 72, 99-117.
- Banakou, E., Dailianis, S., 2010. Involvement of Na⁺/H⁺ exchanger and respiratory burst enzymes NADPH oxidase and NO synthase, in Cd-induced lipid peroxidation and DNA damage in haemocytes of mussels. *Comparative Biochemistry and Physiology* 152C, 346-352.
- Barata, C., Alañon, P., Gutierrez-Alonso, S., Riva, M.C., Fernández, C., Tarazona, J.V., 2008. A *Daphnia magna* feeding bioassay as a cost effective and ecological relevant sublethal toxicity test for environmental risk assessment of toxic effluents. *Science of the Total Environment* 405, 78-86.
- Baršienė J., Lazutka J., Šyvokienė J., Dedonytė, V., Rybakovas, A., Bjornstad, A., Andersen O.K., 2004. Analysis of micronuclei in blue mussels and fish from the Baltic and the North Seas. *Environmental Toxicology* 19, 365-371.
- Baršienė, J., Rybakovas, A., Lang, T., Andreikėnaitė, L., Michailovas, A., 2013. Environmental genotoxicity and cytotoxicity levels in fish from the North Sea offshore region and Atlantic coastal waters. *Marine Pollution Bulletin* 68, 106-116.
- Bartsch, M.R., Waller, D.L., Cope, W.G., Gutreuter, S., 2000. Emersion and thermal tolerances of three species of unionid mussels: survival and behavioral effects. *Journal of Shellfish Research* 19, 233-240.
- Bayne, B.L., Brown, D.A., Burns, K., Dixon, D.R., Ivanovici, A., Livingstone, D.R., Lowe, D.M., Moore, M.N., Stebbing, A.R.D., Widdows, J., 1985. *The Effects of Stress and Pollution on Marine Animals*. Praeger, New York.
- Bayne, B. L., Newell, R. C., 1983. Physiological energetics of marine molluscs, in: Wilbur, K.M., Saleuddin, A. S.M. (Eds.), *The Mollusca Vol. 4, Physiology, Part 1*. Academic Press, New York, pp. 407-515.

- Bebianno, M.J., G eret, F., Hoarau, P., Serafim, M.A., Coelho, M.R., Gnassia-Barelli, M., Rom eo, M., 2004. Biomarkers in *Ruditapes decussatus*: a potential bioindicator species. *Biomarkers* 9, 305-330.
- Bebianno, M.J., Serafim, M.A., 1998. Comparison of metallothionein in response to cadmium in the gills of the bivalve molluscs *Mytilus galloprovincialis* and *Ruditapes decussatus*. *Science of the Total Environment* 214, 123-131.
- Beck, M., B oning, P., Sch uckel, U., Stiehl, T., Schnetger, B., Rullk otter, J., Brumsack, H.-J., 2013. Consistent assessment of trace metal contamination in surface sediments and suspended particulate matter: A case study from the Jade Bay in NW Germany. *Marine Pollution Bulletin* 70, 100-111.
- Beiras, R., Albentosa, M., 2004. Inhibition of embryo development of the commercial bivalves *Ruditapes decussatus* and *Mytilus galloprovincialis* by trace metals; implications for the implementation of seawater quality criteria. *Aquaculture* 230, 205-213.
- Bellas, J., Fern andez, N., Lorenzo, I., Beiras, R., 2008. Integrative assessment of coastal pollution in a R ia coastal system (Galicia, NW Spain): Correspondence between sediment chemistry and toxicity. *Chemosphere* 72, 826-835.
- Bendell, L.I., 2009. Survey of levels of cadmium in oysters, mussels, clams and scallops from the Pacific Northwest coast of Canada. *Food Additives and Contaminants: Part B Surveillance* 2, 131-139.
- Bertin, G., Averbeck, D., 2006. Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). *Biochimie* 88, 1549-1559.
- Bervoets, L., Voets, J., Chu, S.G., Covaci, A., Schepens, P., Blust, R., 2004. Comparison of accumulation of micropollutants between indigenous and transplanted zebra mussels (*Dreissena polymorpha*). *Environmental Toxicology and Chemistry* 23, 1973-1983.
- Besada, V., Andrade, J. M., Schultze, F., Fumega, J., Cambeiro, B., Gonz alez, J.J., 2008. Statistical comparison of trace metal concentrations in wild mussels (*Mytilus galloprovincialis*) in selected sites of Galicia and Gulf of Biscay (Spain). *Journal of Marine Systems* 72, 320-333.

- Birch, G.F., Chang, C.-H., Lee, J.-H., Churchill, L.J., 2013. The use of vintage surficial sediment data and sedimentary cores to determine past and future trends in estuarine metal contamination (Sydney estuary, Australia). *Science of the Total Environment* 454/455, 542-561.
- Bishop, S.H., Ellis, L.L., Burcham, J.M., 1983. Amino acid metabolism in molluscs, in: Hochachka, P. W. (Ed.), *The Mollusca. Metabolic Biochemistry and Molecular Biomechanics*. Vol 1: Academic Press, New York, pp. 243-327.
- Bjornevik, M., Solbakken, V., 2010. Preslaughter stress and subsequent effect on flesh quality in farmed cod. *Aquaculture Research* 41, 467-474.
- Blackmore, G., Wang, W.-X., 2002. Uptake and efflux of Cd and Zn by the green mussel *Perna viridis* after metal preexposure. *Environmental Science & Technology* 36, 989-995.
- Blackmore, G., Wang, W.-X., 2003a. Variations of metal accumulation in marine mussels at different local and global scales. *Environmental Toxicology and Chemistry* 22, 388-395.
- Blackmore, G., Wang, W.-X., 2003b. Inter-population differences in Cd, Cr, Se, and Zn accumulation by the green mussel *Perna viridis* acclimated at different salinities. *Aquatic Toxicology* 62, 205-218.
- Boening, D.W., 1999. An evaluation of bivalves as biomonitors of heavy metals pollution in marine waters. *Environmental Monitoring and Assessment* 55, 459-470.
- Boettcher, M., Grund, S., Keiter, S., Kosmehl, T., Reifferscheid, G., Seitz, N., Soares Rocha, P.S., Hollert, H., Braunbeck, T., 2010. Comparison of *in vitro* and *in situ* genotoxicity in the Danube River by means of the comet and the micronucleus test. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 700, 11-17.
- Bolognesi, C., Hayashi, M., 2011. Micronucleus assay in aquatic animals. *Mutagenesis* 26, 205-213.
- Bolognesi, C., Rabboni, R., Roggiere, P., 1996. Genotoxicity biomarkers in *Mytilus galloprovincialis* as indicators of marine pollutants. *Comparative Biochemistry and Physiology* 113C, 319-323.

- Bonneris, E., Perceval, O., Masson, S., Hare, L., Campbell, P.G.C., 2005. Sub-cellular partitioning of Cd, Cu and Zn in tissues of indigenous unionid bivalves living along a metal exposure gradient and links to metal-induced effects. *Environmental Pollution* 135, 195-208.
- Bourgeault, A., Gourlay-Francé, C., Priadi, C., Ayrault, S., Tusseau-Vuillemin, M.-H., 2011. Bioavailability of particulate metal to zebra mussels: Biodynamic modelling shows that assimilation efficiencies are site-specific. *Environmental Pollution* 159, 3381-3389.
- Bourne, R., 2003. Post-mortem: research spotlight falls on treated timber, August edition *Horticulture news*, Auckland, New Zealand, pp. 21-24.
- Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* 72, 248-254.
- Braunbeck, T., 1998. Cytological alterations in fish hepatocytes following in vivo and in vitro sublethal exposure to xenobiotics—structural biomarkers of environmental contamination, in: Braunbeck, T., Hinton, D.E., Streit, B. (Eds.), *Fish Ecotoxicology*. Birkhauser Verlag, Basel, Switzerland, pp. 61-140.
- Brix, K.V., Keithly, J., Santore, R.C., DeForest, D.K., Tobiasson, S., 2010. Ecological risk assessment of zinc from stormwater runoff to an aquatic ecosystem. *Science of the Total Environment* 408, 1824-1832.
- Brookes, P.S., Yoon, Y., Robotham, J.L., Anders, M.W., Sheu, S., 2004. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *American Journal of Physiology - Cell Physiology* 287, C817-C833.
- Brooks, J.D., Harvie, R.E., 1981. Quality changes during storage of the green-lipped mussel, *Perna canaliculus*. *Food Technology in Australia* 33, 490-495.
- Brousseau, P., Pellerin, J., Morin, Y., Cyr, D., Blakley, B., Boermans, H., Fournier, M., 2000. Flow cytometry as a tool to monitor the disturbance of phagocytosis in the clam *Mya arenaria* hemocytes following in vitro exposure to heavy metals. *Toxicology* 142, 145-156.

- Brown, J.N., Peake, B.M., 2006. Sources of heavy metals and polycyclic aromatic hydrocarbons in urban stormwater runoff. *Science of the Total Environment* 359, 145-155.
- Brown, J., Sander, S., Craw, D., Hunter, K., 2005. Measurement of labile metals in acid rock drainage springs, New Zealand: Field application of anodic stripping voltammetry. *Applied Geochemistry* 20, 1533-1545.
- Brown, K.L., Simmons, S.F., 2003. Precious metals in high-temperature geothermal systems in New Zealand. *Geothermics* 32, 619-625.
- Brunelli, E., Mauceri, A., Maisano, M., Bernabò, I., Giannetto, A., Domenico, E.D., Corapi, B., Tripepi, S., Fasulo, S., 2011. Ultrastructural and immunohistochemical investigation on the gills of the teleost, *Thalassoma pavo* L., exposed to cadmium. *Acta Histochemica* 113, 201-213.
- Buccolieri, A., Buccolieri, G., Cardellicchio, N., Dell'Atti, A., Leo, A.D., Maci, A., 2006. Heavy metals in marine sediments of Taranto Gulf (Ionian Sea, Southern Italy). *Marine Chemistry* 99, 227-235.
- Buckland, S.J., Jones, P.D., Ellis, H.K., Salter, R.T., 1998. Organochlorines in New Zealand: ambient concentrations of selected organochlorines in rivers. New Zealand Ministry for the Environment, Wellington.
- Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. *Methods in Enzymology* 52, 302-310.
- Burger, J., Gochfeld, M., 2006. Locational differences in heavy metals and metalloids in Pacific Blue Mussels *Mytilus [edulis] trossulus* from Adak Island in the Aleutian Chain, Alaska. *Science of the Total Environment* 368, 937-950.
- Burgess, R.M., Berry, W.J., Mount, D.R., Di Toro, D.M., 2013. Mechanistic sediment quality guidelines based on contaminant bioavailability: Equilibrium partitioning sediment benchmarks. *Environmental Toxicology and Chemistry* 32, 102-114.
- Burggraaf, S., Wilkins, A.L., Langdon, A.G., Kim, N.D., 1997. Heavy metals and organic hydrocarbons in sediments from the Waikareao Estuary, Tauranga Harbour, New Zealand. *Bulletin of Environmental Contamination and Toxicology* 58, 871-878.

- Bustamante, P., Miramand, P., 2004. Interspecific and geographical variations of trace element concentrations in Pectinidae from European waters. *Chemosphere* 57, 1355-1362.
- Caglak, E., Cakli, S., Kilinc, B., 2008. Microbiological, chemical and sensory assessment of mussels (*Mytilus galloprovincialis*) stored under modified atmosphere packaging. *European Food Research and Technology* 226, 1293-1299.
- Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *Science of the Total Environment* 247, 295-311.
- Calisi, A., Lionetto, M.G., Caricato, R., Giordano, M.E., Schettino, T., 2008. Morphometric alterations in *Mytilus galloprovincialis* granulocytes: a new biomarker. *Environmental Toxicology and Chemistry* 27, 1435-1441.
- Camacho, A.P., Delgado, M., Hernandez-Reiriz, M.J., Labarta, U., 2003. Energy balance, gonad development and biochemical composition in the clam *Ruditapes decussatus*. *Marine Ecology Progress Series* 258, 133-145.
- Cao, L., Huang, W., Shan, X., Ye, Z., Dou, S., 2012. Tissue-specific accumulation of cadmium and its effects on antioxidative responses in Japanese flounder juveniles. *Environmental Toxicology and Pharmacology* 33, 16-25.
- Castille, F.L., Lawrence, A.L., 1981. The effect of salinity on the osmotic, sodium and chloride concentrations in the hemolymph of euryhaline shrimp of the genus *Penaeus*. *Comparative Biochemistry and Physiology* 68A, 75-80.
- Cattani, O., Serra, R., Isani, G., Raggi, G., Cortesi, P., Carpena, E., 1996. Correlation between metallothionein and energy metabolism in sea bass, *Dicentrarchus labrax*, exposed to cadmium. *Comparative Biochemistry and Physiology* 113C, 193-199.
- Cavas, T., Garanko, N.N., Arkhipchuk, V.V., 2005. Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. *Food and Chemical Toxicology* 43, 569-574.

- Chakraborty, S., Ray, M., Ray, S., 2010. Toxicity of sodium arsenite in the gill of an economically important mollusc of India. *Fish & Shellfish Immunology* 29, 136-148.
- Chan, H.M., 1988. Accumulation and tolerance to cadmium, copper, lead and zinc by the green mussel *Perna viridis*. *Marine Ecology Progress Series* 48, 295-303.
- Chang, M.H., Lin, H.C., Hwang, P.P., 1998. Ca^{2+} uptake and Cd^{2+} accumulation in larvae tilapia (*Oreochromis mossambicus*) acclimated to waterborne Cd^{2+} . *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 274, R1570-R1577.
- Chapman, P.M., Wang, F., 2001. Assessing sediment contamination in estuaries. *Environmental Toxicology and Chemistry* 20, 3-22.
- Chapman, P.M., Wang, F., Janssen, C.R., Goulet, R.R., Kamunde, C.N., 2003. Conducting ecological risk assessment of inorganic metals and metalloids: current status. *Human and Ecological Risk Assessment* 9, 641-697.
- Chapman, P.M., Wang, F., Janssen, C., Persoone, G., Allen, H. E., 1998. Ecotoxicology of metals in aquatic sediments: binding and release, bioavailability, risk assessment, and remediation. *Canadian Journal of Fisheries and Aquatic Sciences* 55, 2221-2243.
- Chen, L.Y., Heath, A.G., Neves, R.J., 2001. An evaluation of air and water transport of freshwater mussels (Bivalvia: Unionidae). *American Malacological Bulletin* 16, 147-154.
- Cheng, T.C., 1988. In vivo effects of heavy metals on cellular defense mechanisms of *Crassostrea virginica*: total and differential haemocyte counts. *Journal of Invertebrate Pathology* 51, 207-214.
- Cherkasov, A.S., Grewal, S., Sokolova, I.M., 2007. Combined effects of temperature and cadmium exposure on haemocyte apoptosis and cadmium accumulation in the eastern oyster *Crassostrea virginica* (Gmelin). *Journal of Thermal Biology* 32, 162-170.
- Cheung, S.G., Cheung, R.Y.H., 1995. Effects of heavy metals on oxygen consumption and ammonia excretion in green mussels (*Perna viridis*). *Marine Pollution Bulletin* 31, 381-386.

- Chong, K., Wang, W.-X., 2001. Comparative studies on the biokinetics of Cd, Cr, and Zn in the green mussel *Perna viridis* and the Manila clam *Ruditapes philippinarum*. *Environmental Pollution* 115, 107-121.
- Chora, S., Starita-Geribaldi, M., Guignonis, J.-M., Samson, M., Roméo, M., Bebianno, M.J., 2009. Effect of cadmium in the clam *Ruditapes decussatus* assessed by proteomic analysis. *Aquatic Toxicology* 94, 300-308.
- Christophersen, G., Román, G., Gallagher, J., Magnesen, T., 2008. Post-transport recovery of cultured scallop (*Pecten maximus*) spat, juveniles and adults. *Aquaculture International* 16, 171-185.
- Christophoridis, C., Dedepsidis, D., Fytianos, K., 2009. Occurrence and distribution of selected heavy metals in the surface sediments of Thermaikos Gulf, N. Greece. Assessment using pollution indicators. *Journal of Hazardous Materials* 168, 1082-1091.
- Cima, F., Matozzo, V., Marin, M.G., Ballarin, L., 2000. Haemocytes of the clam *Tapes philippinarum* (Adams & Reeve, 1850): morphofunctional characterisation. *Fish & Shellfish Immunology* 10, 677-693.
- Clark, Z., Sivakumaran, S., Sharp, K., 2009. The concise New Zealand food composition tables (8th Ed.). New Zealand: New Zealand Institute for Plant & Food Research Ltd. New Zealand Ministry of Health, Wellington.
- Clarke, B.C., Griffiths, C.L., 1990. Ecological energetics of mussels *Choromytilus meridionalis* under simulated intertidal rock pool conditions. *Journal of Experimental Marine Biology and Ecology* 137, 63-77.
- Coleman, J.E., Gettings, P., 1983. Molecular properties and mechanisms of alkaline phosphatase, in: Spiro, T.G. (Ed.), *Zinc Enzymes*. Wiley Interscience, New York, pp.154-217.
- Coles, J.A., Farley, S.R., Pipe, R.K., 1995. Alteration of the immune response of the common marine mussel *Mytilus edulis* resulting from exposure to cadmium. *Diseases of Aquatic Organisms* 22, 59-65.

- Comoglio, L., Amin, O., Botté, S., Marcovecchio, J., 2011. Use of biomarkers in resident organisms as a tool for environmental monitoring in a cold coastal system, Tierra del Fuego Island. *Ecotoxicology and Environmental Safety* 74, 382-393.
- Company, R., Serafim, A., Cosson, R.P., Fiala-Médioni, A., Camus, L., Serrão-Santos, R., Bebianno, M.J., 2010. Sub-lethal effects of cadmium on the antioxidant defence system of the hydrothermal vent mussel *Bathymodiolus azoricus*. *Ecotoxicology and Environmental Safety* 73, 788-795.
- Conover, R.J., 1966. Assimilation of organic matter by zooplankton. *Limnology & Oceanography* 11, 338-354.
- Cooper, S., Bonneris, E., Michaud, A., Pinel-Alloul, B., Campbell, P.G.C., 2013. Influence of a step-change in metal exposure (Cd, Cu, Zn) on metal accumulation and subcellular partitioning in a freshwater bivalves *Pyganodon grandis*: A long-term transplantation experiment between lakes with contrasting ambient metal levels. *Aquatic Toxicology* 132/133, 73-83.
- Cooper, S., Hare, L., Campbell, P.G.C., 2010. Subcellular partitioning of cadmium in the freshwater bivalve, *Pyganodon grandis*, after separate short-term exposures to waterborne or diet-borne metal. *Aquatic Toxicology* 100, 303-312.
- Cope, W.G., Waller, D.L., 1995. Evaluation of freshwater mussel relocation as a conservation and management strategy. *Regulated Rivers: Research & Management* 11, 147-155.
- Costanza, R., 1997. The value of the world's ecosystem services and natural capital. *Nature* 387, 253-260.
- Coughlan, J., 1969. The estimation of filtering rate from the clearance of suspensions. *Marine Biology* 2, 356-358.
- Craw, D., Brown, K., Webster-Brown, J., 2005. Metals derived from gold mining and geothermal sources. In: Moore, T.A., Black, A., Centeno, J.A., Harding, J.S., Trumm, D.A. (Eds.), *Metal Contaminants in New Zealand*. Resolutionz Press, Christchurch, pp. 231-246.

- Croot, P.L., Hunter, K.A., 1998. Trace metal distributions across the continental shelf near Otago Peninsula, New Zealand. *Marine Chemistry* 62, 185-201.
- Cukrov, N., Frančišković-Bilinski, S., Hlača, B., Barišić, D., 2011. A recent history of metal accumulation in the sediments of Rijeka harbor, Adriatic Sea, Croatia. *Marine Pollution Bulletin* 62, 154-167.
- Cuypers, A., Plusquin, M., Remans, T., Jozefczak, M., Keunen, E., Gielen, H., Opdenakker, K., Nair, A.R., Munters, E., Artois, T.J., Nawrot, T., Vangronsveld, J., Smeets, K., 2010. Cadmium stress: an oxidative challenge. *Biometals* 23, 927-940.
- Dabrowska, H., Kopko, O., Turja, R., Lehtonen, K.K., Góra, A., Polak-Juszczak, L., Warzocha, J., Kholodkevich, S., 2013. Sediment contaminants and contaminant levels and biomarkers in caged mussels (*Mytilus trossulus*) in the southern Baltic Sea. *Marine Environmental Research* 84, 1-9.
- de Almeida, E.A., Miyamoto, S., Bainy, A.C.D., Medeiros, M.H.G., Mascio, P.D., 2004. Protective effect of phospholipid hydroperoxide glutathione peroxidase (PHGPx) against lipid peroxidation in mussels *Perna perna* exposed to different metals. *Marine Pollution Bulletin* 49, 386-392.
- De Jonge, M., Teuchies, J., Meire, P., Blust, R., Bervoets, L., 2012. The impact of increased oxygen conditions on metal-contaminated sediments part I: Effects on redox status, sediment geochemistry and metal bioavailability. *Water Research* 46, 2205-2214.
- Depledge, M.H., Aagaard, A., Gyorkos, P., 1995. Assessment of trace metal toxicity using molecular, physiological and behavioural biomarkers. *Marine Pollution Bulletin* 31, 19-27.
- Deudero, S., Box, A., Tejada, S., Tintoré, J., 2009. Stable isotopes and metal contamination in caged marine mussel *Mytilus galloprovincialis*. *Marine Pollution Bulletin* 58, 1025-1031.
- Dietrich, G.J., Dietrich, M., Kowalski, R.K., Dobosz, S., Karol, H., Demianowicz, W., Glogowski, J., 2010. Exposure of rainbow trout milt to mercury and cadmium alters sperm motility parameters and reproductive success. *Aquatic Toxicology* 97, 277-284.

- Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. *Environmental Toxicology and Chemistry* 20, 2383-2396.
- Di Toro, D.M., Mahony, J.D., Hansen, D.J., Scott, K.J., Carlson, A.R., Ankley, G.T., 1992. Acid volatile sulfide predicts the acute toxicity of cadmium and nickel in sediments. *Environmental Science & Technology* 26, 96-101.
- Di Toro, D. M., McGrath, J. A., Hansen, D. J., Berry, W. J., Paquin, P. R., Mathew, R., . Wu, K. B., Santore, R. C., 2005. Predicting sediment metal toxicity using a sediment biotic ligand model: Methodology and initial application. *Environmental Toxicology and Chemistry* 24, 2410-2427.
- Dixon, D.R., Pruski, A.M., Dixon, L.R.J., Jha, A.N., 2002. Marine invertebrate ecogenotoxicity: a methodological overview. *Mutagenesis* 17, 495-507.
- Duarte, C.A., Giarratano, E., Amin, O.A., Comoglio, L.I., 2011. Heavy metal concentrations and biomarkers of oxidative stress in native mussels (*Mytilus edulis chilensis*) from Beagle Channel coast (Tierra del Fuego, Argentina). *Marine Pollution Bulletin* 62, 1895-1904.
- Duchemin, M.B., Auffret, M., Wessel, N., Fortier, M., Morin, Y., Pellerin, J., Fournier, M., 2008. Multiple experimental approaches of immunotoxic effects of mercury chloride in the blue mussel, *Mytilus edulis*, through in vivo, in tubo and in vitro exposures. *Environmental Pollution* 153, 416-423.
- Duquesne, S., Liess, M., Bird, D.J., 2004. Sub-lethal effects of metal exposure: physiological and behavioural responses of the estuarine bivalve *Macoma balthica*. *Marine Environmental Research* 58, 245-250.
- Dyrynda, E.A., Pipe, R.K., Burt, G.R., Ratcliffe, N.A., 1998. Modulations in the immune defences of mussels (*Mytilus edulis*) from contaminated sites in the UK. *Aquatic Toxicology* 42, 169-185.
- Dyrynda, E.A., Pipe, R.K., Ratcliffe, N.A., 1997. Sub-populations of haemocytes in the adult and developing marine mussel, *Mytilus edulis*, identified by use of monoclonal antibodies. *Cell and Tissue Research* 289, 527-536.

- Eagar, S.H., 1999. Distribution of Ostracoda around a coastal sewer outfall: A case study from Wellington, New Zealand. *Journal of the Royal Society of New Zealand* 29, 257-264.
- ECAN (Environment Canterbury), 2007. Erosion and sediment control guidelines for the Canterbury region. Report No. R06/23, Canterbury Regional Council, Christchurch, New Zealand. ISBN No. 1-86937-607-2.
- ECAN (Environment Canterbury) , 2011a. The sediments and biota of the Avon-Heathcote Estuary/Ihutai and tidal reaches of the Avon and Heathcote rivers - Summary report on data collected in 2011. Report No. R12/110, Canterbury Regional Council, Christchurch, New Zealand.
- ECAN (Environment Canterbury), 2011b. Mapping earthquake induced topographical change and liquefaction in the Avon-Heathcote Estuary. Report No. U11/13, Canterbury Regional Council, Christchurch, New Zealand.
- ECAN (Environment Canterbury), 2012. Heavy metals in fish and shellfish from Christchurch rivers and Estuary: 2012 Survey - Report on data collected in 2012 Report No. R12/77, Canterbury Regional Council, Christchurch, New Zealand.
- Edward, F.B., Yap, C.K., Ismail, A., Tan, S.G., 2009. Interspecific variation of heavy metal concentrations in the different parts of tropical intertidal bivalves. *Water, Air, and Soil Pollution* 196, 297-309.
- Einsporn, S., Koehler, A., 2008. Immuno-localisations (GSSP) of subcellular accumulation sites of phenanthrene, aroclor 1254 and lead (Pb) in relation to cytopathologies in the gills and digestive gland of the mussel *Mytilus edulis*. *Marine Environmental Research* 66, 185-186.
- Eisler, R., 1971. Cadmium poisoning in *Fundulus heteroclitus* (Pisces: Cyprinodontidae) and other marine organisms. *Journal of Fisheries Research Board of Canada* 28, 1225-1234.
- Eisler, R., 2010. Molluscs, in: *Compendium of Trace Metals and Marine Biota: Volume 1: Plants and Invertebrates*, Elsevier, 143-397.

- El-Demerdash, F.M., Elagamy, E.I., 1999. Biological effects in *Tilapia nilotica* fish as indicators of pollution by cadmium and mercury. *International Journal of Environmental Health Research* 9, 173-186.
- Ellwood, M.J., 2004. Zinc and cadmium speciation in subantarctic waters east of New Zealand. *Marine Chemistry* 87, 37-58.
- Emmanouil, C., Sheehan, T.M.T., Chipman, J.K., 2007. Macromolecule oxidation and DNA repair in mussel (*Mytilus edulis* L.) gill following exposure to Cd and Cr (VI). *Aquatic Toxicology* 82, 27-35.
- Erickson, R.J., Mount, D.R., Highland, T.L., Hockett, J.R., Jenson, C.T., 2011. The relative importance of waterborne and dietborne arsenic exposure on survival and growth of juvenile rainbow trout. *Aquatic Toxicology* 104, 108-115.
- Erk, M., Ruus, A., Ingebrigtsen, K., Hylland, K., 2005. Cadmium accumulation and Cd-binding proteins in marine invertebrates—A radiotracer study. *Chemosphere* 61, 1651-1664.
- Fairbrother, A., Wenstel, R., Sappington, K., Wood, W., 2007. Framework for metals risk assessment. *Ecotoxicology and Environmental Safety* 68, 145-227.
- Fattorini, D., Notti, A., Regoli, F., 2006. Characterization of arsenic content in marine organisms from temperate, tropical, and polar environments. *Journal of Chemical Ecology* 22, 405-414.
- Faucher, K., Fichet, D., Miramand, P., Lagardère, J-P., 2008. Impact of chronic cadmium exposure at environmental dose on escape behaviour in sea bass (*Dicentrarchus labrax* L.; Teleostei, Moronidae). *Environmental Pollution* 151, 148-157.
- Felten, V., Charmantier, G., Mons, R., Geffard, A., Rousselle, P., Coquery, M., Garric, J., Geffard, O., 2008. Physiological and behavioural responses of *Gammarus pulex* (Crustacea: Amphipoda) exposed to cadmium. *Aquatic Toxicology* 86, 413-425.
- Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E., 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 534, 65-75.

- Firat, O., Cogun, H.Y., Aslanyavrusu, S., Kargin, F., 2009. Antioxidant responses and metal accumulation in tissues of Nile tilapia *Oreochromis niloticus* under Zn, Cd and Zn+Cd exposures. *Journal of Applied Toxicology* 29, 295-301.
- Fisher, N.S., Teyssie, J.-L., Fowler, S.W., Wang, W.-X., 1996. Accumulation and retention of metals in mussels from food and water: a comparison under field and laboratory conditions. *Environmental Science & Technology* 30, 3232-3242.
- Foll, F.L., Rioult, D., Boussa, S., Pasquier, J., Dagher, Z., Leboulenger, F., 2010. Characterisation of *Mytilus edulis* hemocyte subpopulations by single cell time-lapse motility imaging. *Fish & Shellfish Immunology* 28, 372-386.
- Fraser, M., Surette, C., Vaillancourt, C., 2011. Spatial and temporal distribution of heavy metal concentrations in mussels (*Mytilus edulis*) from the Baie des Chaleurs, New Brunswick, Canada. *Marine Pollution Bulletin* 62, 1345-1351.
- Frenzilli, G., Nigro, M., Lyons, B.P., 2009. The Comet assay for the evaluation of genotoxic impact in aquatic environments. *Mutation Research/Reviews in Mutation Research* 681, 80-92.
- Frew, R., Hunter, K., 1995. Cadmium-phosphorus cycling at the subtropical convergence south of New Zealand. *Marine Chemistry* 51, 223-237.
- FSANZ (Food Standards Australia New Zealand) Australia New Zealand Food Standard Code, 2002. "Contaminants and natural toxicants" Food Standard Code: 251.
- Fukunaga, A., Anderson, M.J., 2011. Bioaccumulation of copper, lead and zinc by the bivalves *Macomona liliana* and *Austrovenus stutchburyi*. *Journal of Experimental Marine Biology and Ecology* 396, 244-252.
- Fung, C.N., Lam, J.C.W., Zheng, G.J., Connell, D.W., Monirith, I., Tanabe, S., Richardson, B.J., Lam, P.K.S., 2004. Mussel-based monitoring of trace metal and organic contaminants along the east coast of China using *Perna viridis* and *Mytilus edulis*. *Environmental Pollution* 127, 203-216.
- Gagnon, C., Gagné, F., Turcotte, P., Saulnier, I., Blaise, C., Salazar, M.H., Salazar, S.M., 2006. Exposure of caged mussels to metals in a primary-treated municipal wastewater plume. *Chemosphere* 62, 998-1010.

- Galloway, T.S., Brown, R.J., Browne, M.A., Dissanayake, A., Lowe, D., Jones, M.B., Depledge, M.H., 2004. Ecosystem management bioindicators: the ECOMAN project – a multi-biomarker approach to ecosystem management. *Marine Environmental Research* 58, 233-237.
- Galloway, T.S., Depledge, M.H., 2001. Immunotoxicity in invertebrates: measurement and ecotoxicological relevance. *Ecotoxicology* 10, 5-23.
- Galloway, T.S., Goven, A., 2006. Invertebrate immunotoxicology, in: Luebke, R. (Ed.), *Immunotoxicology and Immunopharmacology*. CRC Press, Boca Raton, pp. 365-383.
- Gao, X., Li, P., 2012. Concentration and fractionation of trace metals in surface sediments of intertidal Bohai Bay, China. *Marine Pollution Bulletin* 64, 1529-1536.
- Gardner, J.P.A., 2000. Where are the mussels on Cook Strait (New Zealand) shores? Low seston quality as a possible factor limiting multi-species distributions. *Marine Ecology Progress Series* 194, 123-132.
- Gardner, J.P.A., 2002. Effects of seston variability on the clearance rate and absorption efficiency of the mussels *Aulacomya maoriana*, *Mytilus galloprovincialis* and *Perna canaliculus* from New Zealand. *Journal of Experimental Marine Biology and Ecology* 268, 83-101.
- Garton, D.W., Berg, D.J., 1989. Genetic variation at the Lap locus and ammonia excretion following salinity transfer in an estuarine snail. *Comparative Biochemistry and Physiology* 92A, 71-74.
- Géret, F., Cosson, R.P., 2002. Induction of specific isoforms of metallothionein in mussel tissues after exposure to cadmium or mercury. *Archives of Environmental Contamination and Toxicology* 42, 36-42.
- Géret, F., Jouan, A., Turpin, V., Bebianno, M.J., Cosson, R.P., 2002. Influence of metal exposure on metallothionein synthesis and lipid peroxidation in two bivalve molluscs: the oyster (*Crassostrea gigas*) and the mussel (*Mytilus edulis*). *Aquatic Living Resources* 15, 61-66.

- Giari, L., Manera, M., Simoni, E., Dezfuli, B.S., 2007. Cellular alterations in different organs of European sea bass *Dicentrarchus labrax* (L.) exposed to cadmium. *Chemosphere* 67, 1171-1181.
- Giarratano, E., Amin, O.A., 2010. Heavy metals monitoring in the southernmost mussel farm of the world (Beagle Channel, Argentina). *Ecotoxicology and Environmental Safety* 73, 1378-1384.
- Giarratano, E., Duarte, C. A., Amin, O.A., 2010. Biomarkers and heavy metal bioaccumulation in mussels transplanted to coastal waters of the Beagle Channel. *Ecotoxicology and Environmental Safety* 73, 270-279.
- Gnaiger, E., 1983. Heat dissipation and energetic efficiency in animal anoxibiosis: Economy contra power. *Journal of Experimental Zoology* 228, 471-490.
- Goldberg, E.D., 1975. The Mussel Watch— A first step in global marine monitoring. *Marine Pollution Bulletin* 6, 111.
- Goldsmith, S.T., Carey, A.E., Lyons, W.B., Hicks, D.M., 2008. Geochemical fluxes and weathering of volcanic terrains on high standing islands: Taranaki and Manawatu-Wanganui regions of New Zealand. *Geochimica et Cosmochimica Acta* 72, 2248-2267.
- Gomez-Mendikute, A., Cajaraville, M.P., 2003. Comparative effects of cadmium, copper, paraquat and benzo[a]pyrene on the actin cytoskeleton and production of reactive oxygen species (ROS) in mussel haemocytes. *Toxicology in Vitro* 17, 539-546.
- Goulet, R.R., Krack, S., Doyle, P.J., Hare, L., Vigneault, B., McGeer, J.C., 2007. Dynamic multipathway modeling of Cd bioaccumulation in *Daphnia magna* using waterborne and dietborne exposures. *Aquatic Toxicology* 81, 117-125.
- Grintzalis, K., Georgiou, C.D., Dailianis, S., 2012. Total thiol redox status as a potent biomarker of PAH-mediated effects on mussels. *Marine Environmental Research* 81, 26-34.
- Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates, in: Smith, W., Chanley, M. (Eds.), *Culture of Marine Invertebrate Animals*. Plenum Press, New York, pp. 29-60.

- Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. Canadian Journal of Microbiology 8, 229-239.
- Gundacker, C., 1999. Tissue-specific heavy metal (Cd, Pb, Cu, Zn) deposition in a natural population of the zebra mussel *Dreissena polymorpha* Pallas. Chemosphere 38, 3339-3356.
- Haffert, L., Craw, D., 2008. Mineralogical controls on environmental mobility of As from historic mine processing residues, NZ. Applied Geochemistry 23, 1467-1483h.
- Hallare, A.V., Schirling, M., Luckenbach, T., Kohler, H.-R., Triebkorn, R., 2005. Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. Journal of Thermal Biology 30, 7-17.
- Hamilton, M.A., Russo, R.C., Thurston, R.V., 1977. Trimmed Spearman-Kärber method for estimating median lethal concentrations in toxicity bioassays. Environmental Science & Technology 11, 714-719.
- Hansen, J.A., Welsh, P.G., Lipton, J., Suedkamp, M.J., 2002. The effects of long-term cadmium exposure on the growth and survival of juvenile bull trout (*Salvelinus confluentus*). Aquatic Toxicology 58, 165-174.
- Hartwig, A., 1994. Role of DNA repair inhibition in lead- and cadmium-induced genotoxicity: a review. Environmental Health Perspectives 102, 45-50.
- Hawkins, A.J.S., Bayne, B.L., 1991. Nutrition of marine mussels: factors influencing the relative utilisation of protein and energy. Aquaculture 94, 177-196.
- Hervé -Fernández, P., Houlbrèque, F., Boisson, F., Mulsow, S., Teyssié, J.-L., Oberhaensli, F., Azemard, S., Jeffree, R., 2010. Cadmium bioaccumulation and retention kinetics in the Chilean blue mussel *Mytilus chilensis*: seawater and food exposure pathways. Aquatic Toxicology 99, 448-456.
- Hicks, D.W., McMahon, R.F., 2002. Respiratory responses to temperature and hypoxia in the non-indigenous brown mussel, *Perna perna* (Bivalvia: Mytilidae), from the Gulf of Mexico. Journal of Experimental Marine Biology and Ecology 277, 61-78.

- Hinton, D.E., Laurén, D.J., 1990. Integrative histopathological effects of environmental stressors on fishes. *American Fisheries Society Symposium* 8, 51-66.
- Hodgkin, E.P., 1994. Estuaries and coastal lagoons., in: Hammond, L.S., Synnot, R.N. (Eds.), *Marine Biology*. Longman Cheshire, Melbourne, pp. 315-332.
- Hogstrand, C., Haux, C., 1991. Binding and detoxification of heavy metals in lower vertebrates with reference to metallothionein. *Comparative Biochemistry and Physiology* 100C, 137-141.
- Hollis, L., McGeer, J.C., McDonald, D.G., Wood, C.M., 2000. Protective effects of calcium against chronic waterborne cadmium exposure to juvenile rainbow trout. *Environmental Toxicology and Chemistry* 19, 2725-2734.
- Honkoop, P.J.C., Bayne, B.L., Underwood, A.J., Svensson, S., 2003. Appropriate experimental design for transplanting mussels (*Mytilus* sp.) in analyses of environmental stress: an example in Sydney Harbour (Australia). *Journal of Experimental Marine Biology and Ecology* 297, 253-268.
- Hutchinson, T.H., Williams, T.D., Eales, G.J., 1994. Toxicity of cadmium, hexavalent chromium and copper to marine fish larvae (*Cyprinodon variegatus*) and copepods (*Tisbe battagliai*). *Marine Environmental Research* 38, 275-290.
- Hyun, S., Lee, C.-H., Lee, T., Choi, J.-W. , 2007. Anthropogenic contributions to heavy metal distributions in the surface sediments of Masan Bay, Korea. *Marine Pollution Bulletin* 54, 1059-1068.
- IARC (International Agency for Research on Cancer) Monographs., 1993, Cadmium, Vol. 58, IARC Press, Lyon, pp. 119-238.
- Ikeda, T., 1977. The effect of laboratory conditions on the extrapolation of experimental measurements to the ecology of marine zooplankton. IV. Changes in respiration and excretion rates of boreal zooplankton species maintained under fed and starved conditions. *Marine Biology* 41, 241-252.
- Inanba, T., Kobayashi, E., Suwazono, Y., Uetani, M., Oishi, M., Nakagawa, H., Nogawa, K., 2005. Estimation of cumulative cadmium intake causing Itai-itai disease. *Toxicology Letters* 159, 192-201.

- Ishihara, T., Kobayashi, E., Okubo, Y., Suwazono, Y., Kido, T., Nishijyo, M., Nakagawa, H., Nogawa, K., 2001. Association between cadmium concentration in rice and mortality in the Jinzu River basin, Japan. *Toxicology* 163, 23-28.
- Issartel, J., Boulo, V., Wallon, S., Geffard, O., Charmantier, G., 2010. Cellular and molecular osmoregulatory responses to cadmium exposure in *Gammarus fossarum* (Crustacea: Amphipoda) *Chemosphere* 81, 701-710.
- Ivanina, A.V., Habinck, E., Sokolova, I.M., 2008. Differential sensitivity to cadmium of key mitochondrial enzymes in the eastern oyster, *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). *Comparative Biochemistry and Physiology* 148C, 72-79.
- Jansen, J.M., Wendelaar Bonga, S.E., Hummel, H., 2007. Differential cold-shock resistance among acclimated European mussel populations. *Marine and Freshwater Behaviour and Physiology* 40, 233-245.
- Järup, L., Åkesson, A., 2009. Current status of cadmium as an environmental health problem. *Toxicology and Applied Pharmacology* 238, 201-208.
- Jeffs, A.G., Holland, R.C., Hooker, S.H., Hayden, B.J., 1999. Overview and bibliography of research on the greenshell mussel, *Perna canaliculus*, from New Zealand waters. *Journal of Shellfish Research* 18, 347-360.
- Jeng, M-S., Jeng, W-L., Hung, T-C., Yeh, C-Y., Tseng, R-J., Meng, P-J., Han, B-C., 2000. Mussel Watch: a review of Cu and other metals in various marine organisms in Taiwan, 1991–98. *Environmental Pollution* 110, 207-215.
- Jha, A. N., Dogra, Y., Turner, A., Millward, G.E., 2005. Impact of low doses of tritium on the marine mussel, *Mytilus edulis*: Genotoxic effects and tissue-specific bioconcentration. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 586, 47-57.
- Jones, O.A.H., Dondero, F., Viarengo, A., Griffin, J.L., 2008. Metabolic profiling of *Mytilus galloprovincialis* and its potential applications for pollution assessment. *Marine Ecology Progress Series* 369, 169-179.
- Jones, H.D., Richards, O.G., Southern, T.A., 1992. Gill dimensions, water pumping rate and body size in the mussel *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology* 155, 213-237.

- Jørgensen, C.B., Larsen, P.S., Riisgård, H.U., 1990. Effects of temperature on the mussel pump. *Marine Ecology and Progress Series* 64, 89-97.
- Ju, Y.-R., Chen, W.-Y., Singh, S., Liao, C.-M., 2011. Trade-offs between elimination and detoxification in rainbow trout and common bivalve molluscs exposed to metal stressors. *Chemosphere* 85, 1048-1056.
- Katsikatsou, M., Anestis, A., Pörtner, H.O., Kampouris, T., Michaelidis, B., 2011. Field studies on the relation between the accumulation of heavy metals and metabolic and HSR in the bearded horse mussel *Modiolus barbatus*. *Comparative Biochemistry and Physiology* 153C, 133-140.
- Ketata, I., Smaoui-Damak, W., Guermazi, F., Rebai, T., Hamza-Chaffai, A., 2007. In situ endocrine disrupting effects of cadmium on the reproduction of *Ruditapes decussatus*. *Comparative Biochemistry and Physiology* 146C, 415-430.
- Kil, I.S., Shin, S.W., Yeo, H.S., Lee, Y.S., Park, J.-W., 2006. Mitochondrial NADP-dependent isocitrate dehydrogenase protects cadmium-induced apoptosis. *Molecular Pharmacology* 70, 1053-1061.
- Kim, M.C., Cho, S.M., Jeong, W.G., 2007. Short-term physiological response of the Pacific oyster, *Crassostrea gigas*, on exposure to varying levels of polycyclic aromatic hydrocarbon. *Aquaculture Research* 38, 1612-1618.
- Kim, N., Fergusson, J., 1993. Concentrations and sources of cadmium, copper, lead and zinc in house dust in Christchurch, New Zealand. *Science of the Total Environment* 138, 1-21.
- Kingett Mitchell Ltd., 2003. A Study of Roof Runoff Quality in Auckland New Zealand: Implications for Stormwater Management. Auckland Regional Council, Auckland, New Zealand.
- Klinck, J.S., Wood, C.M., 2011. In vitro characterization of cadmium transport along the gastro-intestinal tract of freshwater rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* 102, 58-72.

- Kraak, M.H.S., Lavy, D., Peeters, W.H.M., Davids, C., 1992. Chronic ecotoxicity of copper and cadmium to the zebra mussel *Dreissena polymorpha*. Archives of Environmental Contamination and Toxicology 23, 363-369.
- Kumada, H., Kimura, S., Yokote, M., 1980. Accumulation and biological effects of cadmium in rainbow trout. Bulletin of the Japanese Society for the Science of Fish 46, 97-103.
- Kwok, A., Lyons, B.P., Hodges, N.J., Bean, T.P., 2013. Cryopreservation and storage of mussel (*Mytilus* spp.) haemocytes for latent analysis by the Comet assay. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 750, 86-91.
- Lane, T.W., Morel, M.M., 2000. A biological function for cadmium in marine diatoms. Proceedings of the National Academy of Sciences USA 97, 4627-4631.
- Latire, T., Pabic, C.L., Mottin, E., Mottier, A., Costil, K., Koueta, N., Lebel, J-M., Serpentine, A., 2012. Responses of primary cultured haemocytes from the marine gastropod *Haliotis tuberculata* under 10-day exposure to cadmium chloride. Aquatic Toxicology 109, 213-221.
- Lee, A.C., Lin, Y.H., Lin, C.R., Lee, M.C., Chen, Y.P., 2007. Effects of components in seawater on the digging behavior of the hard clam (*Meretrix lusoria*). Aquaculture 272, 636-643.
- Lee, R.F., Steinert, S., 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 544, 43-64.
- Liao, C.M., Chang, C.-F., Yeh, C.-H., Chen, S.-C., Chiang, K.-C., Chio, C.-P., Chou, B.Y.-H., Jou, L.-J., Lien, G.-W., Lin, C.-M., Shen, H.-H., Wu, G.-D., 2006. Metal stresses affect the population dynamics of disease transmission in aquaculture species. Aquaculture 257, 321-332.
- Linton, D.M., Warner, G.F., 2003. Biological indicators in the Caribbean coastal zone and their role in integrated coastal management. Ocean & Coastal Management 46, 261-276.
- Lionetto, M.G., Giordano, M.E., Vilella, S., Schettino, T., 2000. Inhibition of eel enzymatic activities by cadmium. Aquatic Toxicology 48, 561-571.

- Lionetto, M.G., Maffia, M., Cappello, M.S., Giordano, M.E., Storelli, C., Schettino, T., 1998. Effect of cadmium on carbonic anhydrase and Na⁺-K⁺-ATPase in eel, *Anguilla anguilla*, intestine and gills. *Comparative Biochemistry and Physiology* 120A, 89-91.
- Liu, X.-J., Luo, Z., Li, C.-H., Xiong, B.-X., Zhao, Y.-H., Li, X.-D., 2011. Antioxidant responses, hepatic intermediary metabolism, histology and ultrastructure in *Synechogobius hasta* exposed to waterborne cadmium. *Ecotoxicology and Environmental Safety* 74, 1156-1163.
- Liu, J.H., Kueh, C.S.W., 2005. Biomonitoring of heavy metals and trace organics using the intertidal mussel *Perna viridis* in Hong Kong coastal waters. *Marine Pollution Bulletin* 51, 857-875.
- Livingstone, D.R., Widdows, J., Fieth, P., 1979. Aspects of nitrogen metabolism of the common mussel *Mytilus edulis*: adaptation to abrupt and fluctuating changes in salinity. *Marine Biology* 53, 41-55.
- Lizardo-Daudt, H.M., Kennedy, C., 2008. Effects of cadmium chloride on the development of rainbow trout *Oncorhynchus mykiss* early life stages. *Journal of Fish Biology* 73, 702-718.
- Loayza-Muro, R., Elias-Letts, R., 2007. Responses of the mussel *Anodontites trapesialis* (Unionidae) to environmental stressors: effect of pH, temperature and metals on filtration rate. *Environmental Pollution* 149, 209-215.
- Long, A., Li, C., Chen, S., Yan, W., Dang, A., Cheng, Y., Lu, D., 2010. Short-term metal accumulation and MTLP induction in the digestive glands of *Perna viridis* exposed to Zn and Cd. *Journal of Environmental Science* 22, 975-981.
- Lorenzon, S., Giulianini, P.G., Libralato, S., Martinis, M., Ferrero, E.A., 2008. Stress effect of two different transport systems on the physiological profiles of the crab *Cancer pagurus*. *Aquaculture* 278, 156-163.
- Lorenzon, S., Giulianini, P.G., Martinis, M., Ferrero, E.A., 2007. Stress effect of different temperatures and air exposure during transport on physiological profiles in the American lobster *Homarus americanus*. *Comparative Biochemistry and Physiology* 147A, 94-102.

- Loro, V.L., Jorge, M.B., da Silva, K.R., Wood, C.M., 2012. Oxidative stress parameters and antioxidant response to sublethal waterborne zinc in a euryhaline teleost *Fundulus heteroclitus*: Protective effects of salinity. *Aquatic Toxicology* 110/111, 187-193.
- Luoma, S.N., Rainbow, P.S., 2005. Why Is Metal Bioaccumulation So Variable? Biodynamics as a Unifying Concept. *Environmental Science & Technology* 39, 1921-1931.
- Luoma, S.N., Rainbow, P.S., 2008. *Metal Contamination in Aquatic Environments: science and lateral management*. Cambridge University Press, Cambridge.
- Lurman, G.J., Hilton, Z., Ragg, N.L.C., 2013. Energetics of byssus attachment and feeding in the green-lipped mussel *Perna canaliculus*. *Biological Bulletin* 224, 79-88.
- Ma, W., Wang, L., He, Y., Yan, Y., 2008. Tissue-specific cadmium and metallothionein levels in freshwater crab *Sinopotamon henanense* during acute exposure to waterborne cadmium. *Environmental Toxicology* 23, 393-400.
- MacGregor, S.E., Walker, J.M., 1993. Inhibitors of the Na⁺, K⁺ ATPase. *Comparative Biochemistry and Physiology* 105C, 1-9.
- Maguire, J.A., Cashmore, D., Burnell, G.M., 1999. The effect of transportation on the juvenile scallop *Pecten maximus* (L.). *Aquaculture Research* 30, 325-333.
- Mamindy-Pajany, Y., Hurel, C., G ret, F., Galgani, F., Battaglia-Brunet, F., Marmier, N., Rom o, M., 2013. Arsenic in marine sediments from French Mediterranean ports: Geochemical partitioning, bioavailability and ecotoxicology. *Chemosphere* 90, 2730-2736.
- Manyin, T., Rowe, C.L., 2009. Bioenergetic effects of aqueous copper and cadmium on the grass shrimp, *Palaemonetes pugio*. *Comparative Biochemistry and Physiology* 150C, 65-71.
- Marig mez, I., Soto, M., Cajaraville, M.P., Angulo, E., Giamberini, L., 2002. Cellular and subcellular distribution of metals in molluscs. *Microscopic Research and Techniques* 56, 358-392.
- Marig mez, I., Zorita, I., Izagirre, U., Ortiz-Zarragoitia, M., Navarro, P., Etxebarria, N., Orbea, A., Soto, M., Cajaraville, M.P., 2013. Combined use of native and caged

- mussels to assess biological effects of pollution through the integrative biomarker approach. *Aquatic Toxicology* 136/137, 32-48.
- Marinho, C.H., Gil, M.N., Esteves, J.L., 2013. Distribution and origin of trace metals in sediments of a marine park (Northern San Jorge Gulf) from Argentina. *Marine Pollution Bulletin* 72, 260-263.
- Marsden, I.D., 2004. Effects of reduced salinity and seston availability on growth of the New Zealand little-neck clam *Austrovenus stutchburyi*. *Marine Ecology Progress Series* 266, 157-171.
- Marsden, I.D., Weatherhead, M.A., 1998. Effects of aerial exposure on oxygen consumption by the New Zealand mussel *Perna canaliculus* (Gmelin, 1791) from an intertidal habitat. *Journal of Experimental Marine Biology and Ecology* 230, 15-29.
- Martí, N., Aguado, D., Segovia-Martínez, L., Bouzas, A., Seco, A., 2011. Occurrence of priority pollutants in WWTP effluents and Mediterranean coastal waters of Spain. *Marine Pollution Bulletin* 62, 615-625.
- Martinez, M., Del Ramo, J., Torreblanca, A., Pastor, A., Diaz-Mayans, J., 1996. Cadmium toxicity, accumulation and metallothionein induction in *Echinogammarus echinosetosus*. *Journal of Environmental Science and Health A* 31, 1605-1617.
- Martínez, M.L., Intralawan, A., Vázquez, G., Pérez-Maqueo, O., Sutton, P., Landgrave, R., 2007. The coasts of our world: ecological, economic and social importance. *Ecological Economics* 63, 254-272.
- Martínez-Gómez, C., Fernández, B., Benedicto, J., Valdés, J., Campillo, J.A., León, V.M., Vethaak, A.D., 2012. Health status of red mullets from polluted areas of the Spanish Mediterranean coast, with special reference to Portmán (SE Spain). *Marine Environmental Research* 77, 50-59.
- Mazorra, M.T., Rubio, J.A., Blasco, J., 2002. Acid and alkaline phosphatase activities in the clam *Scrobicularia plana*: kinetic characteristics and effects of heavy metals. *Comparative Biochemistry and Physiology* 131B, 241-249.

- McCarthy, J.F., Shugart, L.R., 1990. Biological markers of environmental contamination, in: McCarthy, J.F., Shugart, L.R. (Eds.), Biomarkers of environmental contamination. Lewis Publishers, Boca Raton, Florida, pp. 3-14.
- McCarty, L.S., Power, M., Munkittrick, K.R., 2002. Bioindicators versus biomarkers in ecological risk assessment. *Human and Ecological Risk Assessment* 8, 159-164.
- McCauley, D.J., DeGraeve, G.M., Linton, T.K., 2000. Sediment quality guidelines and assessment: overview and research needs. *Environmental Science & Policy* 3, 133-144.
- McCormick, S.D., 1993. Methods for nonlethal gill biopsy and measurements of Na⁺, K⁺-ATPase activity. *Canadian Journal of Fisheries and Aquatic Science* 50, 656-658.
- McCready, S., Birch, G.F., Long, E.R., 2006. Metallic and organic contaminants in sediments of Sydney Harbour, Australia and vicinity - A chemical dataset for evaluating sediment quality guidelines. *Environment International*, 32, 455-465.
- McDowell, R.W., Taylor, M.D., Stevenson, B.A., 2013. Natural background and anthropogenic contributions of cadmium to New Zealand soils. *Agriculture, Ecosystems and Environment* 165, 80-87.
- McGeer, J.C., Brix, K.V., Skeaff, J.M., DeForest, D.K., Brigham, S.I., Adams, W.J., Green, A., 2003. Inverse relationship between bioconcentration factor and exposure concentration for metals: Implications for hazard assessment of metals in the aquatic environment. *Environmental Toxicology and Chemistry* 22, 1017-1037.
- McGeer, J.C., Henningsen, G., Lanno, R., Fisher, N., Sappington, K., Drexler, J., 2004. Issue paper on the bioavailability and bioaccumulation of metals. US Environmental Protection Agency risk assessment forum. 126. <http://cfpub2.epa.gov/ncea/raf/recordisplay.cfm?deid=86119>.
- McGeer, J.C., Niyogi, S., Smith, D.S., 2011. Cadmium, in: Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), *Fish Physiology: Homeostasis and Toxicology of Non-Essential Metals*. Vol. 31B, Elsevier, New York, pp. 125-184.
- McGeer, J.C., Szebedinszky, C., McDonald, D.G., Wood, C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd, or Zn in rainbow trout. 1: ionoregulatory disturbance and metabolic costs. *Aquatic Toxicology* 50, 233-245.

- McGrath, D., Zhang, C., Carton, O. T., 2004. Geostatistical analyses and hazard assessment on soil lead in Silvermines area, Ireland. *Environmental Pollution* 127, 239-248.
- McLaren, R.G., Naidu, R., Smith, J., Tiller, K.G., 1998. Fractionation and distribution of arsenic in soils contaminated by cattle dip. *Journal of Environmental Quality* 27, 348-354.
- McLeese, D.W., Ray, S., 1986. Toxicity of CdCl₂, CdEDTA, CuCl₂ and CuEDTA to marine invertebrates. *Bulletin of Environmental Contamination and Toxicology* 36, 749-755.
- McLusky, D.S., Elliott, M., 2004. *The Estuarine Ecosystem: Ecology, Threats and Management*, third ed. Oxford University Press, Oxford.
- McMurtrie, S., Kennedy, S., 2012. *Exploring an Estuary. A field guide to the Avon-Heathcote Estuary/Ihutai* Christchurch. Avon-Heathcote Estuary Ihutai Trust, Christchurch.
- Menezes, S., Soares, A.M.V.M., Guilhermino, L., Peck, M.R., 2006. Biomarker responses of the estuarine brown shrimp *Crangon crangon* L. to non-toxic stressors: Temperature, salinity and handling stress effects. *Journal of Experimental Marine Biology and Ecology* 335, 114-122.
- Mersch, J., Wagner, P., Pihan, J.C., 1996. Copper in indigenous and transplanted zebra mussels in relation to changing water concentrations and body weight. *Environmental Toxicology and Chemistry* 15, 886-893.
- Metian, M., Warnau, M., Oberhänsli, F., Bustamante, P., 2009. Delineation of Pb contamination pathways in two Pectinidae: The variegated scallop *Chlamys varia* and the king scallop *Pecten maximus*. *Science of the Total Environment* 407, 3503-3509.
- MfE (Ministry of Health), 2010. *Assessment of Public Health Risk from the Remediation of the former Fruitgrowers Chemical Company site, Mapua, Nelson*. pp 1-70.
- Middaugh, D.P., Floyd, G., 1978. Effect of prehatch and posthatch exposure to cadmium on salinity tolerance of larval grass shrimp, *Palaemonetes pugio*. *Estuaries* 1, 123-125.
- Minhorst, A., Liebezeit, G., 2003. Determination of glycogen in tissues of the blue mussel *Mytilus edulis* L. *Senckenbergiana Maritima* 32, 191-193.

- Mitchelmore, C.L., Birmelin, C., Chipman, J.K., Livingstone, D.R., 1998. Evidence for cytochrome P450 catalysis and free radical involvement in the production of DNA strand breaks by benzo[a]pyrene and nitroaromatics in mussel (*Mytilus edulis* L.) digestive gland cells. *Aquatic Toxicology* 41, 193-212.
- Moncheva, S., Gotsis-Skretas, O., Pagou, K., Krastev, A., 2001. Phytoplankton blooms in Black Sea and Mediterranean coastal ecosystems subjected to anthropogenic eutrophication: Similarities and Differences. *Estuarine, Coastal and Shelf Science* 53, 281-295.
- Moolman, L., Van Vuren, J.H.J., Wepener, V., 2007. Comparative studies on the uptake and effects of cadmium and zinc on the cellular energy allocation of two freshwater gastropods. *Ecotoxicology and Environmental Safety* 68, 443-450.
- Morillo, J., Usero, J., Gracia, I., 2002. Partitioning of metals in sediments from the Odiel River (Spain). *Environment International* 28, 263-271.
- Mosesso, P., Angeletti, D., Pepe, G., Pretti, C., Nascetti, G., Bellacima, R., Cimmaruta, R., Jha, A.N., 2012. The use of cyprinodont fish, *Aphanius fasciatus*, as a sentinel organism to detect complex genotoxic mixtures in the coastal lagoon ecosystem. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 742, 31-36.
- Mroczek, E.K., 2005. Contributions of arsenic and chloride from the Kawerau geothermal field to the Tarawera River, New Zealand. *Geothermics* 34, 218-233.
- Mubiana, V.K., Blust, R., 2007. Effects of temperature on scope for growth and accumulation of Cd, Co, Cu and Pb by the marine bivalve *Mytilus edulis*. *Marine Environmental Research* 63, 219-235.
- Muñoz-Barbosa, A., Gutiérrez-Galindo, E.A., Flores-Muñoz, G., 2000. *Mytilus californianus* as an indicator of heavy metals on the northwest coast of Baja California, Mexico. *Marine Environmental Research* 49, 123-144.
- Nadella, S.R., Fitzpatrick, J.L., Franklin, N., Bucking, C., Smith, S., Wood, C.M., 2009. Toxicity of dissolved Cu, Zn, Ni and Cd to developing embryos of the blue mussel (*Mytilus trossulus*) and the protective effect of dissolved organic carbon. *Comparative Biochemistry and Physiology* 149C, 340-348.

- Naimo, T.J., 1995. A review of the effects of heavy metals on freshwater mussels. *Ecotoxicology* 4, 341-362.
- Najle, R., Elissondo, M., Gentile, S., Gentile, M., Vacarezza, G., Solana, H., 2000. Histopathology of the digestive gland of an Antarctic limpet exposed to cadmium. *Science of the Total Environment* 247, 263-268.
- Navarro, A., Faria, M., Barata, C., Pina, B., 2011. Transcriptional response of stress genes to metal exposure in zebra mussel larvae and adults. *Environmental Pollution* 159, 100-107.
- Navarro, J.M., Urrutia, G.X., Carrasco, C., 2006. Scope for growth versus actual growth in the juvenile predatory gastropod *Chorus giganteus*. *Journal of the Marine Biological Association of the United Kingdom* 86, 1423-1428.
- Nelson, D.L., Cox, M.M., 2004. *Lehninger Principles of Biochemistry*, third Ed. Worth Publishers, New York.
- Nesto, N., Romano, S., Moschino, V., Mauri, M., Da Ros, L., 2007. Bioaccumulation and biomarker responses of trace metals and micro-organic pollutants in mussels and fish from the Lagoon of Venice, Italy. *Marine Pollution Bulletin* 55, 469-484.
- New Zealand Aquaculture Farm Facts, 2010. Aquaculture New Zealand website <http://aquaculture.org.nz/>
- New Zealand Government, 2007. *Our Blue Horizon: the Government's commitment to aquaculture*. Ministry of Economic Development, New Zealand.
- Ng, T.Y.-T., Wang, W.-X., 2005a. Modelling Cd bioaccumulation in two populations of the green mussel *Perna viridis*. *Environmental Toxicology and Chemistry* 24, 2299-2305.
- Ng, T.Y.-T., Wang, W.-X., 2005b. Dynamics of metal subcellular distribution and its relationship with metal uptake in marine mussels. *Environmental Toxicology and Chemistry* 24, 2365-2372.
- Ngo, H.T.T., Gerstmann, S., Frank, H., 2011. Subchronic effects of environment-like cadmium levels on the bivalve *Anodonta anatina* (Linnaeus 1758): II. Effects on energy reserves in relation to calcium metabolism. *Toxicological & Environmental Chemistry* 93, 1802-1814.

- Nigro, M., Falleni, A., Del Barga, I., Scarcelli, V., Lucchesi, P., Regoli, F., Frenzilli, G., 2006. Cellular biomarkers for monitoring estuarine environments: Transplanted versus native mussels. *Aquatic Toxicology* 77, 339-347.
- Niyogi, S., Wood, C.M., 2004. Kinetic analyses of waterborne Ca and Cd transport and their interactions in the gills of rainbow trout (*Oncorhynchus mykiss*) and yellow perch (*Perca flavescens*), two species differing greatly in acute waterborne Cd sensitivity. *Journal of Comparative Physiology* 174B, 243-253.
- Nordberg, G.F., Nogawa, K., Nordberg, M., Friberg, L., 2007. Cadmium, in: Nordberg, G.F., Fowler, B.F., Nordberg, M., Friberg, L. (Eds.), *Handbook on the Toxicology of Metals* Elsevier, Amsterdam, pp. 445-486.
- NZTDS (New Zealand Total Diet Study), 2009. Agricultural compound residues, selected contaminant and nutrient elements. Ministry of Agriculture and Forestry, New Zealand.
- Ocaño-Higuera, V.M., Maeda-Martínez, A.N., Lugo-Sánchez, M.E., García-Sánchez, G., Márquez-Ríos, E., Gómez-Jimenez, S., Pacheco-Aguilar, R., 2011. Effect of emerged shipment on the physiological condition of the adductor muscle in adult giant lion's paw scallop *Nodipecten subnodosus* (Sowerby 1835). *Aquaculture Research* 42, 1087-1095.
- O'Connor, T.P., Lauenstein, G.G., 2006. Trends in chemical concentrations in mussels and oysters collected along the US coast: update to 2003. *Marine Environmental Research* 62, 261-285.
- Olabarrieta, I., L'Azou, B., Yuric, S., Cambar, J., Cajaraville, M.P., 2001. In vitro effects of cadmium on two different animal cell models. *Toxicology in Vitro* 15, 511-517.
- Oliva, M., Vicente, J.J., Gravato, C., Guilhermino, L., Galindo-Riaño, M. D., 2012. Oxidative stress biomarkers in Senegal sole, *Solea senegalensis*, to assess the impact of heavy metal pollution in a Huelva estuary (SW Spain): Seasonal and spatial variation. *Ecotoxicology and Environmental Safety* 75, 151-162.

- Pan, J., Plant, J.A., Voulvoulis, N., Oates, C.J., Ihlenfeld, C., 2010. Cadmium levels in Europe: implications for human health. *Environmental Geochemistry and Health* 32, 1-12.
- Pan, K., Wang, W.-X., 2008. The subcellular fate of cadmium and zinc in the scallop *Chlamys nobilis* during waterborne and dietary metal exposure. *Aquatic Toxicology* 90, 253-260.
- Pan, K., Wang, W.-X., 2012. Reconstructing the biokinetic processes of oysters to counteract the metal challenges: Physiological acclimation. *Environmental Science & Technology* 46, 10765–10771.
- Pane, E.F., Smith, C., McGeer, J.C., Wood, C.M., 2003. Mechanisms of acute and chronic waterborne nickel toxicity in the freshwater cladoceran, *Daphnia magna*. *Environmental Science & Technology* 37, 4382-4389.
- Paquin, P.R., Santore, R.C., Wu, K.B., Kavvadas, C.D., Di Toro, D.M., 2000. The biotic ligand model: a model of the acute toxicity of metals to aquatic life. *Environmental Science & Policy* 3, 175-182.
- Pastoriza, L., Bernardez, M., Sampedro, G., Gabo, M.L., Herrera, J.J.R., 2004. Elevated concentrations of oxygen on the stability of live mussel stored refrigerated. *European Food Research and Technology* 218, 415-419.
- Pavlica, M., Klobučar, G.I.V., Mojaš, N., Erben, R., Papeš, D., 2001. Detection of DNA damage in haemocytes of zebra mussel using comet assay. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 490, 209-214.
- Pedersen, F., Bjorestad, E., Anderson, H. V., Kjølholt, J., Poll, C., 1998. Characterization of sediments from Copenhagen harbour by use of biotests. *Water Science and Technology* 37, 233-240.
- Pekey, H., 2006. The distribution and sources of heavy metals in Izmit Bay surface sediments affected by a polluted stream. *Marine Pollution Bulletin* 52, 1197-1208.
- Penttinen, S., Malk, V., Väisänen, A., Penttinen, O.-P., 2011. Using the critical body residue approach to determine the acute toxicity of cadmium at varying levels of water hardness and dissolved organic carbon concentrations. *Ecotoxicology and Environmental Safety* 74, 1151-1155.

- Phillips, D.J.H., Rainbow, P.S., 1993. Biomonitoring of Aquatic Trace Contaminants. Chapman and Hall, London.
- Pilotto, P.J., Goff, J.R., Weatherburn, D.C., 1998. A contemporary contamination record of stormdrain and harbour sediments, Wellington, New Zealand. *Environmental Geology* 36, 159-166.
- Pinot, F., Kreps, S.E., Bachelet, M., Hainaut, P., Bakonyi, M., Polla, B.S., 2000. Cadmium in the environment: sources, mechanisms of biotoxicity, and biomarkers. *Reviews on Environmental Health* 15, 299-323.
- Pipe, R.K., 1992. Generation of reactive oxygen metabolites by the haemocytes of the mussel *Mytilus edulis*. *Developmental & Comparative Immunology* 16, 111-122.
- Pipe, R.K., Coles, J.A., 1995. Environmental contaminants influencing immune function in marine bivalve molluscs. *Fish & Shellfish Immunology* 5, 581-595.
- Pipe, R.K., Coles, J.A., Carissan, F.M.M., Ramanathan, K., 1999. Copper induced immunomodulation in the marine mussel, *Mytilus edulis*. *Aquatic Toxicology* 46, 43-54.
- Pivovarova, N.B., Lagerspetz, K.Y.H., 1996. Effect of cadmium on the ATPase activity in gills of *Anodonta cygnea* at different assay temperatures. *Journal of Thermal Biology* 21, 77-84.
- Playle, R.C., 2004. Using multiple metal–gill binding models and the toxic unit concept to help reconcile multiple-metal toxicity results. *Aquatic Toxicology* 67, 359-370.
- Porto, J.I.R., Araujo, C.S.O., Feldberg, E., 2005. Mutagenic effects of mercury pollution as revealed by micronucleus test on three Amazonian fish species. *Environmental Research* 97, 287-292.
- Powell, A.W.B., 1979. *The New Zealand Mollusca: Marine Land and Freshwater Shells*. Collins, Auckland.
- Power, A., Sheehan, D., 1996. Seasonal variation in the antioxidant defence systems of gill and digestive gland of the blue mussel, *Mytilus edulis*. *Comparative Biochemistry and Physiology* 114C, 99-103.

- Principato, G.B., Aisa, M.C., Talesa, V., Rosi, G., Giovannini, E., 1985. Characterization of the soluble alkaline phosphatase from hepatopancreas of *Squilla mantis* L. Comparative Biochemistry and Physiology 80B, 801-804.
- Pruski, A.M., Dixon, D.R., 2002. Effects of cadmium on nuclear integrity and DNA repair efficiency in the gill cells of *Mytilus edulis* L. Aquatic Toxicology 57, 127-137.
- Pytharopoulou, S., Grintzalis, K., Sazakli, E., Leotsinidis, M., Georgiou, C.D., Kalpaxis, D.L., 2011. Translational responses and oxidative stress of mussels experimentally exposed to Hg, Cu and Cd: one pattern does not fit at all. Aquatic Toxicology 105, 157-165.
- Qin, Q., Qin, S., Wang, L., Lei, W., 2012. Immune responses and ultrastructural changes of hemocytes in freshwater crab *Sinopotamon henanense* exposed to elevated cadmium. Aquatic Toxicology 106/107, 140-146.
- Rainbow, P.S., 2002. Trace metal concentrations in aquatic invertebrates: why and so what? Environmental Pollution 120, 497-507.
- Rainbow, P.S., 2007. Trace metal bioaccumulation: models, metabolic availability and toxicity. Environment International 33, 576-582.
- Rainbow, P.S., Luoma, S.N., 2011. Metal toxicity uptake and bioaccumulation in aquatic invertebrates—Modelling zinc in crustaceans. Aquatic Toxicology 105, 455-465.
- Rainbow, P.S., Smith, B.D., 2010. Trophic transfer of trace metals: subcellular compartmentalization in bivalve prey and comparative assimilation efficiencies of two invertebrate predators. Journal of Experimental Marine Biology and Ecology 390, 143-148.
- Rajalekshmi, P., Mohandas, A., 1993. Effect of heavy metals on tissue glycogen levels in the freshwater mussel, *Lamellidens corrianus* (Lea). Science of the Total Environment 134, 617-630.
- Rana, S.V.S., Singh, R., 1996. Species differences in glutathione-dependent enzymes in the liver and kidney of two fresh water fishes and their implications for cadmium toxicity. Ichthyological Research 43, 223-229.

- Regoli, F., Frenzilli, G., Bocchetti, R., Annarumma, F., Scarcelli, V., Fattorini, D., Nigro, M., 2004. Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, *Mytilus galloprovincialis*, during a field translocation experiment. *Aquatic Toxicology* 68, 167-178.
- Regoli, F., Principato, G., 1995. Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. *Aquatic Toxicology* 31, 143-164.
- Reyes, A.G., Trompetter, W.J., Britten, K., Searle, J., 2003. Mineral deposits in the Rotokawa geothermal pipelines, New Zealand. *Journal of Volcanology and Geothermal Research* 119, 215-239.
- Richardson, B.J., Garnham, J.S., Fabris, J.G., 1994. Trace metal concentrations in mussels (*Mytilus edulis planulatus* L.) transplanted into Southern Australian waters. *Marine Pollution Bulletin* 28, 392-396.
- Riedel, G.F., Valette-Silver, N., 2002. Differences in the bioaccumulation of arsenic by oysters from south-east coastal U.S. and Chesapeake Bay: Environmental versus genetic control. *Chemosphere* 49, 27-37.
- Risso-de Faverney, C., Orsini, N., de Sousa, G., Rahmani, R., 2004. Cadmium-induced apoptosis through the mitochondrial pathway in rainbow trout hepatocytes: involvement of oxidative stress. *Aquatic Toxicology* 69, 247-258.
- Robson, A.A., Kelly, M.S., Latchford, J.W., 2007. Effect of temperature on the spoilage rate of whole, unprocessed crab: *Carcinus maenas*, *Necora puber* and *Cancer pagurus*. *Food Microbiology* 24, 419-424.
- Roesijadi, G., 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology* 22, 81-113.
- Roesijadi, G., Brubacher, L.L., Unger, M.E., Anderson, R.S., 1997. Metallothionein mRNA induction and generation of reactive oxygen species in molluscan hemocytes exposed to cadmium in vitro. *Comparative Biochemistry and Physiology* 118C, 171-176.

- Rogevich, E.C., Hoang, T.C., Rand, G.M., 2008. The effects of water quality and age on the acute toxicity of copper to the Florida apple snail, *Pomacea paludosa*. *Archives of Environmental Contamination and Toxicology* 54, 690-696.
- Romano, E., Bergamin, L., Magno, M.C., Ausili, A., 2013. Sediment characterization of the highly impacted Augusta harbour (Sicily, Italy): Modern benthic foraminifera in relation to grain-size and sediment geochemistry. *Environmental Sciences: Processes and Impacts* 15, 930-946.
- Roméo, M., Frasila, C., Gnassia-Barelli, M., Damiens, G., Micu, D., Mustata, G., 2005. Biomonitoring of trace metals in the Black Sea (Romania) using mussels *Mytilus galloprovincialis*. *Water Research* 39, 596-604.
- Rose, W.L., Nisbet, R.M., Green, P.G., Norris, S., Fan, T., Smith, E.H., Cherr, G.N., Anderson, S. L., 2006. Using an integrated approach to link biomarker responses and physiological stress to growth impairment of cadmium-exposed larval topsmelt. *Aquatic Toxicology* 80, 298-308.
- Rosenberg, R., Costlow, J.D., 1976. Synergistic effects of cadmium and salinity combined with constant and cycling temperatures on the larval development of two estuarine crab species. *Marine Biology* 38, 291-303.
- Rumsby, A., 2009. Trace elements in the sediment of Waikato West Coast Estuaries. Environment Waikato Technical Report 2009/08, Pattle Delamore Partners Ltd, Environment Waikato, Hamilton.
- Russo, C., Rocco, L., Morescalchi, M.A., Stingo, V., 2004. Assessment of environmental stress by the micronucleus test and the comet assay on the genome of teleost populations from two natural environments. *Ecotoxicology and Environmental Safety* 57, 168-174.
- Saavedra Y, Gonzalez A, Blanco J. 2008. Anatomical distribution of heavy metals in the scallop *Pecten maximus*. *Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment* 25, 1339-1344.
- Sadiq, M., 1992. *Toxic metal chemistry in marine environments*, first ed. Marcel Dekker, New York.

- Sáenz, L.A., Seibert, E. L., Zanette, J., Fiedler, H.D., Curtius, A. J., Ferreira, J.F., Almeida, E.A., Marques, M. R. F., Bainy, A. C. D., 2010. Biochemical biomarkers and metals in *Perna perna* mussels from mariculture zones of Santa Catarina, Brazil. *Ecotoxicology and Environmental Safety* 73, 796-804.
- Sakellari, A., Sotirios Karavoltsos, S., Theodorou, D., Dassenakis, M., Scoullou, M., 2013. *Environmental Monitoring and Assessment* 185, 3383-3395.
- Salviati, L., Hernandez-Rosa, E., Walker, W.F., Sacconi, S., DiMauro, S., Schon, E.A., Davidson, M.M., 2002. Copper supplementation restores cytochrome c oxidase activity in cultured cells from patients with SCO2 mutations. *The Biochemical Journal* 363, 321-327.
- Sanchez-Avila, J., Bonet, J., Velasco, G., Lacorte, S., 2009. Determination and occurrence of phthalates, alkylphenols, bisphenol A, PBDEs, PCBs and PAHs in an industrial sewage grid discharging to a Municipal Wastewater Treatment Plant. *Science of the Total Environment* 407, 4157-4167.
- Sánchez-Marín, P., Santos-Echeandía, J., Nieto-Cid, M., Álvarez-Salgado, X.A., Beiras, R., 2010. Effect of dissolved organic matter (DOM) of contrasting origins on Cu and Pb speciation and toxicity to *Paracentrotus lividus* larvae. *Aquatic Toxicology* 96, 90-102.
- Sarı, E., Çağatay, M.N., 2001. Distributions of heavy metals in the surface sediments of the Gulf of Saros, NE Aegean Sea. *Environmental International* 26, 169-173.
- Sauve, S., Brousseau, P., Pellerin, J., Morin, Y., Senecal, L., Goudreau, P., Fournier, M., 2002. Phagocytic activity of marine and freshwater bivalves: in vitro exposure of hemocytes to metals (Ag, Cd, Hg and Zn). *Aquatic Toxicology* 58, 189-200.
- Schmider, E., Ziegler, M., Danay, E., Beyer, L., Bühner, M., 2010. Is it really robust? Reinvestigating the robustness of ANOVA against violations of the normal distribution assumption. *Methodology* 6, 147-151.
- Schoenmakers, T.J.M., Klaren, P.H.M., Flik, G., Lock, R.A.C., Pang, P.K.T., Wendelaar Bonga, S.E., 1992. Actions of cadmium on basolateral plasma membrane proteins involved in calcium uptake by fish intestine. *Journal of Membrane Biology* 127, 161-172.

- Secor, S. 2009. Specific dynamic action: a review of the postprandial metabolic response. *Journal of Comparative Physiology* 179B, 1-56.
- Şen, H., Sunlu, U., 2007. Effects of cadmium (CdCl₂) on development and hatching of eggs in European squid (*Loligo vulgaris* Lamarck, 1798) (Cephalopoda: Loliginidae). *Environmental Monitoring and Assessment* 133, 371-378.
- Serafim, A., Bebianno, M.J., 2007. Kinetic model of cadmium accumulation and elimination and metallothionein response in *Ruditapes decussatus*. *Environmental Toxicology and Chemistry* 26, 960-969.
- Sheir, S.K., Handy, R.D., 2010. Tissue injury and cellular immune responses to cadmium chloride exposure in the common mussel *Mytilus edulis*: Modulation by lipopolysaccharide. *Archives of Environmental Contamination and Toxicology* 59, 602-613.
- Shi, D., Wang, W.-X., 2005. Uptake of aqueous and dietary metals by mussel *Perna viridis* with different Cd exposure histories. *Environmental Science & Technology* 39, 9363-9369.
- Shumilin, E., Jiménez-Illescas, A.R., López-López, S., 2013. Anthropogenic contamination of metals in sediments of the Santa Rosalía Harbor, Baja California Peninsula. *Bulletin of Environmental Contamination and Toxicology* 90, 333-337.
- Sidoumou, Z., Gnassia-Barelli, M., Siau, Y., Morton, V., Roméo, M., 2006. Heavy metal concentrations in molluscs from the Senegal coast. *Environment International* 32, 384-387.
- Sigel, A., Sigel, H., Sigel, Ronald K.O., 2012. *Cadmium: From Toxicity to Essentiality* Springer, Netherlands.
- Simkiss, K., Mason, A.Z., 1983. Metal ions: metabolic and toxic effects, in: Wilbur, K.M. (Ed.), *Biology of Mollusca*. Academic Press, New York, pp 101-164.
- Simpson, S. L., Batley, G.E., Chariton, A.A., Stauber, J.L., King, C.K., Chapman, J.C., Hyne, R.V., Gale, S.A., Roach, A.C., Maher, W.A., 2005. *Handbook of Sediment Quality Assessment* (CSIRO: Bangor, NSW).

- Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low-levels of DNA damage in individual cells. *Experimental Cell Research* 175, 184-191.
- Slovan, K.A., Scott, G.R., Diao, Z., Rouleau, C., Wood, C.M., McDonald, D.G., 2003. Cadmium affects the social behaviour of rainbow trout, *Oncorhynchus mykiss*. *Aquatic Toxicology* 65,171-185.
- Slooff, W., Canton, J.H., Hermens, J., 1983. Comparison of the susceptibility of 22 freshwater species to 15 chemical compounds. I. (Sub) acute toxicity tests. *Aquatic Toxicology* 4, 113-128.
- Smolders, R., Baillieul, M., Blust, R., 2005. Relationship between the energy status of *Daphnia magna* and its sensitivity to environmental stress. *Aquatic Toxicology* 73, 155-170.
- Smolders, R., Bervoets, L., De Coen, W., Blust, R., 2004. Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. *Environmental Pollution* 129, 99-112.
- Sneddon, R., Bailey, M., 2009. Environmental Monitoring of Impacts of Maintenance dredging Spoil Disposal in Lyttelton Harbour: 2009. Lyttelton Port of Christchurch.
- So, M.K., Zhang, X., Giesy, J.P., Fung, C.N., Fong, H.W., Zheng, J., Kramer, M.J., Yoo, H., Lam, P.K.S., 2005. Organochlorines and dioxin-like compounds in green-lipped mussels *Perna viridis* from Hong Kong mariculture zones. *Marine Pollution Bulletin* 51, 677-687.
- Soares-da-Silva, I.M., Ribeiro, J., Valongo, C., Pinto, R., Vilanova, M., Bleher, R., Machado, J., 2002. Cytometric, morphologic and enzymatic characterisation of haemocytes in *Anodonta cygnea*. *Comparative Biochemistry and Physiology* 132A, 541-553.
- Sobral, P., Widdows, J., 1997. Effects of elevated temperatures on the scope for growth and resistance to air exposure of the clam *Ruditapes decussatus* (L.), from Southern Portugal. *Scientia Marina* 61, 163-171.

- Sokolova, I.M., 2004. Cadmium effects on mitochondrial function are enhanced by elevated temperatures in a marine poikilotherm, *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). *Journal of Experimental Biology* 207, 2639-2648.
- Solaun, O., Rodríguez, J.G., Borja, A., González, M., Saiz-Salinas, J.I., 2013. Biomonitoring of metals under the water framework directive: Detecting temporal trends and abrupt changes, in relation to the removal of pollution sources. *Marine Pollution Bulletin* 67, 26-35.
- Solorzano, L., 1969. Determination of ammonia in natural waters by the phenylhypochlorite method. *Limnology & Oceanography* 14, 799-801.
- Soto, M., Cajaraville, M.P., Marigómez, I., 1996. Tissue and cell distribution of copper, zinc and cadmium in the mussel, *Mytilus galloprovincialis*, determined by autometallography. *Tissue and Cell* 28, 557-568.
- Spann, N., Aldridge, D.C., Griffin, J. L., Jones, O.A.H., 2011. Size-dependent effects of low level cadmium and zinc exposure on the metabolome of the Asian clam, *Corbicula fluminea*. *Aquatic Toxicology* 105, 589-599.
- Spehar, R.L. 1982. Cadmium toxicity to fathead minnows in Lake Superior water and reconstituted water. Memorandum, U.S. Environmental Protection Agency.
- Spehar, R.L., Carlson, A.R., 1984. Derivation of site specific water quality criteria for cadmium and the St. Louis River basin, Duluth, Minnesota. *Environmental Toxicology and Chemistry* 3, 651-665.
- Spencer, K.L., 2002. Spatial variability of metals in the inter-tidal sediments of the Medway Estuary, Kent, UK. *Marine Pollution Bulletin* 44, 933-944.
- Sprung, M., Borcharding, J., 1991. Physiological and morphometric changes in *Dreissena polymorpha* (Mollusca, Bivalvia) during a starvation period. *Malacologia* 33, 179-191.
- Stewart, M., Phillips, N.R., Olsen, G., Hickey, C.W., Tipa, G., 2011. Organochlorines and heavy metals in wild caught food as a potential human health risk to the indigenous Maori population of South Canterbury, New Zealand. *Science of the Total Environment* 409, 2029-2039.

- Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radical Biology & Medicine* 18, 321-336.
- Stohs, S.J., Bagchi, D., Hassoun, E., Bagchi, M., 2001. Oxidative mechanisms in the toxicity of chromium and cadmium ions. *Journal of Environmental Pathology, Toxicology and Oncology* 20, 77-88.
- Storelli, M.M., 2011. Cadmium Exposure from Seafood Intake. *Encyclopedia of Environmental Health*, Elsevier, 456-464.
- Sukasem, P., Tabucanon, M.S., 1993. Monitoring heavy metals in the Gulf of Thailand using mussel watch approach. *Science of the Total Environment* 139/140, 297-305.
- Sunila, I., Lindström, R., 1985. The structure of the interfilamentary junction of the mussels (*Mytilus edulis* L.) gill and its uncoupling by copper and cadmium exposures. *Comparative Biochemistry and Physiology* 81C, 267-272.
- Suzuki, T., Yoshinaka, R., Mizuta, S., Funakoshi, S., Wada, K., 1991. Extracellular matrix formation by amebocytes during epithelial regeneration in the pearl oyster *Pinctada fucata*. *Cell and Tissue Research* 266, 75-82.
- Sze, P.W.C., Lee, S.Y., 1995. The potential role of mucus in the depuration of copper from the mussels *Perna viridis* (L) and *Septifer virgatus* (Wiegmann). *Marine Pollution Bulletin* 31, 390-393.
- Szefer, P., Fowler, S.W., Ikuta, K., Paez Osuna, F., Ali, A.A., Kim, B.-S., Fernandes, H.M., Belzunce, M.-J., Guterstam, B., Kunzendorf, H., Wołowicz, M., Hummel, H., Deslous-Paoli, M., 2006. A comparative assessment of heavy metal accumulation in soft parts and byssus of mussels from subarctic, temperate, subtropical and tropical marine environments. *Environmental Pollution* 139, 70-78.
- Szefer, P., Ikuta, K., Frelek, K., Zdrojewska, I., Nabrzyski, M., 1999. Mercury and other trace metals (Ag, Cr, Co, and Ni) in soft tissue and byssus of *Mytilus edulis* from the east coast of Kyushu Island, Japan. *Science of the Total Environment* 229, 227-234.
- Szefer, P., Kim, B.-S., Kim, C.-K., Kim, E.-H., Lee, C.-B., 2004. Distribution and coassociations of trace elements in soft tissue and byssus of *Mytilus galloprovincialis*

- relative to the surrounding seawater and suspended matter of the southern part of the Korean Peninsula. *Environmental Pollution* 129, 209-228.
- Tavakoly Sany, S.B., Salleh, A., Rezayi, M., Saadati, N., Narimany, L., Monazami Tehrani, G., 2013. Distribution and contamination of heavy metal in the coastal sediments of Port Klang, Selangor, Malaysia. *Water, Air, and Soil Pollution* 224, 1476.
- Taylor, M.D., 1997. Accumulation of cadmium derived from fertilisers in New Zealand soils. *Science of the Total Environment* 208, 123-126.
- Taylor, M.D., Percival, H.J., 2001. Cadmium in soil solutions from a transect of soils away from a fertiliser bin. *Environmental Pollution* 113, 35-40.
- Taylor, R., Smith, I., Cochrane, P., Stephenson, B., Gibbs, N., 1997. *The State of New Zealand's Environment*, Wellington: New Zealand Ministry for the Environment.
- TDC (Tasman District Council) Report, 2010. Impact of discharges from stormwater systems on streams and estuary margins in Richmond: 2010 report.
- TDC (Tasman District Council) Report, 2012. Tasman Coast: Waimea Inlet to Kahurangi Point habitat mapping, ecological risk assessment and monitoring recommendations.
- Theodorakis, C.W., 2001. Integration of genotoxic and population genetic end-points in biomonitoring and risk assessment. *Ecotoxicology* 10, 245-256.
- Thophon, S., Kruatrachue, M., Upatham, E.S., Pokethitiyook, P., Sahaphong, S., Jaritkhuan, S., 2003. Histopathological alterations of white seabass, *Lates calcarifer*, in acute and subchronic cadmium exposure. *Environmental Pollution* 121, 307-320.
- Thornton, I., 1992. Sources and pathways of cadmium in the environment. IARC Scientific Publications Series 118, 149-162.
- Tilton, S.C., Foran, C.M., Benson, W.H., 2003. Effects of cadmium on the reproductive axis of Japanese medaka (*Oryzias latipes*). *Comparative Biochemistry and Physiology* 136C, 265-276.
- Tran, D., Boudou, A., Massabuau, J.C., 2001. How water oxygenation levels influences cadmium accumulation pattern in the Asiatic clam *Corbicula fluminea*: a laboratory and field study. *Environmental Toxicology and Chemistry* 20, 2073-2080.

- Tsangaris, C., Cotou, E., Papathanassiou, E., Nicolaidou, A., 2010. Assessment of contaminant impacts in a semi-enclosed estuary (Amvrakikos Gulf, NW Greece): bioenergetics and biochemical biomarkers in mussels. *Environmental Monitoring and Assessment* 161, 259-269.
- Tsangaris, C., Hatzianestis, I., Catsiki, V-A., Kormas, K A., Stroglyoudi, E., Neofitou, C., Andral, B., Galgani, F., 2011. Active biomonitoring in Greek coastal waters: Application of the integrated biomarker response index in relation to contaminant levels in caged mussels. *Science of the Total Environment* 412/413, 359-365.
- Turja, R., Soirinsuo, A., Budzinski, H., Devier, M.H., Lehtonen, K.K., 2013. Biomarker responses and accumulation of hazardous substances in mussels (*Mytilus trossulus*) transplanted along a pollution gradient close to an oil terminal in the Gulf of Finland (Baltic Sea). *Comparative Biochemistry and Physiology* 157C, 80-92.
- Turner, A., 2000. Trace metals contamination in sediments from U.K. estuaries: an empirical evaluation of the role of hydrous iron and manganese oxides. *Estuarine Coastal and Shelf Science* 50, 355-371.
- UNEP (United Nations Environment Programme), 2002. Human actions leading to coastal degradation. www.unep.org/dewa/vitalwater/article180.html
- UNEP (United Nations Environment Programme), 2006. Marine and coastal ecosystems and human wellbeing: A synthesis report based on the findings of the Millennium Ecosystem Assessment. UNEP.
- UNEP (United Nations Environment Programme), 2008. Draft final review of scientific information on cadmium. http://www.chem.unep.ch/Pb_and_Cd/SR/Draft_final_reviews/Cd_Review/Final_UNEP_Cadmium_review_Nov_2008.doc
- UNEP (United Nations Environment Programme), 2010. Final Review of Scientific Information on Cadmium, Nairobi, Kenya.
- US EPA (U.S. Environmental Protection Agency), 2001. Uptake of Ambient Water Quality Criteria for Cadmium US Environmental Protection Agency. Office of Water, Washington, DC. EPA-822-R-OA-001.

- US EPA (Environmental Protection Agency), 2003. Draft Update of Ambient Water Quality Criteria for Copper. US Environmental Protection Agency. Office of Water, Washington, DC. EPA-822-R03-026.
- Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullou, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety* 64, 178-189.
- Valverde, M., Trejo, C., Rojas, E., 2001. Is the capacity of lead acetate and cadmium chloride to induce genotoxic damage due to direct DNA-metal interaction? *Mutagenesis* 16, 265-270.
- van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* 13, 57-149.
- Vareltzis, K., 1996. Mussels as food. *Fish News* 11, 38-47.
- Vasanthi, L.A., Revathi, P., Arulvasu, C., Munuswamy, N., 2012. Biomarkers of metal toxicity and histology of *Perna viridis* from Ennore estuary, Chennai, south east coast of India. *Ecotoxicology and Environmental Safety* 84, 92-98.
- Verbost, P.M., Van Rooij, J., Flik, G., Lock, R.A.C., Wendelaar Bonga, S.E., 1989. The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. *Journal of Experimental Biology* 145, 185-197.
- Vetillard, A., Bailhache, T., 2004. Cadmium: an endocrine disrupter that affects gene expression in the liver and brain of juvenile rainbow trout. *Biology of Reproduction* 72, 119-206.
- Viarengo, A., Burlando, B., Cavaletto, M., Marchi, B., Ponzano, E., Blasco, J.N., 1999. Role of metallothionein against oxidative stress in the mussel *Mytilus galloprovincialis*. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 277, R1612-R1619.
- Viarengo, A., Canesi, L., Pertica, M., Livingstone, D.R., 1991. Seasonal variations in the antioxidant defense systems and lipid peroxidation of the digestive gland of mussels. *Comparative Biochemistry and Physiology* 100C, 187-190.

- Viarengo, A., Moore, M.N., Pertica, M., Mancinelli, G., Zanicchi, G., Pipe, R.K., 1985. Detoxification of copper in the cells of the digestive gland of mussel: The role of lysosomes and thioneins. *Science of the Total Environment* 44,135-145.
- Viarengo, A., Nott, J.A., 1993. Mechanisms of heavy metal cation homeostasis in marine invertebrates. *Comparative Biochemistry and Physiology* 104C, 355-372.
- Viarengo, A., Pertica, M., Mancinelli, G., Palmero, S., G. Zanicchi, G., Oruyesu, M., 1982. Evaluation of general and specific stress indices in mussels collected from populations subjected to different levels of heavy metal pollution. *Marine Environmental Research* 6, 235-243.
- Viarengo, A., Ponzano, E., Dondero, F., Fabbri, R., 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. *Marine Environmental Research* 44, 69-84.
- Vijver, M.G., van Gestel, C.A., Lanno, R.P., Van Straalen, N.M., Peijnenburg, W.J., 2004. Internal metal sequestration and its ecotoxicological relevance: a review. *Environmental Science & Technology* 38, 4705-4712.
- Villaescusa-Celaya, J.A., Gutiérrez-Galindo, E.A., Flores-Muñoz, G., 2000. Heavy metals in the fine fraction of coastal sediments from Baja California (Mexico) and California (USA). *Environmental Pollution* 108, 453-462.
- Vincent-Hubert, F., Arini, A., Gourlay-Francé, C., 2011. Early genotoxic effects in gill cells and haemocytes of *Dreissena polymorpha* exposed to cadmium, B[a]P and a combination of B[a]P and Cd. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 723, 26-35.
- Vlahogianni, T., Dassenakis, M., Scoullou, M.J., Valavanidis, A., 2007. Integrated use of biomarkers (superoxide dismutase, catalase and lipid peroxidation) in mussels *Mytilus galloprovincialis* for assessing heavy metals' pollution in coastal areas from the Saronikos Gulf of Greece. *Marine Pollution Bulletin* 54, 1361-1371.
- Voets, J., Redeker, E.K., Blust, R., Bervoets, L., 2009. Differences in metal sequestration between zebra mussels from clean and polluted field locations. *Aquatic Toxicology* 93, 53-60.

- Wakimoto, T., Kondo, H., Nii, H., Kimura, K., Egami, Y., Oka, Y., Yoshida, M., Kida, E., Ye, Y.P., Akahoshi, S., Asakawa, T., Matsumura, K., Ishida, H., Nukaya, H., Tsuji, K., Kan, T., Abe, I., 2011. Furan fatty acid as an anti-inflammatory component from the green-lipped mussel *Perna canaliculus*. *Proceedings of the National Academy of Sciences of the United States of America* 108, 17533-17537.
- Walker, C.H., 1995. Biochemical biomarkers in ecotoxicology — some recent developments. *Science of the Total Environment* 171, 189-195.
- Wallace, W. G., Lee, B.-G., Luoma, S. N., 2003. Subcellular compartmentalization of Cd and Zn in two bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal (BDM). *Marine Ecology Progress Series* 249, 183-197.
- Wang, W.-X., Fisher, N.S., 1999. Delineating metal accumulation pathways for marine invertebrates. *Science of the Total Environment* 237/238, 459-472.
- Wang, L., Gallagher, E.P., 2013. Role of Nrf2 antioxidant defense in mitigating cadmium-induced oxidative stress in the olfactory system of zebrafish. *Toxicology and Applied Pharmacology* 266, 177-186.
- Wang, W.-X., Rainbow, P.S., 2005. Influence of metal exposure history on trace metal uptake and accumulation by marine invertebrate. *Ecotoxicology and Environmental Safety* 61, 145-159.
- Wang, W.X., Rainbow, P.S., 2006. Subcellular partitioning and the prediction of cadmium toxicity to aquatic organisms. *Environmental Chemistry* 3, 395-399.
- Wang, Y., Hu, M., Chiang, M.W.L., Shin, P.K.S., Cheung, S.G., 2012. Characterization of subpopulations and immune-related parameters of hemocytes in the green-lipped mussel *Perna viridis*. *Fish & Shellfish Immunology* 32, 381-390.
- Ward, T.J., Robinson, W.E., 2005. Evolution of cadmium resistance in *Daphnia magna*. *Environmental Toxicology and Chemistry* 24, 2341-2349.
- Warwick, J., 1984. A Code of Practice for Mussel Processing. New Zealand Fishing Industry Board, Wellington, New Zealand.

- Watling, H.R., 1983. Comparative study on the effects of metals on the settlement of *Crassostrea gigas*. *Bulletin of Environmental Contamination and Toxicology* 31, 344-351.
- WCRC (West Coast Regional Council), 2000 Regional coastal plan for the West Coast. West Coast Regional Council, New Zealand.
- WCRC (West Coast Regional Council), 2006. RMA Section 35 Report on efficiency And effectiveness of the regional coastal plan for the West Coast. West Coast Regional Council, New Zealand.
- Wells, R.M.G., Baldwin, J., 1995. A comparison of metabolic stress during air exposure in two species of New Zealand abalone, *Haliotis iris* and *Haliotis australis*: implications for the handling and shipping of live animals. *Aquaculture* 134, 361-370.
- Wetzel, M.A., Wahrendorf, D.-S., von der Ohe, P.C., 2013. Sediment pollution in the Elbe estuary and its potential toxicity at different trophic levels. *Science of the Total Environment* 449, 199-207.
- Whaley-Martin, K.J., Koch, I., Moriarty, M., Reimer, K.J., 2012. Arsenic speciation in blue mussels (*Mytilus edulis*) along a highly contaminated arsenic gradient. *Environmental Science & Technology* 46, 3110-3118.
- WHO (World Health Organization), 1992. Cadmium. *Environmental Health Criteria*, Vol. 134. World Health Organization, Geneva.
- WHO (World Health Organization), 1993. *Biomarkers and Risk Assessment: Concepts and Principles*. Geneva: World Health Organization.
- Whyte, A.L.H., Hook, G.R., Greening, G.E., Gibbs-Smith, E., Gardner, J.P.A., 2009. Human dietary exposure to heavy metals via the consumption of greenshell mussels (*Perna canaliculus* Gmelin 1791) from the Bay of Islands, northern New Zealand. *Science of the Total Environment* 407, 4348-4355.
- Widdows, J., 1985. Physiological procedures, in: Bayne, B.L., Brown, D.A., Burns, K., Dixon, D.R., Ivanovici, A., Livingstone, D.R., Lowe, D.M., Moore, M.N., Stebbing, A.R.D., Widdows, J. (Eds.), *The Effects of Stress and Pollution on Marine Animals*. Praeger, New York, pp. 161-178.

- Widdows, J., 2009. Marine and Estuarine Invertebrate Toxicity Tests, in: Calow, P. (Ed.), Handbook of Ecotoxicology. Blackwell Publishing Ltd., Oxford, UK, pp 145-166.
- Widdows, J., Donkin, P., 1992. Mussels and environmental contaminants: bioaccumulation and physiological aspects, in: Gosling, E. (Ed.), The mussel *Mytilus*: ecology, physiology, genetics and culture. Developments in Aquaculture and Fisheries Science, Vol 25. Elsevier, Amsterdam, 383-424.
- Widdows, J., Donkin, P., Brinsley, M.D., Evans, S.V., Salkeld, P.N., Franklin, A., Law, R.J., Waldock, M.J., 1995. Scope for growth and contaminant levels in North Sea mussels *Mytilus edulis*. Marine Ecology Progress Series 127, 131-148.
- Widdows, J., Donkin, P., Staff, F.J., Matthiessen, P., Law, R.J., Allen, Y.T., Thain, J.E., Allchin, C.R., Jones, B.R., 2002. Measurement of stress effects (scope for growth) and contaminant levels in mussels (*Mytilus edulis*) collected from the Irish Sea. Marine Environmental Research 53, 327-356.
- Widdows, J., Johnson, D., 1988. Physiological energetics of *Mytilus edulis*: Scope for growth. Marine Ecology Progress Series 46, 113-121.
- Widdows, J., Staff, F., 2006. Biological effects of contaminants: Measurement of scope for growth in mussels. ICES Techniques in Marine Environmental Science 40, 1-30.
- Williams, C.R., Gallagher, E.P., 2013. Effects of cadmium on olfactory mediated behaviors and molecular biomarkers in coho salmon (*Oncorhynchus kisutch*). Aquatic Toxicology 140/141, 295-302.
- Wong, K.C., Wong, M.H., 2000. Morphological and biochemical changes in the gills of tilapia (*Oreochromis mossambicus*) to ambient cadmium exposure. Aquatic Toxicology 48, 517-527.
- Wood, C.M., 2001. Toxic responses of the gill, in: Schlenk, D.B., Benson, W.H. (Eds.), Target Organ Toxicity in Marine and Freshwater Teleosts. Taylor and Francis, New York, pp. 1-89.
- Wood, C.M., Farrell, A.P., Brauner, C.J., 2011. Fish Physiology: Homeostasis and Toxicology of Non-Essential Metals. Burlington: Elsevier Science, Vol 31B.

- Wootton, E.C., Pipe, R.K., 2003. Structural and functional characterisation of the blood cells of the bivalve mollusc, *Scrobicularia plana*. *Fish & Shellfish Immunology* 15, 249-262.
- Wu, J-P., Chen, H-C., 2005. Metallothionein and heavy metal accumulation in white shrimp *Litopenaeus vannamei* exposed to cadmium and zinc. *Comparative Biochemistry and Physiology* 140C, 383-394.
- Xiao, R., Xie, L.P., Lin, J.Y., Li, C.H., Chen, Q.X., Zhou, H.M., Zhang, R.Q., 2002. Purification and enzymatic characterization of alkaline phosphatase from *Pinctada fucata*. *Journal of Molecular Catalysis B: Enzymatic* 17, 65-74.
- Yap, C.K., Edward, F., Berandah, T., Soon, G., 2010. Similarities and differences of metal distributions in the tissues of molluscs by using multivariate analyses. *Environmental Monitoring and Assessment* 165, 39-53.
- Yap, C.K., Ismail, A., Cheng, W.H., Tan, S.G., 2006. Crystalline style and tissue redistribution in *Perna viridis* as indicators of Cu and Pb bioavailabilities and contamination in coastal waters. *Ecotoxicology and Environmental Safety* 63, 413-423.
- Yap, C.K., Ismail, A., Tan, S.G., 2003a. Can the byssus of green-lipped mussel *Perna viridis* (Linnaeus) from the west coast of Peninsular Malaysia be a biomonitoring organ for Cd, Pb and Zn? Field and laboratory studies. *Environment International* 29, 521-528.
- Yap, C.K., Ismail, A., Tan, S.G., Abdul Rahim, I., 2003b. Can the shell of the green-lipped mussel *Perna viridis* from the west coast of Peninsular Malaysia be a potential biomonitoring material for Cd, Pb and Zn? *Estuarine, Coastal and Shelf Science* 57, 623-630.
- Yap, C.K., Ismail, A., Tan, S.G., 2004a. Heavy metal (Cd, Cu, Pb and Zn) concentrations in the green-lipped mussel *Perna viridis* (Linnaeus) collected from some wild and aquacultural sites in the west coast of Peninsular Malaysia. *Food Chemistry* 84, 569-575.
- Yap, C.K., Ismail, A., Tan, S.G., Ismail, A.R., 2004b. Assessment of Different Soft Tissues of the Green-lipped Mussel *Perna viridis* (Linnaeus) as Biomonitoring Agents of Pb: Field and Laboratory Studies. *Water, Air, and Soil Pollution* 153, 253-268.

- Yusufzai, S.I., Singh, H., Shirdhankar, M.M., 2010. An evaluation of different methods for transportation of the freshwater mussel *Lamellidens corrianus* (Lea 1834). *Aquaculture International* 18, 679-692.
- Zalups, R.K., Ahmad, S., 2003. Molecular handling of cadmium in transporting epithelia. *Toxicology and Applied Pharmacology* 186, 163-188.
- Zhu, C., Pan, J.-M., Pancost, R.D., 2012. Anthropogenic pollution and oxygen depletion in the lower Yangtze River as revealed by sedimentary biomarkers. *Organic Geochemistry* 53, 95-98.
- Zulkifli, S.Z., Mohamat-Yusuff, F., Arai, T., Ismail, A., Miyazaki, N., 2010. An assessment of selected trace elements in intertidal surface sediments collected from the Peninsular Malaysia. *Environmental Monitoring and Assessment* 169, 457-472.

10 Appendices

1. F/2 medium (Guillard, 1975; Guillard and Ryther 1963)

STOCK SOLUTIONS (1L in sterilised distilled water)

1. NaNO₃ stock solution

NaNO₃ 75.0 g L⁻¹

2. NaH₂PO₄ stock solution

NaH₂PO₄ 5.0 g L⁻¹

3. Trace Metals stock solution

Na₂EDTA 4.36 g L⁻¹

FeCl₃•6H₂O 3.15 g L⁻¹

Primary Metals Stocks 1ml of each of the five Trace Metals stock solutions

Primary Trace Metals stock solutions (100 ml)

CuSO₄•5H₂O 1.0 g

ZnSO₄•7H₂O 2.2 g

CoCl₂•6H₂O 1.0 g

MnCl₂•4H₂O 1.8 g

NaMoO₄•2H₂O 0.63 g

4. Vitamin Stock solution (1 L)

Biotin 10 ml of 0.1 mg ml⁻¹ solution (1mg in 10ml)

Vitamin B₁₂ 1ml of 1.0 mg ml⁻¹ solution (1mg in 1ml)

Thiamine HCl 0.2g

Final Medium

To 950 mL of 0.22 µM filtered seawater:

NaNO₃ Stock solution 1.0 ml

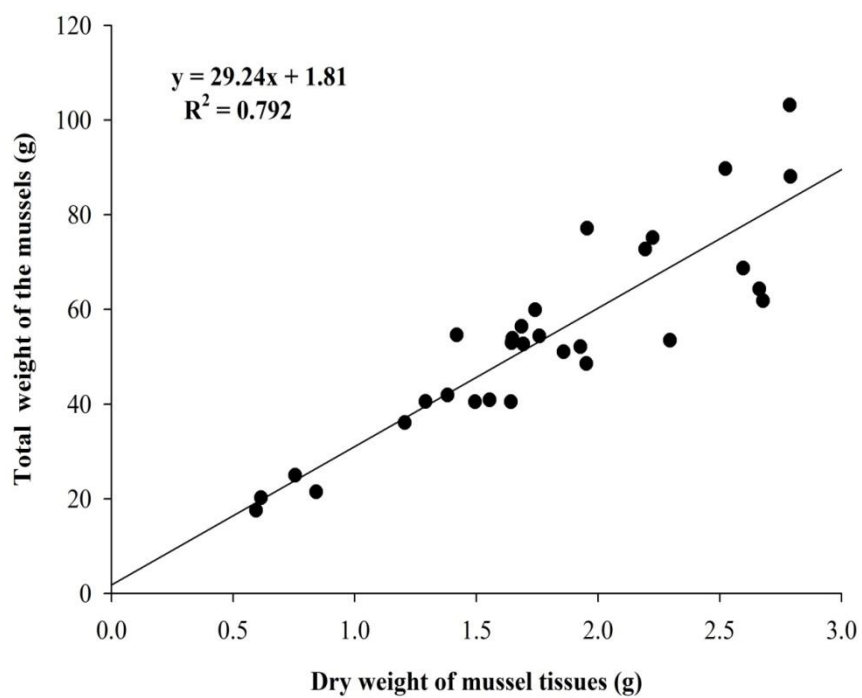
NaH₂PO₄ Stock Solution 1.0 ml

Trace Metals Stock Solution 1.0 ml

Vitamin Stock Solution 0.5 ml

Filter sterilise at 0.22 µM before use and store at 4°C.

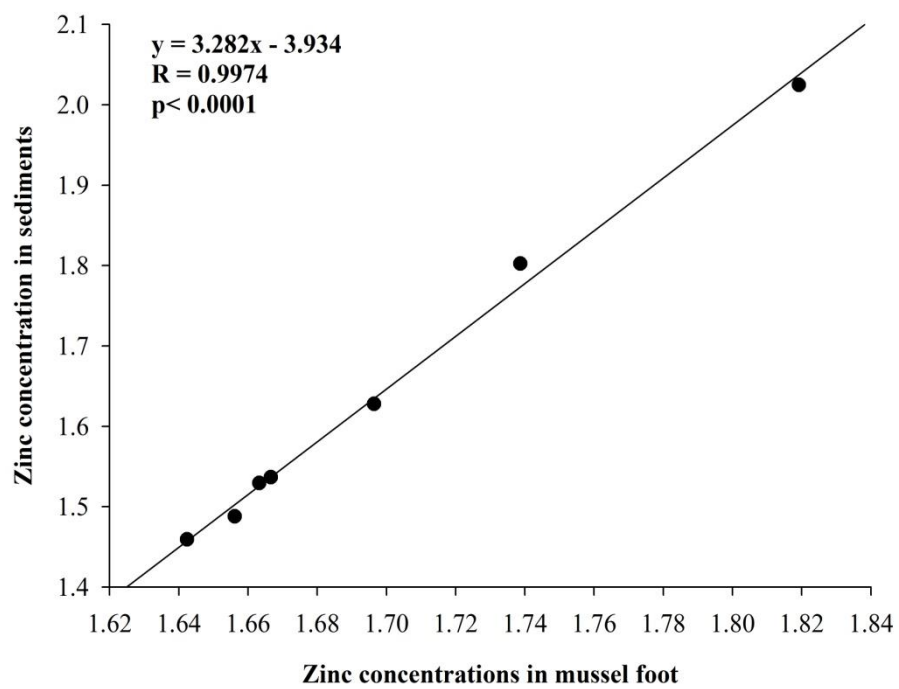
2. Correlation between wet weight and dry weight of mussel tissues



3. Subchronic cadmium exposure (28 d) experiment



4. Correlation between Zn in mussel foot and sediment across all collection sites



5. One-way nested ANOVA results of metal concentrations in the gill and digestive gland of mussels

Gill	Effects	SS	df	MS	F	<i>p</i>	Digestive gland	Effects	SS	df	MS	F	<i>p</i>
Cu	Regions	162	2	81.2	1.971	0.15	Cu	Regions	68.6	2	34.32	3.749	0.031*
	Sites(Regions)	1419	6	236.6	5.745	0.000*		Sites(Regions)	203.1	6	33.85	3.698	0.004*
Zn	Regions	6130	2	3065	2.717	0.076	Zn	Regions	1621	2	810	2.283	0.113
	Sites(Regions)	4.17E+04	6	6948	6.159	0.000*		Sites(Regions)	7804	6	1301	3.665	0.004*
As	Regions	1.6	2	0.81	0.122	0.886	As	Regions	323.3	2	161.7	7.332	0.002*
	Sites(Regions)	238.4	6	39.73	5.959	0.000*		Sites(Regions)	324.1	6	54	2.45	0.038*
Cd	Regions	0.655	2	0.328	1.268	0.29	Cd	Regions	2.652	2	1.326	5.523	0.007*
	Sites(Regions)	3.047	6	0.508	1.966	0.088		Sites(Regions)	2.991	6	0.498	2.076	0.073
Pb	Regions	34.51	2	17.26	15.51	0.000*	Pb	Regions	3.127	2	1.563	4.109	0.022*
	Sites(Regions)	27.59	6	4.6	4.13	0.002*		Sites(Regions)	7.058	6	1.176	3.092	0.012*

6. One-way nested ANOVA results of metal concentrations in the mantle and foot of mussels

Mantle	Effects	SS	df	MS	F	<i>p</i>	Foot	Effects	SS	df	MS	F	<i>p</i>
Cu	Regions	60.1	2	30.07	1.214	0.306	Cu	Regions	26.67	2	13.34	2.233	0.118
	Sites(Regions)	180.5	6	30.09	1.214	0.314		Sites(Regions)	16.73	6	2.79	0.467	0.829
Zn	Regions	785	2	393	2.98	0.059	Zn	Regions	980	2	489.8	2.61	0.084
	Sites(Regions)	8298	6	1383	10.51	0.000*		Sites(Regions)	5224	6	870.6	4.64	0.001*
As	Regions	50.9	2	25.47	6.101	0.004*	As	Regions	13.8	2	6.88	0.627	0.538
	Sites(Regions)	175.6	6	29.27	7.01	0.000*		Sites(Regions)	477.1	6	79.51	7.245	0.000*
Cd	Regions	1.009	2	0.504	21.65	0.000*	Cd	Regions	0.249	2	0.124	4.529	0.016*
	Sites(Regions)	1.181	6	0.197	8.45	0.000*		Sites(Regions)	0.48	6	0.08	2.914	0.016*
Pb	Regions	2.578	2	1.289	8.303	0.001*	Pb	Regions	0.167	2	0.083	1.718	0.19
	Sites(Regions)	3.855	6	0.642	4.139	0.002*		Sites(Regions)	0.544	6	0.091	1.866	0.105

7. One-way nested ANOVA results of metal concentrations in sediments[†]

Sediment	Effects	SS	df	MS	F	<i>p</i>
Cu	Regions	0.604	2	0.302	340.5	0.000*
	Sites(Regions)	0.994	4	0.249	280.3	0.000*
Zn	Regions	696	2	348	0.55	0.589
	Sites(Regions)	1.32E+04	4	3298	5.214	0.009*
As	Regions	11.53	2	5.77	627	0.000*
	Sites(Regions)	6.11E+01	4	15.27	1660	0.000*
Pb	Regions	44.3	2	22.13	116.4	0.000*
	Sites(Regions)	151.1	4	37.77	198.7	0.000*

[†] *Cd concentrations were below detection limit*

8. Publications

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., Waterborne cadmium impacts immunocytotoxic and cytogenotoxic endpoints in green-lipped mussel, *Perna canaliculus*. **Submitted to Aquatic Toxicology.**

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2013. Biochemical biomarker responses of green-lipped mussel, *Perna canaliculus*, to acute and subchronic waterborne cadmium toxicity. **Aquatic Toxicology**, 140/141: 303-313.

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2013. Field-to-laboratory transport protocol impacts subsequent physiological biomarker response in the marine mussel, *Perna canaliculus*. **Comparative Biochemistry and Physiology**, 164A: 84-90.

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2012. Impairment of green-lipped mussel (*Perna canaliculus*) physiology by waterborne cadmium: relationship to tissue bioaccumulation and effect of exposure duration. **Aquatic Toxicology**, 124/125: 114-124.

9. Conference presentations and research outputs

“Mussels monitor metals” UC Research Report 2012, pp 24-25, University of Canterbury 2013.

Chandurvelan, R. – **“Mussel power - fit for monitoring the Kiwi coastline”**, press release – University of Canterbury, November 2012.

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N. **“Mussel power in NZ”: Is *Perna canaliculus* fit for the job?”** oral presentation at the **Annual Biology Conference**, School of Biological Sciences, University of Canterbury, Christchurch, New Zealand, October 17th, 2012. **First place presentation award.**

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N. **“Assessment of metal toxicity effects in New Zealand green-lipped mussels using immunotoxic and cytotoxic biomarkers”** oral presentation at the 8th **Society of Toxicology and Environmental Chemistry Asia-Pacific** conference held at Kumamoto, Japan, 24 - 27th September, 2012. **Student Travel Award Recipient.**

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N. **“Physiological responses of New Zealand green mussels, *Perna canaliculus* to subchronic cadmium exposure”** poster presentation at the 2nd **Society of Toxicology and Environmental Chemistry Australasia** conference held at Brisbane, Australia, 4 - 6th July, 2012.

Chandurvelan, R., Gaw, S., Glover, C.N., Marsden, I.D. **“Changes in physiological biomarker responses of New Zealand green mussels, *Perna canaliculus* in response to acute cadmium exposure”** poster presentation at the **New Zealand Marine Sciences Society** conference held at Stewart Island, South Island, New Zealand, 5 - 8th July, 2011.

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N. “Investigating acute toxicity effects of cadmium in green mussels using biochemical, physiological and chemical biomarkers” oral presentation at the **Annual Biology Conference**, School of Biological Sciences, University of Canterbury, Christchurch, New Zealand 26th October, 2010.