Seasonal Variation in Bivalve Antioxidant Enzymes: Can they be used as indicators of heavy metal contamination?

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Abstract

Seasonal variations in the activity of several enzymes (glutathione reductase (GR), glutathione S-transferase (GST), and glutathione peroxidase (GPx)) involved in the glutathione cellular defence system were measured in whole tissues of the common New Zealand cockle (*Austrovenus stutchburyi*) as biomarkers of oxidative stress. Three sites (Glendowie, Panmure, and Tiraumea) were sampled in Tamaki Estuary, Auckland, New Zealand, that represented a heavy metal contamination gradient. These sites were selected as metal contaminants can affect the activity of antioxidant enzymes by increasing the formation of reactive oxygen species, therefore these enzymes have been proposed as biomarkers of diminished health. Cockles from the three sites were expected to show differences in enzyme activities due to the gradient in metal levels. Sites were sampled monthly over a thirteen month period, from April 2007 – April 2008. Environmental co-variables (sediment silt-clay fraction, organic matter content, chlorophyll *a*, median grain size, cockle condition index, water temperature and salinity) were also measured in order to relate biomarker activities to them. From these measures it was established that a gradient existed in sediment properties, water parameters, and condition index, among the three study sites. Condition index significantly correlated with both GPx ($r = 0.761$) and GST ($r = 0.721$) activity, therefore condition index provides a good measure of overall health. The silt-clay fraction of the sediment also showed a significant negative correlation with both GPx ($r = -0.558$) and GST ($r = -0.498$). GPx was the only enzyme to have significant correlations with metal concentrations including, Pb sediment concentration ($r = -0.561$), Cu sediment concentration ($r =$

-0.539) and Cu tissue concentration (r = -0.530). Therefore this enzyme showed the most promise as a biomarker of contamination. The highest GPx and GST activities were measured in cockles from Glendowie, this site was characterised by lower concentrations of metals. There was seasonality observed in the activity of GPx and GST. The activity of these enzymes greatly increased from January onwards, and remained elevated for the remainder of the sampling period (until April 2008) this increase however, was more pronounced at Glendowie. The increase in enzyme activity in January could have been the result of a number of reasons including 1) changes in the cockles metabolic status due to gonadal development and spawning 2) a combination of environmental co-variables not measured in this study, or 3) a recovery from a stress event that occurred prior to sampling. Both GST and GPx showed promise as biomarkers of contamination; however GR did not as there were little differences in enzyme activities among sites. Further annual sampling at these sites should be carried out to determine if the increased GST and GPx activities in summer were the result of an abnormal event or a typical seasonal variation that occurs annually.

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1 Introduction

Environmental monitoring programmes are fundamental and integral components for assessing the biological conditions of ecosystems in the face of ecological change. It is important that environmental monitoring programmes incorporate all necessary measures across all levels of biological organisation before evaluations and interpretations regarding the health of an ecosystem can be reached (Lam and Gray, 2003). The use of biomarkers is a relatively new approach that relies on changes occurring at lower levels of biological organisation (for example; cell, tissue and organ) before changes occur at higher levels of biological organisation (for example at the community level), (Clements, 2000). Therefore biomarkers can be used in a predictive way and help establish cause-effect links. This thesis focuses on the use of certain antioxidant enzymes as biomarkers of diminished health in a bivalve mollusc species living in an urbanised estuary in Auckland, and the potential use of these enzymes in environmental monitoring programmes.

1.1 Contamination in Urban Estuaries

Estuaries are semi-enclosed bodies of water where salt water from the ocean mixes with fresh water from streams and rivers flowing into it (Mann, 2000); they provide a free connection to the sea and often support high levels of biological diversity including high abundances of fish and shellfish (Neilson and Cronin, 1979). Estuaries are unique marine environments often with fluctuating environmental conditions such as, salinity, pH, and water levels. These chemical gradients are the result of fresh water mixing with sea water (Knox, 1986).

Estuarine sediments often have a gradient of muddy to sandy sediments from the upper to the lower reaches of the estuary (Knox, 1986).

Estuaries are receiving environments, with storm water runoff from surrounding areas providing a significant input of organic and inorganic material into these environments (Mann, 2000). Land run off from industries, agriculture, residential areas, and domestic waste poses a major threat to estuaries. This is because contaminants tend to accumulate in sheltered coastal areas where there is little movement of deposited material; therefore estuaries act as sinks for contaminants (Morrisey *et al.,* 2003). Estuaries are often places that are surrounded by highly urbanised areas with high populations of people (Hale and Guardia, 2001). As worldwide populations increase there is a consequent increase in anthropogenic activities surrounding estuaries. This has resulted in an increase in inputs of contaminants entering estuarine ecosystems. As a result contamination of estuarine environments is increasing globally (Cajaraville *et al*., 2000).

Contamination in estuarine environments is of concern as diverse ranges of biota inhabit these estuaries, of which many are economically and culturally important to humans. Plants and benthic organisms play a major role in estuarine food chains. These organisms are often exposed to contaminants entering estuaries including copper, lead, zinc, organic compounds and polycyclic aromatic hydrocarbons (Morrisey *et al*., 2003). Via consumption, these contaminants can eventually be bio-accumulated in organism tissues and passed up the food chain to fish, birds, mammals and humans (Rand *et al.,* 1995). When organisms at lower levels of the food web in estuarine environments are detrimentally affected, the

entire ecosystem is adversely impacted. For this reason it is important to develop ecological monitoring tools and methods to identify and assess the risks involved from contaminants entering estuarine environments (Cajaraville *et al*., 2000).

Environmental monitoring in its early phase generally consisted of basic testing of physical and chemical water and sediment parameters such as, salinity, temperature, dissolved oxygen concentration, sediment grain size, organics, and heavy metal concentrations (Lam and Gray, 2003). These approaches only give information on levels of contaminants but fail to include the effects these contaminants may have on the biota. Assessment of environmental quality usually involves studies that focus on higher levels of biological organisation, for example species diversity and community structure. These studies can be useful for providing indices, for example; of stress factors. However, studies of community structure require extensive baseline information and can only pick up on ecological disturbances when for example, changes in community structure have become significantly noticeable. By this stage it is likely that some species may have already disappeared (Vasseur & Cossu-Leguille, 2003).

Recently scientists have suggested that there is a need for new environmental monitoring procedures that focus more on the effects of contaminants rather than the levels of contaminants (Lam and Gray, 2003). Researchers in the last decade have started to search for methods and tools that may provide a greater sensitivity of the effects of contamination on biota in estuarine ecosystems and how they interact with pollutants (Etxeberria *et al*., 1995; Black *et al*; 1996; Cosson, 2000). One area of interest has been the activities of enzymes involved in the

detoxification of xenobiotics (Sheehan and Power, 1999). These enzymes can provide an assessment of the physiological state of an organism. Measuring these enzymes provides an indicator of the effects contaminants in the marine environment are having on biota. These indicators are termed biomarkers (Chevre *et al*., 2003).

1.2 Biomarkers

The ecological health of organisms can be monitored at a number of different biological levels including sub-cellular, cellular, organ, individual, population, community and ecosystem responses (Clements, 2000). Whereas most environmental monitoring and toxicology studies focus on higher levels of biological organisation including population and ecosystem effects (Rand *et al*., 1995), biomarkers tend to focus on sub-organismal levels of biological organisation for example biochemical, cellular and molecular changes (Lam and Gray, 2003). Biomarkers can be defined as "the use of physiological, biochemical, and histological changes as "indicators" of exposure and/or effects of xenobiotics at the suborganismal or organismal level" (Rand *et al*., 1995). The use of biomarkers has been proposed as a sensitive early warning detection tool for monitoring the health of organisms (McCarthy and Shugart, 1990; Cajaraville *et al*., 2000). An important assumed feature of biomarkers is that they allow changes to be anticipated that may be occurring at higher levels of biological organisation, for example population or ecosystem responses. The justification for using biomarkers is that they allow for early warning of diminished health before irreversible damaging effects occur and can therefore be used in a predictive way (Cajaraville *et al*., 2000). Accordingly biomarkers can be defined

as “short term indicators of long term biological effects” as they rely on changes occurring at lower levels of biological organisation before higher levels of biological organisation are affected (Cajaraville *et al*., 2000).

Organisms have employed a number of strategies at the cellular level to protect themselves and minimise the harmful effects of environmental contaminants such as metallic and organic compounds. Sequestration, antioxidant defence systems, binding proteins and detoxification processes are the major defence mechanisms used by organisms (Manduzio *et al*., 2004). One of the causes of cellular damage is believed to be the result of a build up of reactive oxygen species (ROS) which all aerobic organisms are exposed to. ROS are ions or small molecules that include free radicals, reactive anions and peroxides. These reactive species can cause tissue damage by a variety of mechanisms including lipid peroxidation, DNA damage, protein damage, and oxidation of important enzymes (Chappie, 1997). Antioxidant systems, present in all aerobic cells, function to protect cells against the damaging effects of oxyradical generation by helping the cells and tissues to maintain low levels of ROS (Manduzio *et al*., 2004). There is a continuous cycle in all aerobic cells that involves ROS production, oxidation and antioxidant depletion. An imbalance in this cycle results in oxidative stress (Torres *et al*., 2002).

Oxidative stress is one type of toxicity caused by exposure to contaminants. To minimise the effects of ROS, living organisms employ a variety of antioxidant and detoxification systems. Common biological antioxidant enzyme systems include superoxide dismutase, catalase, glutathione peroxidase, and glutathione

reductase. Variations in antioxidant enzyme activities have been proposed as biomarkers of oxidative stress (Tsangaris *et al*., 2007; Livingstone, 2001). When an animal is living in a stressed environment the activity of antioxidant enzymes may be either inhibited or increased. An animal trying to overcome a stressed state and prevent toxicity may show an increase in enzyme activity, whereas low activities can either signify low levels of environmental stress or the deficiency may be due to high environmental stress, overwhelming the cell's defence system (Cossu *et al*., 2000). While it is straightforward to identify several defence systems, the correct interpretation of different responses is a challenge. Consequently the activities of antioxidant enzymes can prove to be useful biomarkers of both exposure to contaminants and toxicity (Stegeman *et al.,* 1992; Walker, 1995; Cossu *et al*., 1997; Doyotte *et al*., 1997).

There are a range of antioxidant defences present in organisms. These include low molecular weight compounds, for example ascorbic acid, Vitamin E, and the associated enzymes that are involved in the maintenance of the antioxidant defence (Manduzio *et al*., 2004; Winston, 1991). Among these enzymes, the superoxide dismutase's (Figure 1.2) are one of the most important enzymes in antioxidant defence in all cells exposed to oxygen. Superoxide dismutase is a metalloenzyme that has a metal ion in their active site which is reduced by superoxide, then oxidised releasing hydrogen peroxide (Mannervik & Josephy 2005). As a result superoxide dismutase catalyses the dismutation of superoxide into oxygen and hydrogen peroxide. For this enzyme to be effective, it requires the assistance of other enzymes involved in the antioxidant defence system. These include catalase, glutathione peroxidases, and glutathione reductase.

Glutathione peroxidase (GPx) (EC 1.11.1.9) is a tetrameric protein that comes from a family of enzymes which function to protect the organism from oxidative stress by elimination of ROS. It does this by catalyzing the reduction of hydrogen peroxide to water by oxidising reduced glutathione (GSH) into its oxidised form (GSSG) (Mannervik 1985; Rice-Evans *et al*., 1991).

Glutathione reductase (GR) (EC 1.8.1.7) is an important intracellular antioxidant enzyme that is a member of the pyridine-nucleotide disulfide oxidoreductase family of flavoenzymes (Muller, 2002). GR catalyzes the reduction of GSSG to GSH in the presence of NADPH (Mannervik & Josephy 2005).

Glutathione S- transferase (GST) (E.C 2.5.1.18) is an enzyme found in the cytosolic fraction that is associated with the detoxification metabolism of xenobiotics and is the most important of the phase II enzymes. Phase II enzymes are responsible for inactivating compounds prior to their excretion. GST catalyzes the conjugation of glutathione with electrophiles via the sulfhydryl group (Mannervik & Josephy 2005). GST facilitates the conjugation of glutathione to foreign compounds and aids in the removal of ROS from the organism. Because of this GSTs are expected to respond to changes in levels of contaminants in the marine environment studied (Manduzio *et al*., 2004).

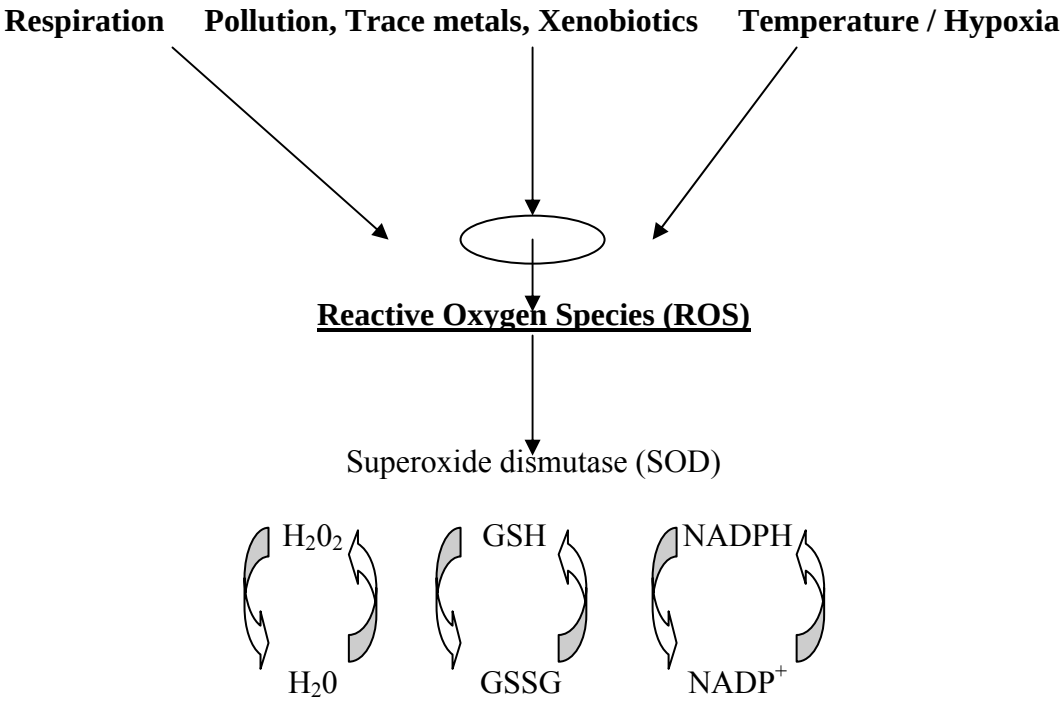

Figure 1.2 Cellular generation of ROS and the antioxidant defence system that acts to remove it.

Bivalve molluscs are useful organisms to assess the effects of contaminants in aquatic environments and are used globally for monitoring coastal ecosystems (Goldberg, 1975). Bivalves are easy to collect, have a wide geographical distribution (Verlecar *et al*., 2007) and are generally abundant in estuarine environments which can often have high contamination levels in urban settings (Manduzio *et al*., 2004). They are sessile and sedentary, so are likely to reflect changes in the environmental conditions they occupy (Manduzio *et al*., 2004; Phillips and Rainbow, 1989). A range of antioxidant defences are present in bivalve molluscs, making them appropriate organisms to use when measuring antioxidant enzymes as biomarkers of diminished health (Verlecar *et al*., 2007).

A number of studies have looked at antioxidant defence system enzymes in bivalve species as biomarkers to predict diminished health (Table 1). Most studies have used a suite of enzymes (including GR, GST, MT, GPx, CAT, GSSG, GSH and SOD) to measure changes in enzyme activity as a consequence of environmental pollution. These studies have produced varied results but have shown that these enzymes can respond differently to varying levels of contaminants. Some studies showed that as levels of contamination increased antioxidant enzyme activities decreased (Box *et al*., 2007; Cossu *et al*., 1997; Cossu *et al*., 2000; Geret *et al*., 2002; Osman., *et al* 2007; Regoli, 1998; Vasseur and Leguille, 200), whereas other studies showed the opposite trend with enzyme activities increased in organisms collected from more polluted sites (Cheung *et al*., 2001; Giguere *et al*., 2002; Manduzio *et al*., 2004; Morales-Casselles *et al*., 2008; Torres e al., 2002; Tsangaris *et al*., 2006). These studies have shown that changes in antioxidant defence system enzymes can be used as a measure of stress in marine organisms, which may result for example, from heavy metal contamination. Since exposure to contamination is just one of the many factors affecting biomarker responses, other environmental variables need to be carefully considered when interpreting biomarker results.

Morales-Caselles *et al*., (2008) were able to demonstrate that laboratory based exposure tests are not representative of natural ecosystems with multiple stressors. They showed this by exposing laboratory animals to the same contaminants that animals in the field were exposed to, and gained different results when compared to field results. This study showed how field studies allowed for the inclusion of alternative sources of stress, and how field studies incorporate all parameters

related to natural settings (Morales-Caselles *et al*., 2008). Morales-Caselles *et al*., (2008) highlighted the importance of field studies when identifying cause-effect relationships between organisms, biomarker responses, and contaminants.

Table 1. Studies carried out measuring antioxidant enzymes and detoxifying proteins in bivalves (GST = glutathione S- transferase, GR = glutathione reductase, CAT = catalase, GSH = reduced glutathione, GSSG = oxidised glutathione, SOD= superoxide dismutase, MDA = malondialdehyde, MT = metallothionein, PChE = propionylcholinesterase).

Author	Year	Species Studied	Study Type	Enzymes studied	Sampling Frequency	Results
Banni *et al*.,	2008	Clams- *Ruditapes decussatus*	Field	CAT, lipid peroxidation using MDA neutral lipid and lipofuscin accumulation.	Clams were collected from 7 sites from January – December.	Neutral lipids and lipofuscin more sensitive to stress.
Box *et al*.,	2007	Mussel – *Mytilus galloprovincialis*	Field	CAT, GPx, GR, SOD, and MDA	Mussels were transplanted at 8 stations left for 3 months then collected and measured.	CAT- higher at polluted sites GPx- no difference between stations. GR- lower activity at non-polluted sites. SOD- polluted areas had higher activity.
Bocchetti *et al*.,	2008	*Tapes philippinarum* and *Mytilus galloprovincialis*	Field	MT, CAT, GST, GPx, GR, peroxisomal enzymes, acetylcholinesterase	Samples collected on a seasonal basis	CAT, GST and GPx showed lowest activities in the summer.
Bodin *et al*.,	2004	*Mytilus galloprovincialis*	Field	AChE, DNA adducts, benzo [a]pyrene hydroxylase, heat-shock proteins, MT, P-glycoprotein-mediated multixenobiotic resistance.	Mussels were transplanted into the Mediterranean sea and samples were measured monthly from April 1999- March 2001	Relationship found between metal concentrations and MT and HSP70.
Cossu *et al*.,	2000	*Unio tumidus*	Field	GSH, GSSG, GPx, GR, MDA,	Animals transplanted from a control site to 4 contaminated sites and measured after 15 days of exposure.	GR, GPx and GSH were depleted at highly polluted sites.

Author	Year	Species Studied	Study Type	Enzymes studied	Sampling Frequency	Results
Cossu *et al*.,	1997	*Unio tumidus*	Field	GPX, GR, CAT, SOD, GSH, GSSG, lipid peroxidation	Control mussels transplanted for 15 and 30 days to polluted sites	All antioxidant enzyme activities significantly decreased in polluted sites.
Cheung *et al*.,	2001	*Perna viridis*	Field	GST, SOD, CAT, GPx, GR, NADPH-DT-diaphorase, GSH and lipid peroxidation	Mussels transplanted from a clean site to various polluted sites in Hong Kong, and measured after 30 day field exposure	GR, GST, and GPX, all increased with increasing toxicant.
De Luca-Abbott *et al*.,	2005	*Perna viridis* and *Ruditapes philippinarum*	Field	GST, CAT, GPx, GSH,	Animals sourced from a clean site, depurated for 8 days, and transplanted to polluted sites in Hong Kong and measured after 14 and 28 days of exposure.	GPx did not vary, GST was higher in clams than mussels.
Geret *et al*.,	2002	*Ruditapes decussatus*	Field	SOD, CAT and GPx	Collected bivalves from 4 sites of the Ria Formosa	GPx inhibited in gills of clams at contaminated sites. CAT activity was higher in the gills of clams from contaminated sites.
Giguere *et al*.,	2003	*Pyganodon grandis*	Field	MT, MDA, GPx, GR	Collected bivalves from 10 lakes.	GPx & GR levels increased with exposure to Cu.
Leinio and Lehtonen	2005	*Mytilus edulis* and *Macoma balthica*	Field	MT, acetylcholinesterase, CAT, GST	Bivalves were collected monthly between April and November 2001 from 2 sites.	Significant seasonal variation in all biomarkers. In *M. edulis* GST activity was related to Secchi depth.
Manduzio *et al*.,	2004	*Mytilus edulis*	Field	GST, GPx, GR and 3 isoforms of Cu/Zn-superoxide dismutase.	Samples were collected in January, June, November, and December at 4 sites	GST activity was higher in mussels living near the outlet of a cooling system of the power plant. GPx showed no difference in mussels from 4 sites. GR showed lower levels in the winter.

Author	Year	Species Studied	Study Type	Enzymes studied	Sampling Frequency	Results
Niyogi *et al*.,	2001	*Saccostrea cucullata*	Field	GPx, CAT, SOD,	Collected animals from a polluted site and a control site, every fortnight in the pre-monsoon, monsoon, and post monsoon period.	Enzyme activities highest in pre-monsoon period, with a gradual decrease from monsoon-post monsoon. Salinity and pH increased greatly in monsoon period.
Osman *et al*.,	2007	*Dreissena polymorpha*	Lab	GST, CAT, GSH, GSSG	Animals exposed to contaminated sediments for 24 hours and 7 days	CAT reduced in contaminated sediments.
Regoli	1998	*Mytilus galloprovincialis*	Field	GST, GR, glyoxalase I. glyoxalase II, GPx, CAT, SOD.	Measured bivalves from 2 sites, a polluted and non-polluted. Animals measured for 10 months.	Higher enzymatic activity in summer. Mussels from polluted site showed lower GST activity.
Torres *et al*.,	2002	*Mytella guyanensis*	Field	SOD, CAT, GR, GST, GPx	Collected mussels from 3 sites (one non-polluted) during the high summer period.	Enzymes (except SOD) showed increased activities at polluted sites.
Tsangaris *et al*.,	2007	*Mytilus galloprovincialis*	Field	GPx, acetylcholinesterase	Native mussels and farmed mussels transplanted to an area affected by a ferro-nickel smelting plant, and to a reference site.	GPx activity was higher in transplanted mussels compared to reference sites.
Verlecar *et al*	2007	*Perna viridis*	Field	Lipid peroxidation, hydrogen peroxide, SOD, CAT, GPx, GST, GR, GSH, ascorbic acid.	Measured enzymes in Feb, May, Aug, and Nov.	Showed seasonal variation in antioxidant enzymes. Antioxidant enzymes vary in tissues with respect to season.
Vidal *et al*	2002	Two populations of *Corbicula fluminea*	Field	CAT, PChE, NADPH-cytochrome c reductase, NADH-cytochrome c reductase, GST	Clams were collected every two or three months for two years (from July 1998 – July 2000).	Seasonal variations in CAT, PChE, NADH- cytochrome c reductase and lipids.

Seasonality is a typical characteristic of estuarine environments. Many abiotic and biotic factors vary with season and influence metabolic activities of marine invertebrates. Changes in metabolic activities of an organism can put stress on the organism; which in turn may lead to an increase in the generation of ROS. Levels of oxidative stress may also fluctuate throughout the year due to seasonal changes in reproductive status, growth, water temperature and nutrient availability (Sheehan and Power, 1999). For this reason biomarker levels in natural populations of organisms can be expected to fluctuate due to physiological adaptations caused by seasonality (Verlecar *et al.*, 2007). Preferably, the ideal biomarker would not vary seasonally in response to abiotic and biotic factors, and would only respond to changes in exposure to contaminants. Therefore it is important to define the natural seasonal changes in biomarkers to validate their use as a biomarker of exposure to contaminants (Sheehan and Power, 1999).

Of the many studies cited in Table 1, six (Vidal *et al*., 2002; Manduzio *et al*., 2004; Leinio and Lehtonen, 2005; Regoli., 1998; Bocchetti *et al*., 2008; Verlecar *et al*., 2007) accounted for seasonal variability in antioxidant enzymes and metallothionein responses. These studies confirmed that seasonal fluctuations do influence biomarker responses and emphasised the importance of taking seasonality into account when measuring biomarker activities. To validate the use of antioxidant enzymes as biomarkers of heavy metal contamination and as an ecological monitoring tool, it is important to define the natural range of biomarker activities by taking into account their seasonal and natural variations (Cajaraville *et al.,* 2000; Viarengo *et al*., 1991; Blasco *et al*., 1993; Sole & Albaiges, 1995)

before implementing the use of biomarkers in field studies and in environmental monitoring programmes (Cajaraville *et al.,* 2000).

Manduzio *et al*., (2004), looked at the seasonal variation in the activity levels of GST, GPx, GR, and three isoforms of Cu/Zn-superoxide dismutase in gills and digestive glands in the blue mussel (*Mytilus edulis*). In this study animals were collected from four sites with different levels of pollution from the Le Havre Harbour on the north-west coast of France. Enzyme responses were measured four times throughout the year to capture response variability associated with seasonality that might be occurring. They were able to show that variability in enzyme activities was dependent on tissue type, with gills having less inter-individual variability for this species. GST consistently showed higher activities at the site located near the outlet of the cooling system of a power plant, compared to the reference site. GPx and GR showed strong seasonality with lower activities in the winter and higher activities in the summer. The investigators were able to conclude from this study that there was a correlation between antioxidant defences and seasonality and this can be related to food consumption and reproductive status (Manduzio *et al*., 2004).

To test the applicability of antioxidant enzymes as biomarkers of diminished health, an appropriately designed study of animals living in their natural environment needs to be conducted to assess changes occurring in field-typical conditions (Depledge and Fossi, 1994; De Coen *et al*., 2006; Martin-Diaz *et al*., 2007). Relationships can then be established between environmental contaminants and biomarker responses taking seasonality into account to quantify

the scale of natural fluctuations. To be able to use antioxidant enzyme biomarkers as an ecological monitoring tool in New Zealand, a study of a New Zealand species living in an urbanised (or presumably impacted) marine environment was deemed necessary. To my knowledge no study of these biomarkers has been carried out in New Zealand. For certain, no study has ever been carried out monitoring antioxidant enzymes in *Austrovenus stutchburyi*, the common New Zealand cockle.

1.3 Aims and Objectives

The purpose of this study was to measure the response of three antioxidant enzymes (glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione S-transferase (GST)) in the suspension feeding bivalve *A.stutchburyi* along a heavy metal contamination gradient in an urbanised estuary. The main objectives of this study were to 1) monitor natural variations in biomarker activities over a one year period along a heavy metal gradient, 2) determine correlations between biomarker responses, environmental co-variables and condition index, and 3) quantify the seasonal variability of these antioxidant enzymes to evaluate their application as an ecological monitoring tool.

It is hypothesized that cockle enzyme responses will vary with season and location. This variation is expected to be explained by a number of environmental variables, including; sediment metal contamination, cockle tissue heavy metal concentrations, several other sediment properties (organic matter content, grain size, chlorophyll *a,* silt-clay fraction) and the condition of the cockles.

This study hopes to quantify metal-specific biomarker responses in cockles and thereby improve the ability to detect sub-lethal adverse effects of metal contamination on estuarine organisms. This study also hopes to identify a sensitive and robust indicator of metal contamination and diminished cockle health that can be used to anticipate changes at higher levels of biological organisation e.g. population level. High frequency monitoring of antioxidant enzyme activity in estuarine bivalves has only been conducted rarely. The high frequency sampling with simultaneous measurement of biomarkers and a large variety of environmental data collected in this present study makes this study valuable and unique. I was interested in collecting this large data set of environmental co-variables to enable a cause-effect link between antioxidant enzymes, contaminant levels and environmental data.

2 Materials and Methods

2.1 Study Site

As a setting for this study I chose the Tamaki Estuary (Figure 2.1), a semi-enclosed body of water, 17 km in length, located on the eastern side of Auckland city. The 11,500 ha catchment surrounding the estuary consists of a diverse range of urbanised and industrialised premises including more than 600 industrial establishments, suburban housing, marinas, power plants, arterial traffic routes, and some farming (Abrahim and Parker 2002; Abrahim *et al.*, 2007).

Samples were collected from three low-gradient intertidal sites in the Tamaki Estuary. Monitoring undertaken by the Auckland Regional Council (ARC) had indicated a gradient in the concentration of heavy metals in the surface sediments from the upper to the lower reaches of the estuary existed, and that heavy metal concentrations have been increasing (ARC, 1990, 1992, 1995, 1999). Based on these studies, three sites were selected along the estuary to represent a contamination gradient. Among these; Tiraumea, at the upper reaches of the estuary was characterised by high sediment metal concentrations of more than 100 ppm zinc. Panmure wharf the middle sampling site had intermediate sediment metal concentrations of 50-100 ppm zinc, and Glendowie, at the lower reaches of the estuary, had the lowest sediment metal concentrations of < 50 ppm zinc. The specific collection locations chosen were situated in the mid-intertidal area of the sand flat and had similar emersion periods. I chose to monitor cockles from three sites within the same estuary to minimise extraneous variability due to weather patterns, and food webs for example.

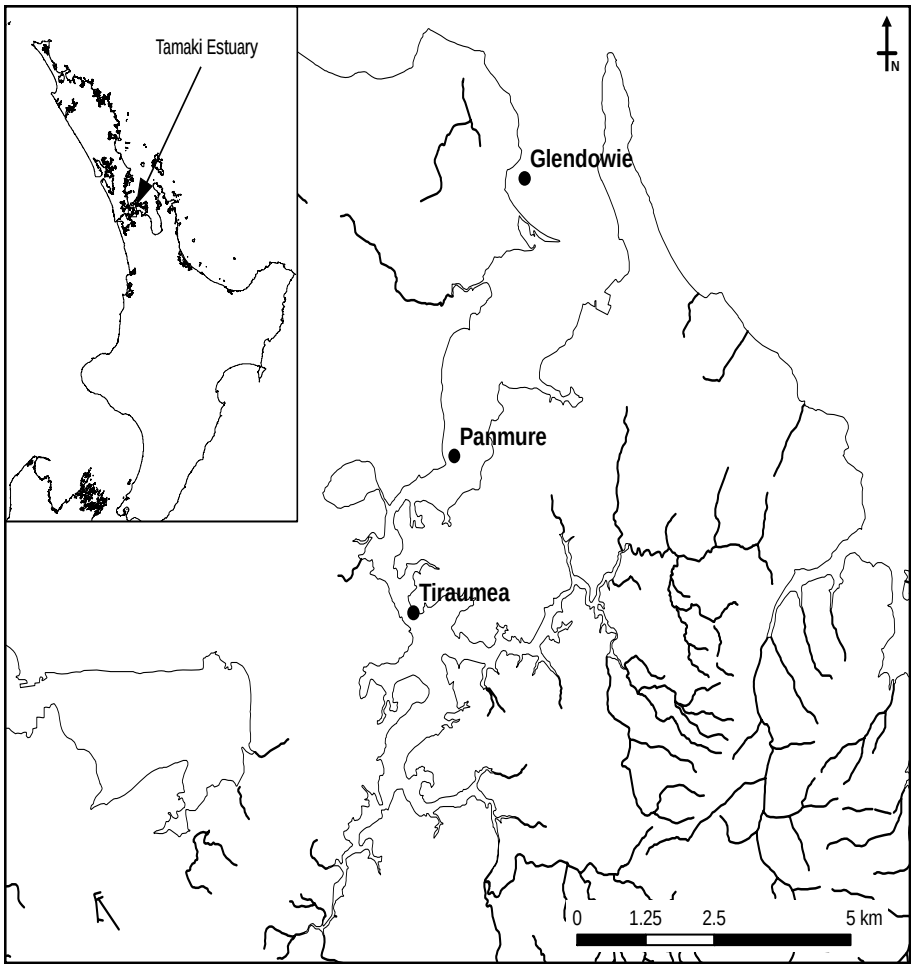

Figure 2.1 Tamaki estuary and location of three sampling sites.

2.2 Study Species

Antioxidant enzyme levels were measured in *A. stutchburyi* (the common New Zealand cockle) from three sites along the Tamaki estuary. *A. stutchburyi* is a suspension feeding bivalve mollusc that lives between the mid and low water of sheltered coastal areas. Cockles are potentially exposed to both contaminants from the water and the sediments as they filter-feed microscopic algae at the sediment-water interface (De Luca-Abbott, 2001; Boening, 1999).

2.3 Sampling Protocol

At each site an 8 x 10 m area was marked out and divided into 1 m^2 plots. Sampling was conducted monthly for 13 months (April 2007 – April 2008) from four randomly selected plots (replicates) per site. Replicates were sampled only once, so on completion of field sampling the majority of the replicates had been sampled from.

From each plot approximately 30 cockles (11-30 mm in shell length) were collected from a 0.25 m^2 area and immediately frozen using dry ice (ca. -80 ºC). Four surface (0-1 cm) sediment samples were also collected from each plot using a 50 ml syringe core and pooled for grain size, organic content, chlorophyll *a* and heavy metal analysis. On return to the laboratory cockles for antioxidant enzyme analysis (approximately 10 from each plot) were stored at -80 ºC to minimise enzyme degradation. All other samples were stored at -20 ºC.

2.4 Laboratory Analysis

2.4.1 Sediment analysis

It was hypothesized that the magnitude of cockle antioxidant enzyme activity would be related to overall cockle health, environmental variables, and ultimately to differences in metal contaminant exposure. Therefore biomarker responses may be the result of variables other than metals and it is important to measure these environmental co-variables to determine their influence on biomarker responses. Consequently chlorophyll *a* content, grain size, the silt-clay fraction, and percentage of organic matter were measured in the sediments to quantify co-

variation with biomarker responses. Chlorophyll *a* (μg / g dry weight) content of the sediment samples was determined using the 90 % acetone extraction method of Arar and Collins (1997) on a Turner Designs 10-AU Flurometer. To quantify the grain size of the surface sediments, samples were pre-treated with 10% hydrogen peroxide to remove organic matter. Sediment grain size was then measured with a Malvern Mastersizer-S, and the median grain size determined (Singer *et al.*, 1988). From this analysis the silt-clay fraction of the sediment was calculated as the fraction < 63 μm and expressed as a percentage. Furthermore, sediment organic matter was determined as loss on ignition by quantifying the percentage of weight loss on sediments dried for 24 h at 60 ºC followed by combustion at 550 ºC for 4 h. Organic matter content was expressed as a percentage of bulk sediment dry weight.

2.4.2 Sample Preparation and Biomarker analysis

Antioxidant enzyme activity was determined in whole cockles to minimise thawing and dissection artefacts. Cockles were removed from the -80 freezer and stored on dry ice at all times during preparation. For each cockle the frozen wet weight (including shell) was recorded, and the shell length, width and height (in mm) measured. The tissue from 6-10 cockles (from the same replicate plot) was placed into a mortar that contained liquid nitrogen to prevent thawing and ground into a fine powder. The ground tissue was transferred into a glass container, labelled, and stored at -80 ºC until analysis of antioxidant enzymes.

For determining total dissolved protein content and activities of the three antioxidant enzymes a liquid tissue homogenate was prepared, following a

modified protocol based on Mannervik *et al.,* (1985). The three enzyme activities and protein content were measured on the same day of preparation of the liquid homogenate. To make the liquid tissue homogenates for biomarker analysis approximately 0.5 grams of ground cockle tissue was placed into a 16 ml polypropylene centrifuge tube and the weight was recorded. 2 ml of homogenisation buffer (100 mM K_2HPO_4-KH_2PO4, pH 7.6, 1 mM EDTA, 100 mM KCL, 20% glycerol) was added and the contents shaken or vortexed vigorously for at least 5 s. Samples were then centrifuged on a Sorvall RC5C centrifuge at 9500 rpm for 30 minutes at 2-4 ºC. The homogenate was then decanted into clean pre-labelled 2 ml microtubes and this extract was subsequently used for enzyme assays and for determining the amount of dissolved total protein.

To obtain the protein concentration a 1/10 dilution was made using 100 µl of the homogenate and 900 µl of homogenisation buffer. Protein concentration of the samples was determined on microplates using the Bradford (1976) method using bovine serum albumin (BSA) as a standard. Each replicate was run in quadruplicate and values averaged.

Glutathione reductase (GR), glutathione S-transferase (GST), and glutathione peroxidase (GPx) were the three antioxidant enzymes measured in cockle tissues collected. I chose to measure these enzymes as they are antioxidant enzymes involved in the defence against reactive oxygen species (ROS) and organic xenobiotics. Previous studies (Table 1) had also shown that these enzymes respond to changes in contamination exposure in bivalves. Oxidative stress can

be the result of a number stressors including metal exposure. It has been shown that environmental contaminants may intensify oxidative stress in aquatic organisms (Winston, 1991). The three sites chosen represented a four-fold contamination gradient, therefore it was expected that these enzymes would respond differently at the three sites due to the varying degree of contaminant exposure in the cockles environments.

GR activity was determined using a modified Sigma-Aldrich EC 1.6.4.2 procedure based on the method of Mavis and Stellwagen (1968). Oxidation of 0.8 mM NADPH by the enzyme was measured at 340 nm at 25 ºC for 5 min in the presence of 30 mM oxidised glutathione (GSSG) on a microplate reader. The buffer used was a 100 mM phosphate buffer (K_2HPO_4-KH_2PO_4) pH 7.6, 1 mM EDTA. GR activity was expressed as units / mg of protein where one unit will reduce 1.0 µmole of oxidised glutathione per minute at pH 7.6 at 25ºC.

$$\text{ß–NADPH + GSSG} \xrightarrow{\text{Glutathione Reductase}} \text{ß–NADP + 2 GSH}$$

GPx activity was measured using a modified Sigma- Aldrich EC 1.11.1.9 procedure based on the method of Wendel (1980). GPx catalyzes the peroxidation of reduced glutathione (GSH) to GSSG. Tert-butyl hydroperoxide (t-Bu-OOH) was used as the oxidant. This assay measured GPx indirectly by following the back reaction in which GSSG is reduced to GSH, in the presence of an excess amount of GR. The amount of NADPH consumed in this reaction is proportional to the amount of GSSG produced by GPx. Thus GPx activity is measured by the decrease in absorbance of NADPH at 340nm for 5 min at 25 ºC. For this assay a 100 mM phosphate buffer was used (K_2HPO_4-KH_2PO_4) pH 7.0, 1

mM EDTA. GPx activity was expressed as units / mg of protein where one unit will catalyze the oxidation by t-Bu-OOH of 1.0 µmole of reduced glutathione to oxidised glutathione per minute at pH 7.0 at 25ºC.

$$2\ GSH + H_2O_2 \xrightarrow{\text{Glutathione Peroxidase}} GSSG + 2\ H_2O$$

$$GSSG + \text{ß—NADPH} \xrightarrow{\text{Glutathione Reductase}} \text{ß—NADP} + 2\ GSH$$

GST was measured using a modified Sigma-Aldrich procedure EC 2.5.1.18 based on the method of Habig *et al*., (1974). GST was measured at 340 nm by measuring the conjugation of 75 mM GSH and 30 mM 1-chloro-2,4-dinitobenzene (CDNB). For this assay, a 100 mM phosphate buffer (K_2HPO_4-KH_2PO_4) pH 6.5, 1 mM EDTA was used. GST activity was expressed as units / mg of protein where one unit will conjugate 1.0 µmole of CDNB with reduced glutathione per minute at pH 6.5 at 25ºC.

$$GSH + CDNB \xrightarrow{\text{Glutathione S-Transferase}} \text{G-CDNB Conjugate}$$

For all three enzymes measured (GR, GPx, and GST) each replicate sample was repeated four times and averaged (n = 4). All biomarker results were normalised by protein content and were expressed as units / mg of protein. This was done by dividing the enzyme activity by the amount of protein determined using the Bradford assay described earlier.

2.4.3 Heavy Metal Analysis

Heavy metal analysis was carried out on sediments and tissue samples of *A. stutchburyi* using inductively coupled plasma-mass spectrometry (ICP-MS model ELAN DRC). I used the same ground tissue samples used for biomarker analyses so I could directly compare metal levels with biomarker results. 0.5 grams of wet tissue was placed into a 50 ml centrifuge tube and dissociated with tetramethylammonium hydroxide. Samples were then heated in a water bath at 65ºC for 1 h, vortexed, and heated for another hour. These were again vortexed and placed into an ice water bath for 30 min. 0.5 ml of cold 50 % hydrogen peroxide was then added to each sample and they were then refrigerated overnight. The following day samples were vortexed and 2.5 ml of 16 M HNO3 acid was added. All samples were then heated at 100 ºC for 1 h, then cooled and made up to 50 ml with reagent water and mixed. Samples were then filtered using a Millipore Steriflip filtration unit, into a clean 50 ml tube. The final solution was analyzed by ICP-MS using calibration standards prepared with the digestion reagents. Measurements were expressed as µg metal / g wet tissue (Martin *et al*., 1994).

Sediment metal analysis was carried out by ICP-MS. Approximately 0.5 g of dry sediment was added to polycarbonate (PC) centrifuge tubes. 5 ml of 7.7 M HNO3 and 15 ml of 2.34 M HCL acid were added to the sediments, and closed tubes were placed into a rack and left in a fume hood overnight. The next day samples were placed into a 90ºC water bath for 2 h. When samples had cooled to room temperature 0.25 ml of the samples was transferred into a 15 ml vulcan tube and diluted with milli-Q water, then stored in a freezer until ICP-MS analysis. Metal

concentrations were expressed as ppm / gram of dry weight (Martin *et al.*, 1994) Measurements of metal concentrations were carried out for every second month of the sampling period including April, June, August, October, December, February, and April 2008. I measured sediment and tissue samples for a suite of metals but have only presented the results of copper, lead and zinc. This is because at high concentrations these metals are highly toxic to marine and estuarine organisms (Bryan, 1971). Blank and reference samples were also run; these were made by following the same procedure but without sediment / tissue added. The detection limits were; 0.50 for copper, 1.00 for zinc, and 0.20 for lead.

2.4.4 Condition Index analysis

Condition index was determined as a proxy of organism health, and to compare the health of the animals among sites and over time (Crosby and Gale, 1990, Davenport and Chen, 1987). This was carried out independent of enzyme biomarker activities to determine any correlation between biomarker activities and condition of the animals. Condition index was measured monthly from April 2007 - April 2008 on 20 individuals per site (5 individuals per replicate). Individual frozen cockles were weighed (including shell) and their length, width and height measured in mm. Tissue samples from individual animals were removed from their shells and dried overnight at 60ºC in aluminium trays. The condition index was calculated by using the length, width and height to estimate the total volume of the cockle (assuming the shape of a spheroid). The cockle dry weight was then multiplied by 100 and divided by the estimated total (shell) volume.

$$\text{Estimated shell volume } (cm^3) = \frac{4 * \pi * L * W * H}{3 * 8000}$$

$$\text{Condition index} = \frac{\text{dry weight} * 100}{\text{Estimated total volume}}$$

(Crosby and Gale, 1990; Davenport and Chen, 1987).

2.5 Statistical analysis

All values expressed are means ± standard error. A two-way analysis of variance (ANOVA) was used to test for differences among sites within sample months for the three antioxidant enzymes measured (GPx, GR, and GST) and condition index. Significant differences ($p < 0.05$) among sites were further analysed by post-hoc Tukey honestly significant difference (HSD) tests.

To test for seasonality, enzymes that showed a significant interaction term ($p < 0.05$) between site and date were further tested by grouping months (three months per seasonal group) into seasons (summer, winter, spring and autumn) and analysed using a one-way ANOVA to test for a significant effect of season (using the average monthly enzyme activity at each site). Sites that showed a significant effect of season were further analysed by post-hoc Tukey HSD tests to determine if there were significant differences among seasons. To minimise sampling effort different combinations of individual months (using the four actual enzyme activities obtained for each site) were also selected to represent each season and analysed by one-way ANOVA. They were then analysed by post-hoc Tukey HSD tests when a significant interaction term resulted. Enzymes that did not show a significant interaction term between site and date were pooled (all sites), and

grouped (three months per seasonal group) into seasons. A one-way ANOVA was then used to determine if there was a significant effect of season followed by post-hoc testing where necessary.

Pearson correlation coefficients were calculated in STATISTICA to determine correlations between enzyme activities, condition index, heavy metal concentrations in sediments and tissues, and silt-clay fraction. Pearson's correlations were also carried out to determine correlations between environmental variables, sediment and tissue metal concentrations.

3 Results

3.1 Water Properties

Differences in water temperature and salinity existed among the three study sites (Figure 3.1a, b). Average salinity was the highest at Glendowie (29.9), followed by Panmure (29.0) and Tiraumea (25.0) (Figure 3.1a). This gradient among the three study sites is typically what you would expect from the upper to the lower reaches of an estuary. At Glendowie salinity values ranged from 19.8 – 33.7, at Panmure from 22.5 -33.0 and at Tiraumea from 15.0 – 33.1.

Water temperature was consistently higher at Glendowie compared to the other two sites with an average water temperature of 21.0 ºC during the sampling period, whereas Panmure had an average temperature of 18.0 ºC and Tiraumea had an average water temperature of 17.8 ºC (Figure 3.1b). Water temperature showed expected seasonal fluctuations, with the water temperature increasing from June 2007 – March 2008 at all three sites. At Glendowie the water temperature ranged from 11.7 -27.0 ºC, at Panmure from 10.5 – 23 .1 ºC, and at Tiraumea from 10.3 -23.9 ºC.

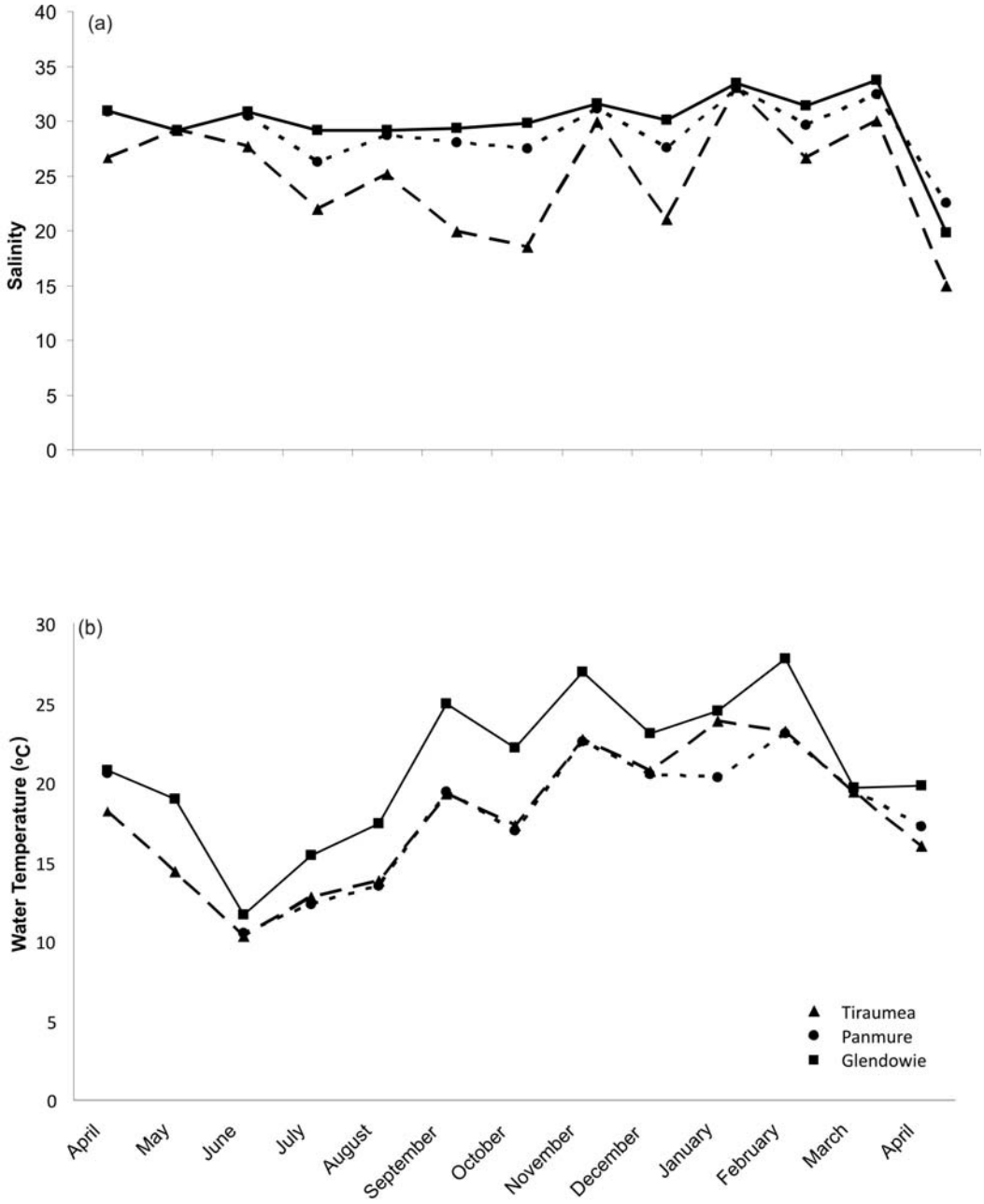

Figure 3.1 (a) Salinity and (b) water temperature (°C) at the low tide mark from the three study sites in the Tamaki Estuary from April 2007 – April 2008.

3.2 Sediment Properties

The average grain size revealed a clear gradient among the three sites; average grain size was considerably greater (coarser) at Glendowie ($\bar{x}$ = 239.9 μm) compared to Panmure ($\bar{x}$ = 161.4 μm) and Tiraumea ($\bar{x}$ = 94.2 μm) (Figure 3.2a). Tiraumea had on average the lowest grain size, although there was a marked increase in grain size at this location from November onwards. Median grain size at Tiraumea ranged from 11.6 -173.5 μm whereas the range was much smaller at Panmure (115.3 – 200.2 μm), and Glendowie (195.3 – 267.3 μm).

Tiraumea consistently had the highest silt-clay fraction ($\bar{x}$ = 47.9 %), followed by Panmure ($\bar{x}$ = 24.3 %), and then Glendowie ($\bar{x}$ = 9.6 %) (Figure 3.2b). There was a marked decrease in the silt-clay fraction at Tiraumea from November onwards. This correlated with the rapid rise in median grain size values. Pearson correlations showed there was a strong negative correlation between silt-clay and median grain size (r = -0.921) (Table 2), as the silt-clay fraction increased; median grain size decreased. A similar pattern was observed at the other two sites however the silt-clay fraction remained more constant throughout the sampling period, when compared with Tiraumea. Silt-clay values ranged from 25.5- 74.8 % at Tiraumea, 10.4 – 39.4 % at Panmure, and 5.1 - 21.9 % at Glendowie.

Tiraumea consistently had a higher percentage of organic matter content when compared to the other sites (Figure 3.2c). The average percentage of organic content at Tiraumea was 3.7 %, this was much higher than Panmure ($\bar{x}$ = 2.1 %) and Glendowie ($\bar{x}$ = 1.4 %). Organic matter content ranged from 0.8 – 1.9 % at Glendowie, from 1.4 – 2.9 % at Panmure and from 3.3 – 4.6 % at Tiraumea.

There was a significant correlation between organic matter content and the silt-clay fraction (r = 0.475), and a significant correlation between organic matter content and median grain size (r = 0.522) (Table 2).

Chlorophyll *a* concentrations varied considerably throughout the sampling period at all three sites (Figure 3.2d). Concentrations of chlorophyll *a* ranged from 5.0 - 14.4 µg / g dw at Glendowie, from 6.0 – 21.7 µg / g dw at Panmure, and from 7.0 – 20.0 µg / g dw at Tiraumea. Glendowie consistently had on average the lowest concentrations of chlorophyll *a* ($\bar{x}$ = 9.79 µg / g dw) compared with Panmure ($\bar{x}$ = 12.7 µg / g dw) and Tiraumea ($\bar{x}$ = 12.2 µg / g dw). Chlorophyll *a* concentrations fluctuated seasonally, with an increase in chlorophyll *a* levels at all three sites from November 2007 onwards.

Table 2. Pearson's correlation coefficients (r) between measured environmental variables, cockle condition index, and sediment and tissue, heavy metal concentrations. Significant correlations (* p< 0.001, **p<0.01) are shown in bold.

	Silt-Clay	***Condition index***	***Chlorophyll a***	***Pb sediment***	***Cu sediment***	***Zn sediment***	***Cu tissue***	***Zn tissue***	***Organics***	***Median grain size***
Silt-Clay	1.00									
Condition index	**-0.372****	1.00								
Chlorophyll a	-0.131	0.116	1.00							
Pb Sediment	**0.796***	**-0.461***	-0.061	1.00						
Cu sediment	**0.781***	**-0.417****	0.021	**0.963***	1.00					
Zn sediment	0.148	-0.105	0.092	**0.356****	**0.349****	1.00				
Cu Tissue	0.063	-0.158	0.116	0.042	0.014	-0.125	1.00			
Zn Tissue	0.136	-0.216	-0.135	0.115	0.123	-0.218	0.166	1.00		
Organics	**0.475***	-0.239	0.063	**0.759****	**0.762***	**0.577***	0.082	-0.102	1.00	
Median grain size	**-0.921***	**0.465***	0.074	**-0.836****	**-0.793***	-0.178	-0.156	-0.154	**0.522****	1.00

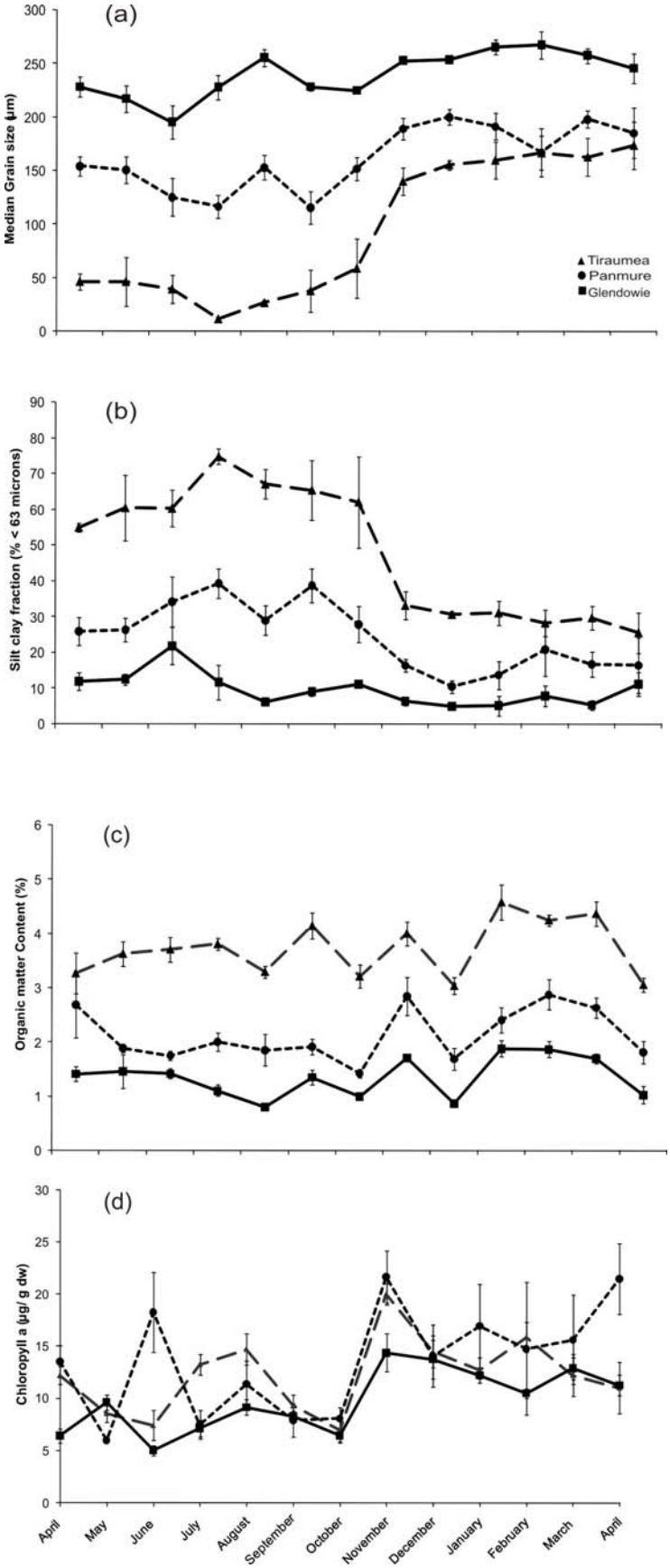

Figure 3.2 (a) Median grain size (µm), (b) silt-clay fraction (%), (c) organic matter content (%) and (d) chlorophyll *a* (µg / g dw) content, of the sediments from the study sites in the Tamaki Estuary from April 2007 – April 2008. Values represent means ± SE (n = 4).

3.3 Sediment Heavy Metals

Heavy metal sediment concentrations of zinc (Zn) (Figure 3.3a), copper (Cu) (Figure 3.3b), and lead (Pb) (Figure 3.3c) showed a clear gradient; Glendowie consistently had the lowest metal concentrations, Tiraumea always had the highest metal concentrations, and Panmure had intermediate sediment metal concentrations. Tiraumea had a much higher mean Zn sediment concentration ($\bar{x}$ = 162 ppm dry weight), than Panmure ($\bar{x}$ = 63 ppm dry weight) and Glendowie ($\bar{x}$ = 45 ppm dry weight). Zn sediment concentrations had the largest range at Tiraumea (142 – 180 ppm dry weight), compared to Panmure (52 – 69 ppm dry weight) and Glendowie (35 – 52 ppm dry weight). The average Cu concentration in the sediments at Tiraumea was 15 ppm dry weight. This was over two times greater than the average concentration at Panmure ($\bar{x}$ = 7 ppm dry weight), and three times greater than the average concentration at Glendowie ($\bar{x}$ = 5 ppm dry weight). The average Pb sediment concentration at Tiraumea ($\bar{x}$ = 15 ppm dry weight) was over two times greater than the average value at Glendowie ($\bar{x}$ = 6 ppm dry weight), whereas Panmure had an average Pb concentration falling between Glendowie and Tiraumea ($\bar{x}$ = 12 ppm dry weight). At Tiraumea there was a decrease in Zn, Cu and Pb concentrations from October 2007 which coincided with a decrease in the silt-clay fraction (Figure 3.2b, Table 2). The decrease in metal concentrations at Tiraumea was also inversely correlated with the median grain size (Figure 3.2a, Table 2); as sediment metal concentrations decreased, median grain size increased.

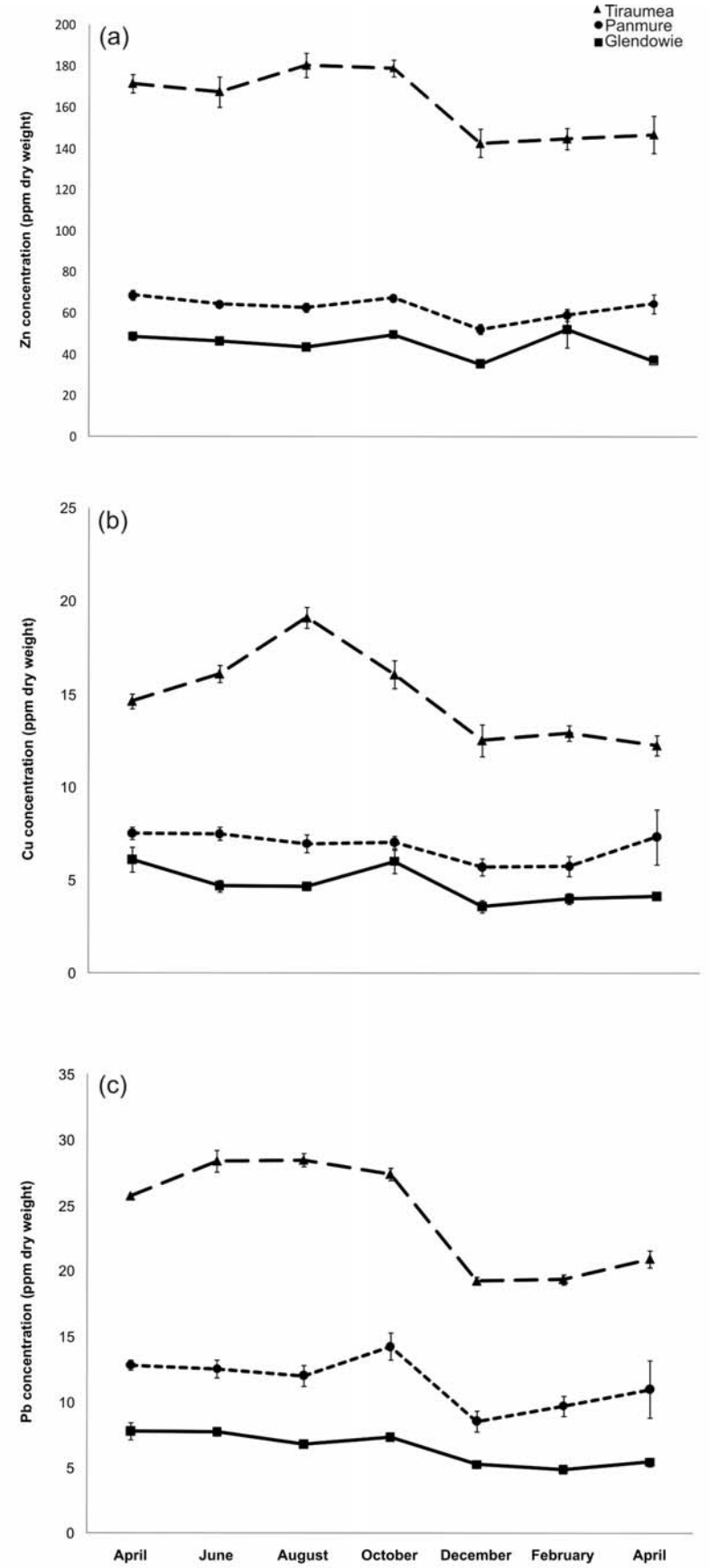

Figure 3.3 (a) Zn, (b) Cu, and (c) Pb (ppm dry weight) sediment concentrations from the study sites in the Tamaki Estuary from April 2007 – April 2008. Values represent means ± SE. (n=4).

3.4 *Austrovenus stutchburyi* Analysis

3.4.1 Heavy Metal Tissue Concentration

Lead concentrations in cockle tissues were below detection limits. Cu tissue concentrations in cockle tissue from all three study sites fluctuated approximately two-fold throughout the sampling period (Figure 3.4.1a). At Panmure Cu concentrations decreased from June 2007 – December 2007 followed by an increase in February 2008. Cockles from Glendowie showed a more constant trend and had consistently lower concentrations ($\bar{x}$ = 0.9 µg Cu / g wet tissue) compared to Panmure ($\bar{x}$ = 1.4 µg Cu / g wet tissue) and Tiraumea ($\bar{x}$ = 1.1 µg Cu / g wet tissue) during the sampling period. Cu concentrations in cockles from Tiraumea had a peak occurring in the month of December 2007. Zn tissue concentrations were less variable than Cu tissue concentrations. All zinc tissue concentrations ranged between 6 – 10 µg Zn / g wet tissue (Figure 3.4.1b). Panmure had the highest average concentration ($\bar{x}$ = 8.0 µg Zn / g wet tissue) followed by Glendowie ($\bar{x}$ = 7.9 µg Zn / g wet tissue) then Tiraumea ($\bar{x}$ = 7.8 µg Zn / g wet tissue).

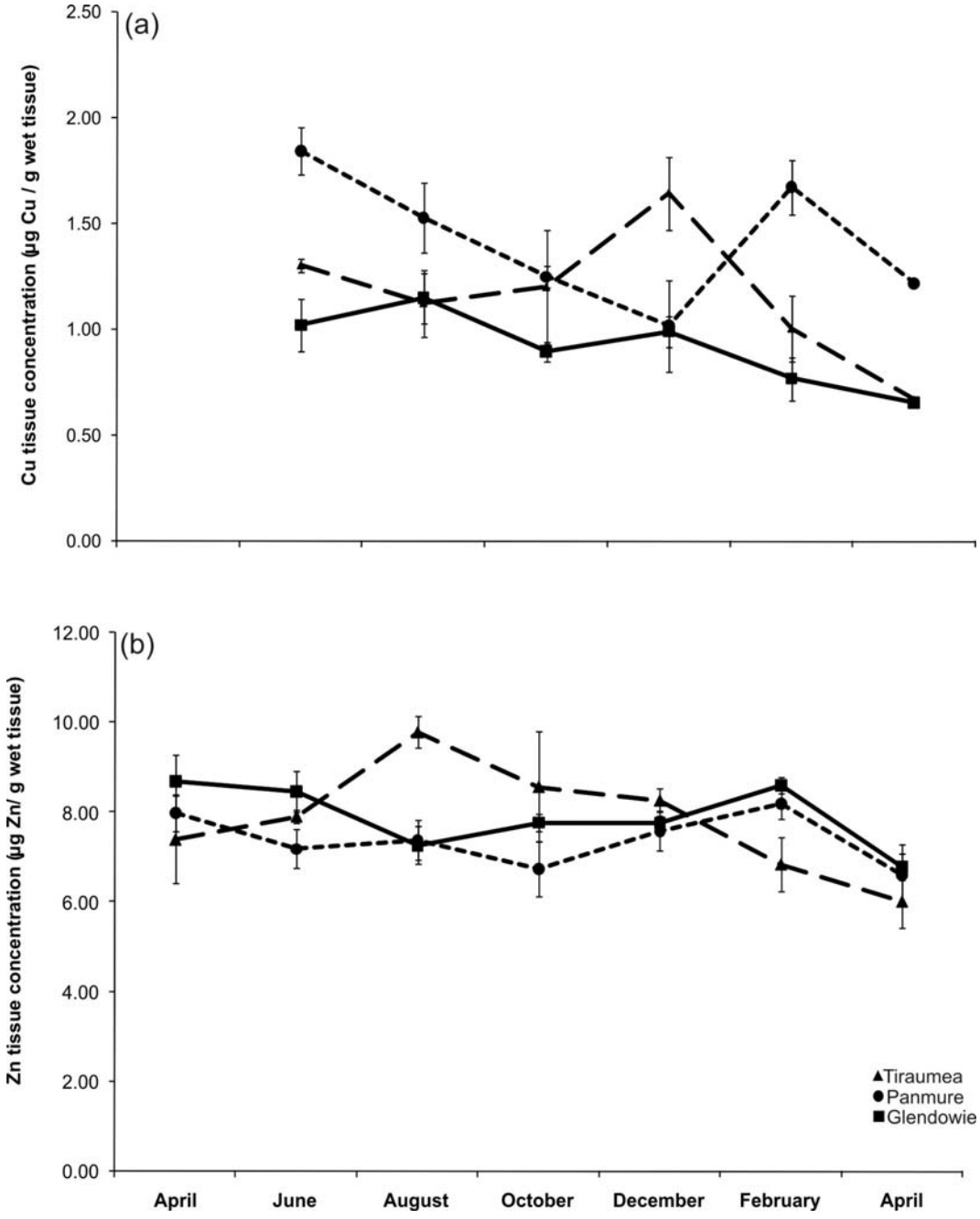

Fig 3.4.1 (a) Cu and (b) Zn tissue concentrations (mg/ g wet tissue) in cockles from the study sites in the Tamaki Estuary from April 2007 – April 2008. Values represent means ± SE. (n=4)

3.4.2 Condition Index

Condition index of cockles during the sampling period showed little seasonal fluctuations (Figure 3.4.2). Glendowie consistently had the highest condition index ($\bar{x}$ = 3.9), compared to Panmure ($\bar{x}$ = 3.4) and Tiraumea ($\bar{x}$ = 3.5). Condition index at all three sites ranged from 2.6 – 4.8 over the sampling period. A two-way ANOVA (Table 3) showed there was a significant interaction term between site and date. Therefore Tukey HSD post-hoc tests were carried out to test if these differences were significant among sites (Table 4). Post-hoc tests showed that condition index from cockles at Glendowie was significantly different and higher than the other study sites for five months during the sampling period. A Pearson's correlation analysis revealed that cockle condition index had significant negative correlations with lead sediment concentrations (r = -0.461), copper sediment concentration (r = -0.417) and silt-clay fraction (r = -0.372), and a positive correlation with median grain size (r = 0.465) (Table 2).

Table 3. Results of a two way analysis of variance, testing the effects of site and sample date on condition index. Significant effects ($p < 0.05$) are indicated in bold.

Condition Index Factor	***SS***	***Deg. Freedom***	***MS***	***F***	***P***
Site	0.08	1	0.09	1.10	0.30
Date	9.79	11	0.89	11.14	**<0.0001**
Site * Date	5.99	23	0.26	3.27	**<0.0001**

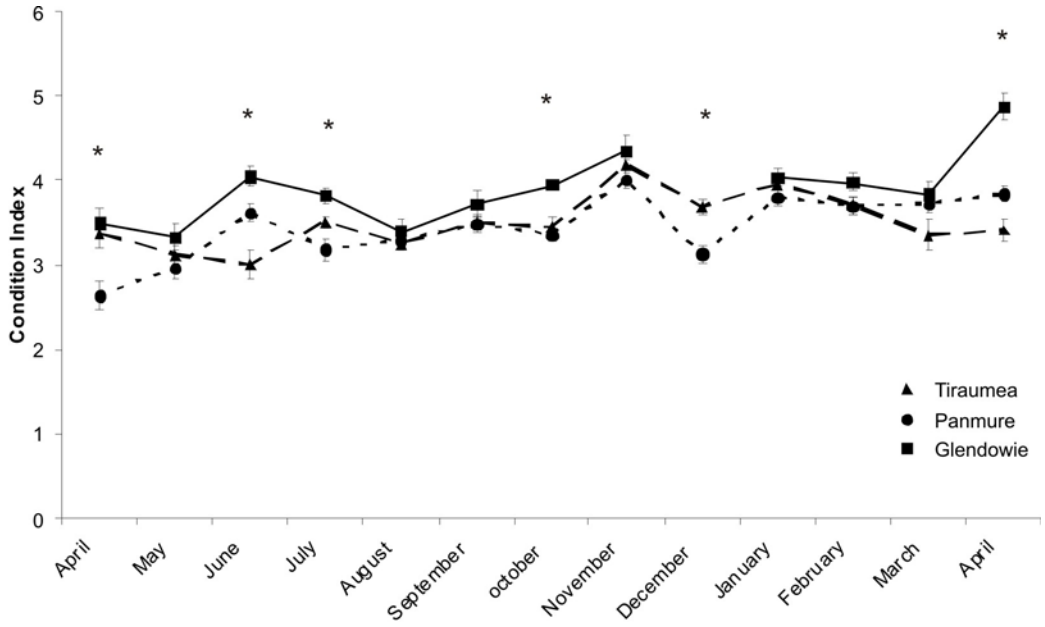

Figure 3.4.2 Condition index of cockles from the three study sites; Glendowie, Panmure, and Tiraumea in the Tamaki Estuary, from April 2007 – April 2008. Values represent means ± SE (n=20). Asterisks denote sample dates where condition index values were significantly different ($p < 0.05$) among sites. See Table 4 for results of post-hoc tests. (Missing value at Glendowie in December 2007 was due to a lack of cockles available at this site during the time of collection).

Table 4. Results of post-hoc Tukey tests ($p < 0.05$) to determine differences between sites within sample months for condition index of cockles from the Tamaki Estuary. (G= Glendowie, P= Panmure, and T= Tiraumea). NS = no significant difference.

April	May	June	July	August	September	October	November	December	January	February	March	April
G>P	NS	G>T	G>P	G>T	NS	G>T	NS	P>T	NS	NS	NS	G>T
P=T		P=T	P=T	G>P		G>P		G=T				G>P
G=T		G=P	G=T	T=P		T=P		P=G				T=P

3.5 Antioxidant Enzyme Analysis

Glutathione peroxidase (GPx) activity in cockle tissues varied approximately three-fold from 0.0026 – 0.0061 units / mg protein (Figure 3.5a) among the three sampling sites, over the sampling period. Glendowie showed the highest GPx activity compared to the other two sites (Figure 3.6). The average GPx activity at Glendowie during the sampling period was 0.0044 units / mg protein, this was higher than Panmure ($\bar{x}$ = 0.0032 units / mg protein) and Tiraumea ($\bar{x}$ = 0.0030 units / mg protein). There was a considerable increase in GPx activity at Glendowie in January 2008 and GPx activity remained elevated for the remainder of the sampling period. From January 2008 – April 2008 GPx activity at Glendowie ranged from 0.0049 – 0.0057 units / mg protein. This was approximately 1.5 times higher than the average GPx activity from April 2007 – December 2007, which was 0.0038 units / mg protein.

A two-way ANOVA (Table 5) showed that site, date and the interaction term for GPx were all significant factors influencing GPx activity, therefore the activity of GPx varied through time but differently at each study site. Post-hoc Tukey tests (Table 9) showed Glendowie had significantly different and higher GPx activities than the other two sites (Panmure and Tiraumea) in April 2007 – August 2007, October, and from February 2008 onwards.

The significant interaction term between site and date meant each site could be analysed separately to determine if the activity of GPx varied seasonally. This was done by grouping months (three months per season) and using a one-way

ANOVA to test if there was a significant effect of season, (summer = January, February, and March; autumn = April (2007), May and June; winter = July, August and September; and spring = October, November, and December). At Glendowie there was a significant effect ($p = 0.022$) of season and post-hoc tests revealed summer was significantly different (higher) than spring. However there was no significant seasonal effect at Tiraumea ($p = 0.493$) or Panmure ($p = 0.080$).

It was evident that the summer months (from January onwards) were significantly higher than the other months (Figure 3.5a) at Glendowie. To determine if the summer months were driving the seasonal response at Glendowie, a one-way ANOVA was carried out excluding the summer months from the analysis. No significant effect of season ($p = 0.602$) was found; therefore it could be concluded that it was the summer months driving the seasonal response at Glendowie.

To confirm the hypothesis that it was all of the summer months driving the seasonal response at Glendowie (not just one atypical month), several seasonal combinations were tested. Firstly, one month was selected to represent each season (e.g. summer represented by January, etc.) and a one-way ANOVA used to determine if season showed a significant effect. For each combination summer was included in one analysis, and excluded in a subsequent analysis, and the results compared (Table 6). In the majority of the combinations tested there was only a significant effect of season when the summer months were included in the analysis. Therefore it could be concluded that it was a combination of the

majority of the summer months (from January onwards) that were driving the seasonal response seen at Glendowie.

Table 5. Results of a two-way analysis of variance, testing the effects of site and sample date on enzyme activity. Significant effects ($p < 0.05$) are indicated in bold.

Enzyme Factor	SS	Deg. Freedom	MS	F	P
GPx					
Site	0.539	2	0.269	64.14	< **0.0001**
Date	0.636	12	0.053	12.62	< **0.0001**
Site*Date	0.311	24	0.013	3.08	< **0.0001**
GST					
Site	0.007	2	0.003	17.47	<**0.0001**
Date	0.061	12	0.005	26.01	<**0.0001**
Site*Date	0.008	24	0.000	1.66	**0.040**
GR					
Site	0.116	2	0.58	1.10	0.337
Date	4.975	12	0.415	7.82	<**0.0001**
Site*Date	1.601	24	0.067	1.26	0.210

Table 6. One-way ANOVA results to determine if a significant seasonal ($p < 0.05$) effect was found when a variety of combinations of seasons were compared, including and excluding summer months for GPx at Glendowie. Combination 1 = April, July, October and January; combination 2 = May, August, November, and February; combination 3 = June, September, December and March. Significant effects ($p < 0.05$) are indicated in bold

Combination	*Summer month*	*P value*	*Post-hoc result*
1	Included	0.444	
	Excluded	0.358	
2	Included	< **0.00**	Summer significantly higher than all other seasons
	Excluded	0.171	
3	Included	< **0.000**	Summer significantly higher than all other seasons
	Excluded	0.171	

GPx activity had a positive significant (r = 0.761) correlation with condition index (Table 10). GPx activity also showed significant negative correlations with sediment lead (r = -0.561) and copper (r = -0.539) concentration, copper tissue (r = -0.530) concentration, and the silt-clay fraction (r = -0.558) of the sediment. Not only did GPx activity have significant correlations with sediment and tissue metal data, but it also showed a slight significant positive correlation with the water temperature (r = 0.375).

Glutathione S-transferase (GST) activity in cockle tissues showed a similar pattern to GPx activity; it was the highest at Glendowie ($\bar{x}$ = 0.098 units / mg protein) (Figure 3.5b) followed by Panmure ($\bar{x}$ = 0.089 units / mg protein) and lastly Tiraumea ($\bar{x}$ = 0.082 units / mg protein). GST activity varied between 0.054 – 0.140 units / mg protein among the three sites, during the sampling period. Tiraumea had the smallest range (0.053 – 0.109 units / mg protein), compared to Glendowie (0.062 – 0.136 units / mg protein) and Panmure (0.056 – 0.136 units / mg protein) which showed similar ranges (Figure 3.6). GST activity followed similar trends at all three sites. Cockles had increased GST activity from January onwards, this was more pronounced at Glendowie. The average GST activity at Glendowie from January to April 2008 was 0.129 units / mg protein. The increase of GST activity from January onwards correlated with the increase in GPx activity. GST activity at all three study sites showed an increasing trend during the sampling period. GST activity at all three study sites were almost twice as high at the end of the sampling period compared to the beginning of the sampling period.

A two-way ANOVA showed that site, date and the interaction term were all significant (Table 5) therefore a post-hoc Tukey test was carried out to compare differences between sites within sample months (Table 9). Post-hoc tests showed that Glendowie had significantly different and higher GST activity compared with the other two study sites (Panmure and Tiraumea) in March 2008.

To examine any seasonal differences in enzyme activity among sites, months were grouped according to season (as described above) and analysed using a one-way ANOVA to test if there was a significant effect of season. Seasonality was significant at both Panmure ($p = 0.039$) and Glendowie ($p = 0.005$), however there was no significant effect of season at Tiraumea ($p = 0.138$). Post-hoc tests revealed that at Panmure, summer was significantly higher than autumn, and at Glendowie, summer was significantly higher than autumn and spring.

It was visible that the summer months (Fig 3.5b) had higher GST activity levels compared to the other months at Panmure and Glendowie. Therefore, to determine if it was exclusively the summer months driving the seasonal effect at these two sites, multiple one way ANOVA analyses were carried out. However in these analyses one month was picked to represent each season and a variety of combinations were tested (as described above) (Table 7 and 8).

At Glendowie, when summer was included in the analyses, it consistently came out significantly higher than the other seasons (Table 7). However there was still a significant effect of season when summer months were excluded from the analyses. Therefore it could be concluded that at Glendowie it was not

exclusively the summer months (January, February, and March) driving the seasonal effect but a combination of all the seasons. Consequently, by picking four months to represent each season (in an effort to minimise sampling effort) a realistic result can not be reached. This is due to the large monthly variability of GST activity at Glendowie. This was not the case at Panmure however, where summer consistently came out significantly higher than the other seasons (in all three tested combinations) and there was no significant effect of season when summer was removed from the analyses (Table 8). Therefore it could be concluded that it was solely the summer months determining the seasonal effect at Panmure

Table 7. One-way ANOVA results to determine if a significant seasonal ($p < 0.05$) effect was found when a variety of combinations of seasons were compared, including and excluding summer months for GST at Glendowie. Combination 1 = April, July, October and January; combination 2 = May, August, November, and February; combination 3 = June, September, December and March. Significant effects ($p < 0.05$) are indicated in bold.

Combination	***Summer month***	***P value***	***Post-hoc result***
1	Included	**< 0.000**	Summer significantly higher than autumn and spring. Autumn significantly lower than all seasons
	Excluded	**0.002**	Autumn significantly lower than winter and spring
2	Included	**0.005**	Summer significantly higher than spring
	Excluded	**0.042**	Spring significantly lower than autumn.
3	Included	**<0.000**	Summer significantly higher than all seasons. Spring significantly lower than autumn and winter.
	Excluded	**0.003**	Spring significantly lower than autumn and winter.

Table 8. One-way ANOVA results to determine if a significant seasonal ($p < 0.05$) effect was found when a variety of combinations of seasons were compared, including and excluding summer months for GST at Panmure. Combination 1 = April, July, October and January; combination 2 = May, August, November, and February; combination 3 = June, September, December and March. Significant effects ($p < 0.05$) are indicated in bold

Combination	*Summer month*	*P value*	*Post-hoc result*
1	Included	**< 0.001**	Summer significantly higher than all other seasons.
	Excluded	0.061	
2	Included	**0.012**	Summer significantly higher than autumn and winter.
	Excluded	0.643	
3	Included	**<0.010**	Summer significantly higher than spring.
	Excluded	0.078	

GST activity had a positive significant correlation with condition index ($r = 0.721$) (Table 10) and a significant negative correlation with the silt-clay fraction ($r = 0.498$).

Glutathione reductase (GR) activity at all three sites showed a fluctuating trend over the sampling period (Figure 3.5c). Panmure had slightly higher average GR activities during the sampling period ($\bar{x}$ = 0.0096 units / mg protein) followed by Glendowie ($\bar{x}$ = 0.0095 units / mg protein) and Tiraumea ($\bar{x}$ = 0.0090 units / mg protein) however theses differences among sites were not statistically significant ($p = 0.337$) during the sampling period. GR activities showed a considerable increase in January; this was more pronounced at Panmure. At all three study sites the majority of GR activity ranged from 0.0080 -0.0120 units / mg protein (Figure 3.6).

A two- way ANOVA (Table 5) showed that date contributed to variations in GR activity whereas location did not. Since location was found to be non-significant all sites were pooled together to test for seasonality to examine whether GR activity showed significant seasonal variability across all three sites during the sampling period. Months were seasonally grouped (three months per season) (as described earlier) and analysed using a one-way ANOVA to test if there was a significant effect of season. The one-way ANOVA revealed there was no significant effect of season ($p = 0.154$). GR activity did not show any significant correlations with any of the environmental co-variables measured (Table 10).

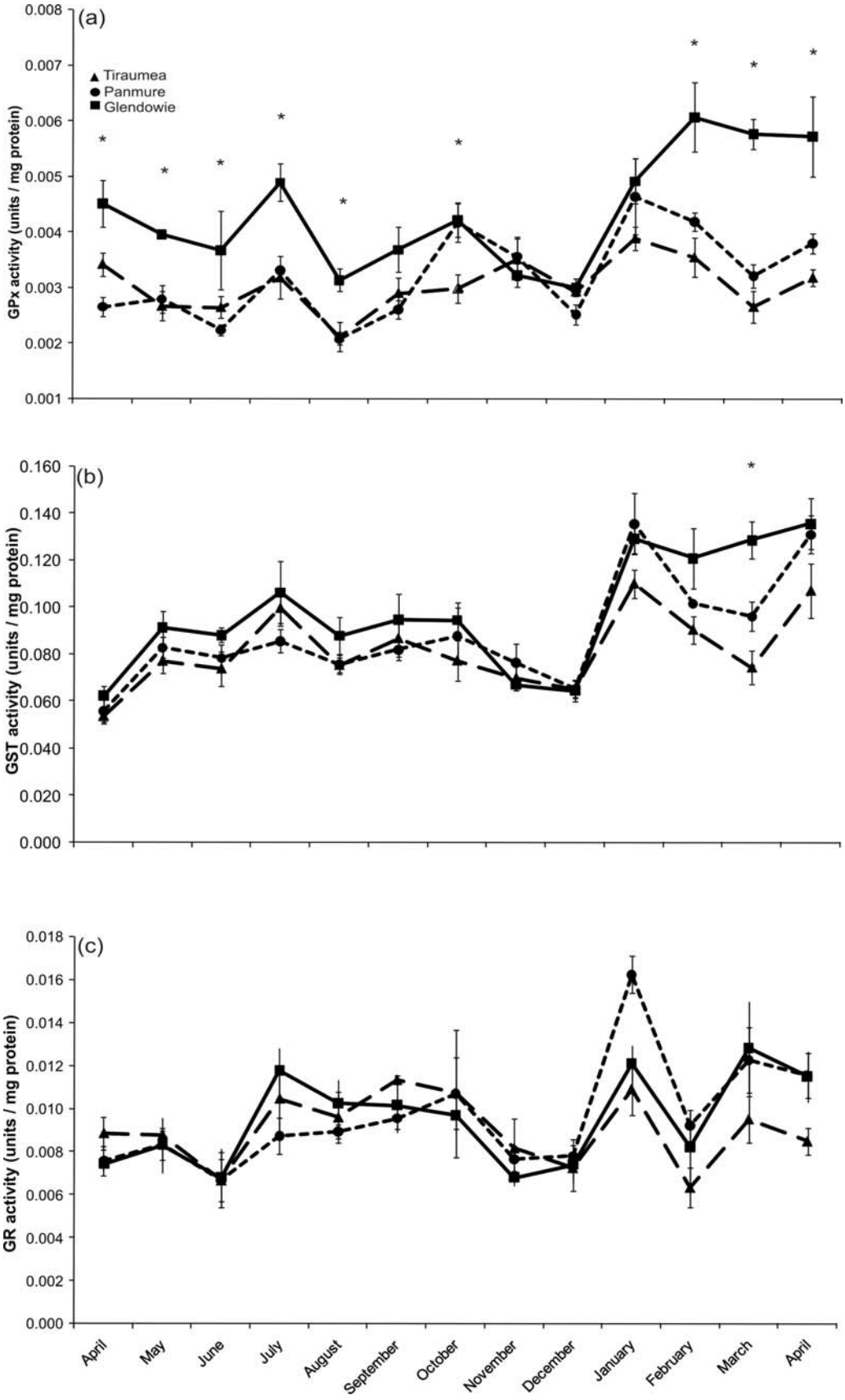

Figure 3.5 (a) GPx, (b) GST, and (c) GR activity (units / mg protein) at Glendowie, Panmure, and Tiraumea, from April 2007 – April 2008. Values represent means ± S.E (n=4). Asterisks denote sample dates where activity levels were significantly different ($p < 0.05$) among sites. See Table 9 for results of post-hoc tests.

Table 9. Results of post-hoc Tukey test to determine differences between sites within sample months for the antioxidant enzymes, GPx, GR, and GST. (G= Glendowie, P= Panmure, and T= Tiraumea). NS = no significant difference.

	April 2007	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**	**January**	**February**	**March**	**April 2008**
GPx	G>T G>P T=P	G>T G>P T=P	G>T G>P T=P	G>T G>P T=P	G>T G>P T=P	NS	G>T T=P G=P	NS	NS	NS	G>T G>P T=P	G>T G>P T=P	G>T G>P T=P
GST	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	G>T G>P T=P	NS
GR	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

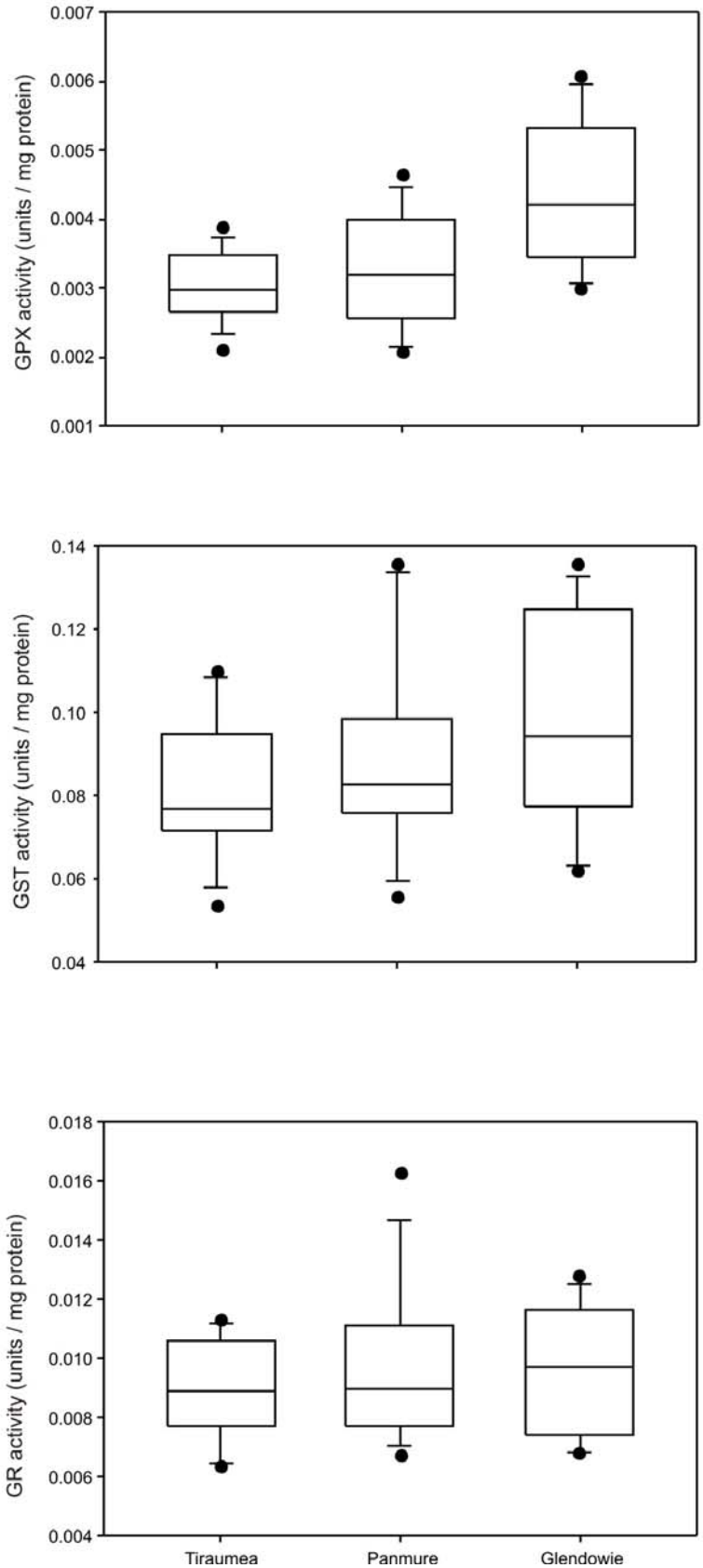

Figure 3.6 Box and whisker plots of GPx, GR and GST activity (units / mg protein) at Tiraumea, Panmure and Glendowie from April 2007 – April 2008. Box denotes 25%, median (50%), and 75 percentile, whiskers indicate 10 % and 90 %, and dots show outliers.

Table 10. Pearson correlation coefficients (r) between enzyme activities, condition index, water salinity and temperature, heavy metal concentrations in sediments and tissues, and the silt-clay fraction. Significant correlations (* p< 0.001, ** p < 0.01, *** p <0.05) are shown in bold.

	Condition Index	**Pb Sediment**	**Cu Sediment**	**Zn Sediment**	**Cu Tissue**	**Zn Tissue**	**Silt-Clay**	**Temp**	**Salinity**
GPx	**0.761***	**-0.561***	**-0.539****	-0.461	**-0.530*****	-0.331	**-0.558****	**0.375***	0.197
GR	0.265	-0.178	-0.177	-0.225	-0.236	-0.089	-0.140	0.035	0.023
GST	**0.721***	-0.469	-0.443	-0.403	-0.512	-0.410	**-0.498****	0.119	0.004

4 Discussion

In this study the influences of contamination, environmental co-variables, and seasonality were examined on three different cellular defence system enzymes (glutathione S-transferase (GST), glutathione reductase (GR), and glutathione peroxidise (GPx)) in the common New Zealand cockle *Austrovenus stutchburyi*. Three sites varying in sediment metal concentrations along the Tamaki Estuary were selected because previous studies (Table 1) had demonstrated that contaminants can affect the activity of antioxidant enzymes, responding as biomarkers of oxidative stress. An imbalance in reactive oxygen species (ROS) production and removal can result in oxidative stress; and metal contaminants are known to increase the formation of ROS therefore causing an imbalance in this cycle (Geret *et al*., 2002; Torres *et al*., 2002). Antioxidant enzyme systems act as protective agents against damaging effects of oxyradicals and other ROS by helping the cells eliminate and maintain low levels of ROS (Manduzio *et al.*, 2004; Geret *et al*., 2002). Therefore measuring different enzymes involved in the cellular defence system has been proposed as biomarkers of oxidative stress and can indicate effects of contaminants (Di Giulio, *et al*., 1989; Livingstone, 1993; Winston and Di Giulio 1991).

4.1 Exposure biomarkers and their relationship to metal concentrations

Firstly this study aimed to determine if varying levels of heavy metal contamination in the sediments at the three selected study sites along the Tamaki Estuary would influence biomarker activities in cockles. Heavy metal sediment

analysis confirmed the existence of a contamination gradient; a four-fold difference in metal levels existed from the upper to the lower reaches of the estuary. It was expected that the presence of metal contaminants would cause variations in enzyme activities in cockles from different locations as metals are a source of oxidative stress (Santovito *et al.,* 2005). GPx activity showed negative correlations with sediment lead (r = -0.561) and copper (r = -0.539) concentrations and copper tissue (r = -0.530) concentrations. In other words, the lower the concentrations of lead and copper, the higher the activity of GPx, indicating that cockles living in environments with lower levels of metal contaminants will have higher GPx activity. This finding is supported by other studies (Cossu *et al*., 2000; Cossu *et al*., 1997; Geret *et al*., 2002) that also found higher GPx activity levels in invertebrates living in less polluted environments. Glendowie – the least metal contaminated site, had the overall highest average GPx activity (0.0044 units / mg protein) compared to the other two study sites (Panmure and Tiraumea) which had lower GPx activities and higher metal concentrations. GPx was the only biomarker measured that showed a significant correlation with metal concentration. GR activity showed no significant correlation with any of the measured metal contaminants (Pb sediment r = -0.178, Cu sediment r = -0.177, Zn sediment r = -0.225). GR activity showed similar activity at all three sites over the sampling period, despite varying metal concentrations in sediments and cockle tissues. Since the observed variations in enzyme activity could not be related to metal concentrations this limits the use of GR as a potential biomarker of metal contamination.

Similar to GPx, GST activity was on average the highest at Glendowie ($\bar{x}$ = 0.098 units / mg protein) over the sampling period. Assuming that these enzymes respond to different degrees of contaminant exposure, this suggests that the healthier cockles were characterised by higher GPx and GST enzyme activities. Cockles at Glendowie, having higher enzyme activities, may be producing more of the enzymes to remove ROS and remain in a healthy state (Cossu *et al*., 2000). Tiraumea (with the highest sediment metal concentrations) showed the lowest enzyme activities for both GST and GPx, suggesting animals living in a more stressed environment have lower enzyme activities. A deficiency in these cellular defence enzymes might weaken the capability of an organism to neutralize the generation of ROS. This allows build up of ROS as antioxidant enzyme activities become depleted and cockles become inundated (Regoli and Principato, 1995; Frenzilli et al., 2004). Cockles might then have a lower ability to prevent cellular damage due to the build up of ROS, hence living in a stressed state. This enzyme deficiency is a likely result of the organism living in a contaminated environment (Cossu *et al*., 2000). This finding is supported by many other studies (Cossu *et al*., 2000; Cossu *et al*., 1997; Geret *et al*., 2002; Osman *et al*., 2007; Regoli, 1998) that similarly showed lower enzymatic activities (including GR, GPx and GST among others) in bivalves from highly polluted sites and higher activities in bivalves living in cleaner environments. Differences in condition index also confirmed this finding; Glendowie cockles had the highest condition index when compared to the other sites, suggesting that these animals are in a better growing condition (can invest more energy into growth) when compared with animals from the other two sites. Condition index had moderately strong significant correlations with both GPx (r = 0.761) and GST (r= 0.721) activity, showing that

as the condition index of an animal increases so too does the activity level of GPx and GST. This corroborates the hypothesis that; the healthier the animal, the higher its cellular defence enzyme activities will be.

The increase in GST and GPx enzyme activities in January at Glendowie (and for the remainder of the sampling period) could indicate that the sampled populations of cockles living at this site experienced improved growing conditions and overall vigour. Or perhaps these increases reflected a recovery from a previous stress event, occurring before April 2007. Therefore there is potential for future use of these (GST, GPx) enzymes as biomarkers for monitoring the health of populations over time. The higher GPx and GST enzyme activities at Glendowie, characterised by lower metal levels, and the lower enzyme activities at Tiraumea (characterised by higher metal levels), confirms the initial hypothesis of a relationship between biomarker activities and heavy metal sediment concentrations; i.e. the higher the sediment heavy metal levels the lower the enzyme activity. This hypothesis however, was only true for both GPx and GST.

4.2 Environmental co-variables

If the response of the enzyme activities was solely driven by heavy metal concentrations then you would expect either a large increase or decrease in metal concentrations and changes in condition index from January onwards at Glendowie (when the large increase in enzyme activity occurred). Condition index provides a useful measure of growth and general physiological performance, thus the condition index of populations is expected to fluctuate due

to varying levels of oxidative stress brought about by contaminants (Cantelmo-Cristini *et* al., 1985; Sujatha *et al.,* 1995; Black and Belin, 1998). Neither condition index nor metal concentrations showed any significant increases or decreases in January 2008 – April 2008. Therefore it is likely that it is not solely metals driving the antioxidant enzyme response. Rather the enzymatic response is very likely to be due to a combination of different variables. For this reason a number of environmental co-variables were measured to quantify co-variation with biomarker responses.

Analysis of sediment characteristics, and water parameters clearly established that each of the three sites possessed differences in grain size, silt-clay fraction, organic matter content, chlorophyll *a* content, and water temperature and salinity. However only the silt-clay fraction showed a moderate negative correlation with both GST ($r = -0.498$) and GPx ($r = -0.558$) activity.

A high silt-clay fraction in marine sediments is known to have adverse effects on macro-benthic communities. Terrigenous sediments, derived from increased sedimentation, (due to changes in land use and extreme weather events) entering marine environments increase the amount of silt in the sediments. An increase in terrigenous sediments is known to adversely impact benthic organisms. For example; by smothering the organisms, altering the organism's environment by changing the grain size of the sediments, and by increasing the amount of suspended solids in the water, this in turn can clog feeding structures of suspension feeders (Norkko *et al*., 2002). It is possible that the silt-clay fraction of the sediment was one factor influencing enzyme activities of both GPx and

GST, as there was a significant negative correlation with both these enzymes. To determine if the silt-clay fraction was driving enzyme responses, an appropriately designed lab test should be carried out with one population of cockles living in sediments with high silt-clay, and another population with low silt clay (both with low metal contamination). A decrease in enzyme activities from the population living in sediments with a high-silt clay fraction could suggest that in fact it is the high silt-clay fraction of the sediments causing stress related enzyme responses.

Organic matter content showed a strong gradient among sites with Tiraumea having the highest organic matter content. This is typical for a site located in the upper reaches of an estuary (Knox, 1986). Organic matter deposits under weak current conditions can become integrated into sediment deposits as detritus. These tend to block the interstices between sediment grains causing grains to bind together, giving sediments a cohesive muddy characteristic. As to be expected there was an inverse relationship between organic content and grain size; the higher the organic content the smaller the grain size of the sediments (Knox, 1986).

While sediment properties and water parameters only had a very minor influence on enzymatic activity some other, unmeasured factors, are worth considering. In December 2007 there were two reported spills into the Tamaki Estuary from the surrounding catchment. One released 10,000 litres of 91 octane petrol, and a second released concrete from a construction project that washed down storm water drains. Both of these spills occurred in the watershed above the Glendowie site. The petrol spill was detected only several days after its occurrence (personal

communication, 2008, Nigel Clarke, ARC pollution response team) making it likely that a significant amount of petrol reached the Estuary. The spill originated from an underground leak below a service station which resulted in an estimated 10-12,000 litres of petrol migrating through the ground to the storm water system. The petrol spill most likely reached the Estuary due to the large quantity of petrol released. The pollution response team at the Auckland Regional Council (ARC) received notification of the spill on the 20th December 2007. However they believe the spill occurred two days prior to their notification. Because of the late notification it is uncertain how much petrol actually reached the Estuary (personal communication, 2008, Nigel Clarke, ARC pollution response team). The unusually high GST and GPx enzyme activities seen at Glendowie from January onwards rather than being due to changes in metal concentrations could therefore be a response to these other contaminants released from the spills.

To confirm this hypothesis sediment samples were analysed for total petroleum hydrocarbon (TPH) content for December (before spill) and January (after spill) from the three study sites. This analysis found TPH concentrations to be undetectable at all three sites (data not shown). Either the oil spill was diluted very quickly, did not reach the study site at Glendowie, or did not accumulate in the sediment. To test the effects of petroleum on cockle antioxidant enzymes an appropriately designed laboratory test should be carried out exposing cockles to varying concentrations of petroleum. Enzyme activities could then be measured and responses compared to cockles not exposed to petroleum (control populations). If enzyme activities increased significantly in animals living in environments with high exposure to petroleum, this could explain the high

activities of GST and GPx seen at Glendowie from January onwards. However a well controlled study would need to be carried out to confirm this hypothesis, to separate petroleum-related effects from natural variability in responses. This would be one area that needs to be explored further to test this hypothesis, as only a few studies (Morales-Caselles *et al*., 2008, Prevodnik *et al*., 2007, Domasio *et al*., 2007) have been carried out measuring stress responses in organisms exposed to oil spills. One study by Morales-Caselles *et al* 2008 attempted to see if caged organisms exposed to sediments affected by oil spills showed a sublethal response. The study proved inconclusive; as the authors carried out lab and field exposures and gained different results. They were able to link certain sediment bound chemicals including lead to biomarker responses, but concluded there were additional, unmeasured factors in the field exposures causing biomarker responses. However another study by Domasio *et al*., 2007 showed that CAT levels were significantly depressed in fish from the site impacted by an oil spill and concluded that the fish from this site were more likely to be suffering from oxidative stress when compared to sites not affected by an oil spill.

4.3 Biomarkers and seasonality

Lastly, this study aimed to quantify the seasonal variability of the measured cellular defence enzymes and evaluate their application as an ecological monitoring tool. A one-way ANOVA showed there was significant seasonal variation in the enzyme activity of cockle GPx at Glendowie ($p= 0.022$), and GST activity at both Panmure ($p = 0.039$) and Glendowie ($p = 0.005$). There was no significant effect of season on GR activity ($p = 0.154$) over the sampling period. For the first nine months of sampling there were little temporal variations in

enzyme activities at all three study sites. However at Glendowie, GPx activity was significantly higher than the other sites for the majority of the sampled months. There was subsequently, an increase in GPx and GST enzyme activities in January at both Glendowie and Panmure, and these activity levels remained elevated for the remainder of the sampling period (until April 2008). In this period of elevated enzyme activity (January 2008 – April 2008), Glendowie continued to have the highest GPx and GST activities.

Multiple one-way ANOVAs revealed that it was the summer months (January, February, and March) that were driving the high seasonal variation of GPx activity at Glendowie and it was evident that GST activity also increased during the summer months. Thus, the activity of GPx is less variable from autumn to spring, and the only significant variability in GPx activity at Glendowie occurred from January onwards. Regoli (1998) also found the same results, with biochemical parameters (GST, glyoxalase I, glyoxalase II, and GPx) increasing in the mussel (*Mytilus galloprovincialis*) during the summer months; this was also when the trace metal concentrations decreased. Regoli (1998) also measured gill condition index and found that condition index was greater in populations from less polluted sites, however condition index only varied moderately throughout the sampling period showing little seasonal effects. Regoli (1998) also found no differences between sites (classified as polluted and non-polluted) for GR. Viarengo *et al*., (1991) also reported results in agreement with the results presented in this study. Viarengo *et al*., (1991) were able to relate the reduction of antioxidant defence in winter and increases in summer to changes in metabolic status. It is likely that the increased GPx and GST enzyme activities during

summer are likely due to changes in the metabolic status of the organisms caused by changes in reproductive status, gonadal development and spawning (Regoli, 1998)

In this study only one seasonal cycle was measured (from April 2007 – April 2008), consequently the results are inconclusive in determining whether the elevated enzyme (GPx and GST) activities are part of a typical annual cycle or an anomalous occurrence. To solve this problem, another yearly study could be conducted repeating the measurements of this present study. From these results it could then be determined if the observed increase in 2008 was a typical seasonal phenomenon or an anomaly. A study of this kind would then confirm if the results presented in this study are a realistic representation of seasonal variation of biomarker activities or an atypical result due to some unknown variable.

4.4 Methods critique

For this study the suspension feeding bivalve *Austrovenus stutchburyi* was the study organism selected. This bivalve was selected for a variety of reasons including, its wide geographical distribution, sessile nature, ease to collect and abundance. However it may be useful in future studies to choose a bivalve with a different mode of feeding, for example a deposit feeder (e.g. *Macomona liliana*). Suspension feeders feed off deposits from the water whereas deposit feeders feed off organic material incorporated in the sediments, and therefore may be more affected by metals, and may show differences in enzyme activities (Manduzio *et al*., 2004; Phillips and Rainbow, 1989).

A number of steps were taken in the analysis of biomarkers to ensure comparable results during the study period. All cockles were processed following a strict operating procedure; this included freezing them immediately on dry ice while in the field, and then transferring cockles to a -80ºC freezer on return to the lab where they remained at until biomarker analysis. Individual cockles were ground in the same manner and processed as quickly as possible to minimise thawing. Rather than using specific tissues whole cockles were analysed to minimise thawing and enzyme degradation that might have resulted from lengthy dissections. All frozen whole and ground cockles were stored in the same clean plastic zip lock bags (when whole) and the same glass containers (when ground) to ensure there were no differences in storage conditions that could influence biomarker results. When measuring cockle tissues for biomarker enzyme activities sample months were picked from the freezer at random, to minimise the effect of the day of analysis as a factor influencing results. The three antioxidant enzymes were always measured in the same order starting with GR followed by GPx then GST and were all analysed on the same day. To minimise inter-individual variability, tissue from approximately 8-10 cockles from each site was pooled for one replicate sample, and four replicates from each site were analysed per month. This maximised the likelihood of obtaining a measurement of enzymatic activity that was representative of the cockle population inhabiting the sample site, and this minimised the chance of measurements being confounded by extreme, individual variability. Nonetheless, enzyme degradation is one issue that could have occurred while samples were stored for a long period of time (e.g. several months), but was not directly quantified in this study. One way to assess whether samples are maintaining their enzyme activities throughout storage would

be to analyse the same sample at various time points over the storage duration and determine whether enzyme activity decreased or maintained its activity.

Most of the studies referred to in Table 1, instead of analysing bivalve whole tissues used specific organs including digestive glands and the gills. It has been shown in some studies (Cossu *et al.,* 1997, Cossu *et al.,* 2002) that using these specific tissues can provide a more sensitive measure (Cossu *et al.,* 1997) of enzyme activities. For example the gills, which are directly exposed to contaminants in the water and can show a more direct response to water quality than total tissue. To use these specific tissues, they first need to be isolated by dissection. The drawback of this is that this process would invariably lead to thawing of samples which could have an influence on enzyme activities. Studies (Cossu *et al.,* 1997; Cossu *et al.,* 2002*)* have also shown that it depends on the bioavailability of the contaminant and the route of uptake to the animal that will influence biomarker tissue specific results. For example contaminants could enter via consumption therefore measuring the digestive glands may be more sensitive, whereas contaminants in the water may filter through the gills making the gills a more sensitive measure (Cossu *et al.,* 2002).

4.5 Future recommendations

To be effective and robust, environmental monitoring programmes should incorporate a variety of measures of biological integrity including; ecosystem, population and taxonomy richness studies, environmental information (including water and sediment data) and analysis of biomarkers, in order to efficiently evaluate the health of ecosystems. A suite of biomarkers should be used, as it is

generally an assortment of multiple factors influencing the quality of an environment (Vassuer and Cossu-Leguille, 2003). Different biomarkers can be more sensitive to different xenobiotics therefore measuring a collection of biomarkers will likely give a better indication of the probable factors influencing ecosystem health. A number of other biomarkers (besides the ones outlined in this study) have shown promise over the last decade including binding proteins such as metallothioneins, heat-shock proteins that respond to stress and detoxification enzymes for example the Cytochrome P-450 system (Sheehan and Power, 1999). Metallothioneins give a good measure of the availability of metals in the environment as they covalently bind metals, hence blocking their reactivity. A variety of additional biomarkers should therefore be measured as well as GST and GPx to better characterise the degree and impact of contaminant exposure, and also to determine the effects the contaminants are having on the studied organism. By using a multimarker approach, the most appropriate biomarkers can be determined and used in future monitoring studies. Any study of biomarkers will need to include a quantification of their seasonal variability as it has been shown in the present study and in a number of others (Vidal *et al*., 2002, Manduzio *et al*., 2004, Leinio and Lehtonen, 2005, Regoli., 1997, Bocchetti *et al*., 2008, and Verlecar *et al*., 2007) that biomarkers do vary seasonally.

4.6 Summary

This study represents the first field study of its kind to measure three enzymes involved in the cellular glutathione defence system in the cockle (*Austrovenus stutchburyi*), over a one year period in a New Zealand estuary. In this study seasonal variations occurred in the activities of glutathione peroxidase (GPx) and

glutathione S-transferase (GST) in the common New Zealand cockle (*Austrovenus stutchburyi*). GPx and GST levels greatly increased from January onwards (until April 2008). The increase was more pronounced at Glendowie (the least metal contaminated site) for both GPx and GST. At Panmure GST activity also increased from January onwards, whereas at Tiraumea there were less noticeable changes. The seasonal variation recorded may be due to a number of abiotic and biotic variables (previously mentioned) that vary with the season. However silt-clay fraction was the only measured environmental co-variable to show a correlation with enzyme activities (GPX and GST), therefore it is likely that the silt-clay fraction of the sediments is having some influence on enzyme activities in cockles. One other possibility is that the seasonal variation observed may be due to some unknown and unmeasured environmental variable, such as petroleum contaminations. The activity of GR did not vary seasonally or show differences among sites.

The three study sites showed a gradient in levels of sediment metal concentrations (Zn, Cu, & Pb). Glendowie was the least metal contaminated, followed by Panmure, and then Tiraumea with the highest concentrations of metals. There was a relationship between the concentrations of contaminants and the activity of GPx including Pb sediment concentration ($r = -0.561$), Cu sediment concentration ($r = -0.539$) and Cu tissue concentration ($r = -0.530$). Therefore it could be concluded that, the lower the concentration of metal contaminants the higher the GPx activity tends to be. Although the activity of GST was also higher at Glendowie, only significant negative correlations with metal concentrations were observed for GPx.

In conclusion by measuring a variety of enzymes this study showed that GR showed little use as a biomarker of diminished health. However both GST and GPx showed promise, as they both showed consistent differences among sites (with cockles from more pristine/less impacted sites generally having higher enzyme activities and condition index), and for GPx; this could be related to the extent of metal concentration. Both GST and GPx varied seasonally with elevated activities occurring during the summer months. To minimise seasonal variation in GST and GPX activity in cockles, it is recommended that these biomarkers be analysed in autumn-spring.

5 References

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