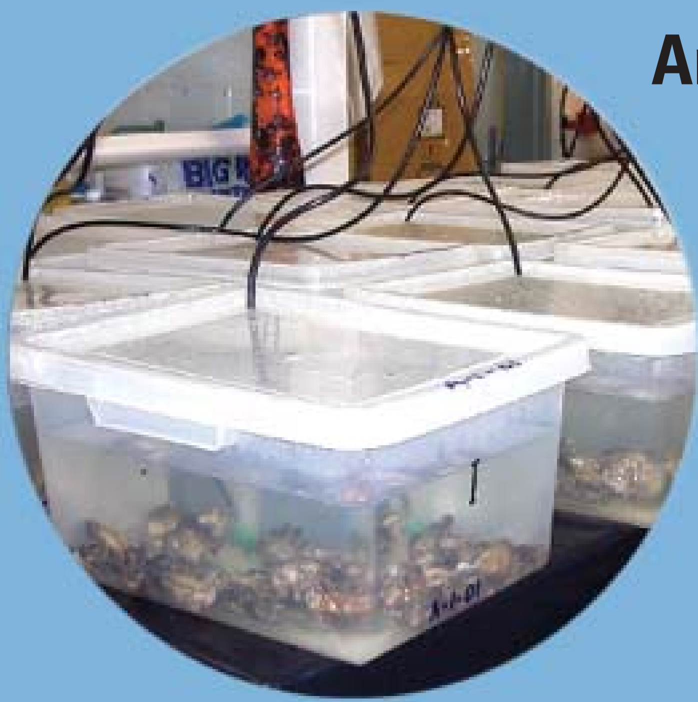
Cooperative Research Centre for Coastal Zone, Estuary & Waterway Management

Technical Report 70



Antioxidant enzymes as biomarkers of environmental stress in oysters in Port Curtis

L. Andersen, W.H.L. Siu, E.W.K. Ching, C.T. Kwok, F. Melville, C. Plummer, A. Storey and P.K.S. Lam

May 2006

香港城市大學 City University of Hong Kong

The University of Western Australia

Central Queensland University Where Students Come First.

CRC for Coastal Zone Estuary & Waterway Management

Antioxidant enzymes as biomarkers of environmental stress in oysters in Port Curtis

Leonie Andersen[1], William H.L. Siu[2], Eric W.K. Ching[2], C.T. Kwok[2], Felicity Melville[1], Clayton Plummer[1], Andrew Storey[3] and Paul K.S. Lam[2]

[1]Centre for Environmental Management, Central Queensland University (l.andersen@cqu.edu.au)
[2]Centre for Coastal Pollution and Conservation, City University of Hong Kong
[3]School of Animal Biology (M092), University of Western Australia

May 2006

CRC for Coastal Zone Estuary & Waterway Management

Antioxidant enzymes as biomarkers of environmental stress in oysters in Port Curtis

Copyright © 2006:
Cooperative Research Centre for Coastal Zone, Estuary and Waterway Management

Written by:

Leonie Andersen
William H.L. Siu
Eric W.K. Ching
C.T. Kwok
Felicity Melville
Clayton Plummer
Andrew Storey
Paul K.S. Lam

Published by the Cooperative Research Centre for Coastal Zone, Estuary
and Waterway Management (Coastal CRC)

Indooroopilly Sciences Centre
80 Meiers Road
Indooroopilly Qld 4068
Australia

www.coastal.crc.org.au

The text of this publication may be copied and distributed for research and educational purposes with proper acknowledgment. Photos cannot be reproduced without permission of the copyright holder.

Disclaimer: The information in this report was current at the time of publication. While the report was prepared with care by the authors, the Coastal CRC and its partner organisations accept no liability for any matters arising from its contents.

National Library of Australia Cataloguing-in-Publication data
Antioxidant enzymes as biomarkers of environmental stress in oysters in Port Curtis

QNRM06143
ISBN 1 921017 24 4 (print)
ISBN 1 921017 25 2 (online)

Executive summary

Background and rationale

The estuarine embayment of Port Curtis is Queensland's largest multi-cargo port and the fifth largest port in Australia. It supports major industries in the Gladstone area including the world's largest alumina refinery and Australia's largest aluminium smelter. Because the estuary is one of the Coastal CRC's three key study areas, research by the CRC contaminants team focused firstly on identifying contaminants of concern in a screening-level risk assessment. Although enrichment of some metals in marine organisms was recorded, subsequent projects focused on assessment of organism health to determine if environmental harm had occurred. There was a need to demonstrate a relationship between exposure to a contaminant and an adverse ecological effect.

The objective of the current study was to examine the use of biomarkers as a measure of pollution-induced 'stress' in oysters (*Saccostrea glomerata*) transplanted into Port Curtis from a clean area. Biomarkers are defined as a biological response that can be related to exposure to an environmental contaminant. In a broad context they can include measuring such endpoints as reproduction and growth, or behavioural changes; however, the biomarkers chosen in this study measured effects at a cellular level. Exposure to pollutants causes the production of potent oxidants and free radicals capable of damaging important cell components such as proteins and DNA. In response, the cell initiates antioxidant enzyme systems and produces free radical scavengers in order to prevent cellular injury and maintain cell homeostasis. The induced biomarker response can then be measured and related to measured concentrations of the contaminant the oyster is exposed to.

Methods

The study assessed a number of biomarker responses including antioxidant enzymes [catalase (CAT) and glutathione-s-transferase (GST)], a free radical scavenger [glutathione (GSH)] and a measurement of cell damage [lipid peroxidation (LPO)] in both gill tissue and digestive glands of oysters, in relation to exposure to metals in both field and laboratory conditions. An intensive sampling strategy was enlisted in order to identify initial transient changes in biomarker responses during the exposure periods.

In the field study, oysters from an oyster lease from a clean area were deployed at two sites: an impacted site in the inner harbour area of Port Curtis, and an oceanic reference site outside Port Curtis. Subsamples of oysters were collected over a 29-day period and biomarker and metal concentrations in the tissues measured on each occasion. In the laboratory experiment, oysters were exposed to five concentrations of copper ranging from background sea water (controls) up to the addition of 30 µg/L of copper for 21 days, followed by a clean-water purging phase of seven days. A similar strategy of sub sampling of oysters for measurements of biomarker and copper concentrations occurred as in the field study.

Results and discussion

Patterns of metal accumulation in field oysters were similar to that observed in other deployment studies conducted in Port Curtis. Copper, zinc and to a lesser extent aluminium were the three main metals identified as having accumulated to a greater degree in Site 1 (impact) oysters compared to those at Site 2 (reference), although there was an overall decline from baseline at both sites for aluminium. Other metals displayed few convincing trends. There were greater accumulations of arsenic at the reference site but this does not necessarily imply contamination, and may indicate antagonism for uptake of arsenic at Site 1 in the presence of elevated copper and zinc. Metal accumulation in the transplanted oysters showed similar patterns to those of resident oysters collected from the same sites indicating that the oysters deployed over a shorter time period reflected the ambient concentrations of metals at those sites.

In the laboratory bioassay, copper accumulated to a greater degree in the higher treatment groups and depuration or purging of copper was observed when the oysters were returned to untreated sea water. Due to oysters in both the field and the laboratory bioassay starting with the same baseline concentrations of metals, there was a lag period before the oysters at each site or in each copper treatment began to separate in terms of the metals they accumulated. This may have prevented significant differences in copper concentrations between treatment groups.

Biomarker responses were observed in both the field and the laboratory oysters. An initial induction response of LPO, GST and GSH in both tissues at Site 2 (reference) may have been related to exposure to toxins from a harmful algal bloom (*Trichodesmium erythraeum*), which was observed at the oceanic sites. Blue-green algae are known to produce toxins that affect biomarker responses

and this may have been the case at the reference site. Handling or transportation stress may have also had an effect on CAT concentrations, indicating that contaminants are not the only stressors to alter biomarker reactions. It appears that although metals in Port Curtis may induce biomarker responses in oysters, the stress effect may be no more than the oysters would experience from natural stressors present in the environment.

Changes in LPO and CAT concentrations in field oysters were related to a number of metals, namely aluminium, cadmium, chromium, copper, lead and nickel, with the majority of responses occurring at the impact site, although the relationships weren't strong. LPO was more elevated in resident oysters, which had higher metal concentrations, compared with deployed oysters. Increase in LPO in both tissues indicates some cell damage may be occurring. Not all metals caused a continual induction of biomarker response with some biomarkers (CAT and GST) declining or levelling out once a certain threshold of metal concentrations was reached. This indicates that either the oyster has acclimated to the new environmental conditions and adaptation has occurred, or a point has been reached where breakdown of the enzyme response has occurred. In the relatively uncontaminated conditions of Port Curtis where dissolved metal concentrations are below regulatory concern, it is likely that oysters have adapted to their new environment.

Copper exposure induced marked biomarker responses in oysters in the laboratory. Initial stimulation of biomarkers—in particular GST and GSH—was followed by a decline in concentrations after a certain exposure period. Similar to the field experiment, this could represent adaptation of the oyster to the new exposure conditions. GSH binds with copper to assist in excretion from the cell and is also used as a substrate in the production of GST. Therefore, a decline in GSH could be expected as the free radical scavenger is 'used up' while protecting the cell from copper-induced damage. The decline in biomarker response was followed by a restimulation of response during the depuration phase for CAT and GST, indicating increase in production of the two enzymes to assist in detoxification or depuration of copper from the cell. Repeated measurements of biomarker responses during exposure or depuration assisted in identifying transient temporal changes in biomarkers in both the field and laboratory conditions.

Causal relationships were identified between biomarker responses and accumulated copper concentrations for GSH and GST. The relationships were stronger as the exposure concentrations increased indicating that more marked

biomarker responses are observed with greater copper accumulations. Therefore greater exposure concentrations caused a more dramatic cellular response. The controlled laboratory environment does not simulate environmental realism where synergistic or antagonistic effects from different metals or other unmeasured contaminants may affect biomarker response.

Conclusions

Significant biomarker responses were evident in both the field and laboratory experiments, although responses were variable. Although lipid peroxidation (LPO) in the field increased as oyster metal concentrations increased, responses were not as dramatic as those recorded for other more polluted environments. After initial stimulation there may also be adaptation or acclimation of biomarker responses to new exposure conditions. Under controlled laboratory conditions, other biomarkers such as glutathione (GSH) and glutathione-s-transferase (GST) exhibited clear, logical responses to copper exposure, the response being greater in the higher exposure groups. The exposure concentrations required to produce a marked response were, however, many times greater than what would be considered as ‘average’ for Port Curtis. Therefore perhaps contaminant effects in Port Curtis are not significant enough to cause detectable changes in this particular suite of biomarker responses.

Biomarker responses in the field were also observed at the reference site and may have been induced by natural stressors. This indicates that ‘stress’ caused by accumulation of metals in Port Curtis may not be any more detrimental than ‘stress’ caused by natural ecological events. Enrichment of metals in the biota of Port Curtis may not necessarily be causing environmental harm. However, it is important to understand the details of temporal changes of biological responses and therefore recognise the limitations of the use of biomarkers in biomonitoring programs. Other bioindicator species would need to be assessed before a firm conclusion could be drawn on the ecological health of Port Curtis organisms.

Table of contents

1. Introduction 1

1.1. Background 1
1.2. Oysters in environmental monitoring 3
1.3. Use of biomarkers in environmental monitoring 4
1.4. Common biomarkers used in environmental monitoring 5
1.5. Objectives of study 6

2. Methodology 9

2.1. Field studies 9
2.2. Laboratory bioassay 11
2.3. Oyster analysis 13
2.3.1. Biomarker analysis 13
2.3.2. Metal analysis 13
2.4. Statistical analysis 14
2.4.1. General 14
2.4.2. Field oyster metal concentrations 14
2.4.3. Field oyster biomarker concentrations 15
2.4.4. Field oyster metal and enzyme concentration comparisons 16
2.4.5. Laboratory oyster copper concentrations 16
2.4.6. Laboratory oyster biomarker concentrations 16
2.4.7. Laboratory oyster metal and enzyme concentration comparison 17

3. Results 19

3.1. Field results 19
3.1.1. Physicochemical properties 19
3.1.2. Oyster metal concentrations 19
3.1.3. Oyster biomarker concentrations 26
3.1.4. Comparison of metal and biomarker concentrations 34
3.2. Laboratory bioassay 44
3.2.1. Oyster copper concentrations 44
3.2.2. Oyster enzyme concentrations 47
3.2.3. Comparison of copper and enzyme concentrations 60

4. Discussion 63

4.1. Oyster metal accumulation 63
4.1.1. Field study 63
4.1.2. Laboratory copper bioassay 64
4.2. Biomarker responses to metal concentrations 65
4.2.1. Field study 65
4.2.2. Laboratory bioassay 67
4.3. Use of biomarkers in oysters 70

5. References 71

6. Appendixes 77

List of figures

Figure 1. Location of Sites 1 and 2 for field experiments in Port Curtis harbour. 2

Figure. 2. Generalized scheme depicting relationships between cellular responses and higher level effects (adapted from Ringwood *et al* (1999). 5

Figure 3. Mean ±1SE concentration of (a) Cu and b) Zn in oysters from Site 1 and Site 2 over time (29 days deployment) including baseline concentrations.............................. 22

Figure 4. Mean ±1SE concentration of (a) As and (b) Cd in oysters from Site 1 and Site 2 over time (29 days deployment) including baseline concentrations.............................. 23

Figure 5. Mean ±1SE concentration of (a) Pb and (b) Al in oysters from Site 1 and Site 2 over time (29 days deployment) including baseline concentrations.............................. 24

Figure 6. Mean ±1SE concentration of (a) Cr and (b) Ni in oysters from Site 1 and Site 2 over time (29 days deployment) including baseline concentrations.............................. 25

Figure 7. Mean ±1SE concentration (µmol/g) of CAT in (a) gill and (b) hepatopancreas in oysters from Site 1 and Site 2 over time (29 days deployment) including baseline concentrations. .... **Error! Bookmark not defined.**

Figure 8. Mean ±1SE concentration (µmol/g) of LPO in (a) gill and (b) hepatopancreas in oysters from Site 1 and Site 2 over time (29 days deployment) including baseline concentrations. 31

Figure 9. Mean ±1SE concentration (µmol/g) of GST in (a) gill and (b) hepatopancreas in oysters from Site 1 and Site 2 over time (29 days deployment) including baseline concentrations. 32

Figure 10. Mean ±1SE concentration (µmol/g) of GSH in (a) gill and (b) hepatopancreas in oysters from Site 1 and Site 2 over time (29 days deployment) including baseline concentrations. 33

Figure 11. Regression of mean CAT activity over time at Site 1 and 2.................................. 34

Figure 12. Regression of mean CAT concentration against mean oyster (a) copper and (b) zinc concentrations at Site 1 and 2 37

Figure 13. Regression of mean CAT concentration against mean oyster (a) lead and (b) aluminium concentrations at Site 1 and 2 34

Figure 14. Regression of mean CAT concentration against mean oyster (a) chromium and (b) nickel concentrations at Site 1 and 2 39

Figure 15. Regression of mean CAT concentration against mean oyster cadmium concentrations at Site 1 and 2 40

Figure 16. Regression of mean LPO concentration against mean oyster cadmium concentrations at Site 1 and 2. 40

Figure 17. Regression of mean LPO concentration against mean oyster (a) lead and (b) aluminium concentrations at Site 1 and 2 41

Figure 18. Regression of mean LPO concentration against mean oyster (a) chromium and (b) nickel concentrations at Site 1 and 2 42

Figure 19. Regression of mean GST concentration against mean oyster (a) chromium and (b) aluminium concentrations at Site 1 and 2 43

Figure 20. Regression of mean GST concentration against mean oyster cadmium concentrations at Site 1 and 2 44

Figure 21. Accumulation in copper exposed oysters from the five treatment concentrations 46

Figure 22. Mean ±1 SE concentration (μmol/g) of CAT in (a) gill and (b) hepatopancreas in oysters in the five spiked treatments (0, 3.75, 7.5, 15 and 30 μg/L) including baseline concentrations. 51

Figure 23. Mean ±1 SE concentration (μmol/g) of LPO in (a) gill and (b) hepatopancreas in oysters in the five spiked treatments (0, 3.75, 7.5, 15 and 30 μg/L) including baseline concentrations. 52

Figure 24. Mean ±1 SE concentration (μmol/g) of GST in (a) gill and (b) hepatopancreas in oysters in the five spiked treatments (0, 3.75, 7.5, 15 and 30 μg/L) including baseline concentrations. 53

Figure 25. Mean ±1 SE concentration (μmol/g) of GSH in (a) gill and (b) hepatopancreas in oysters in the five spiked treatments (0, 3.75, 7.5, 15 and 30 μg/L) including baseline concentrations 54

Figure 26. Regression of mean GSH concentration in gills against time (a) 23 days and (b) 28 days in each treatment (0, 3.75, 7.5, 15 and 30 μg/L) including baseline 56

Figure 27. Regression of mean GSH concentration in hepatopancreas against time (a) 23 days and (b) 28 days in each treatment (0, 3.75, 7.5, 15 and 30 μg/L) including baseline 57

Figure 28. Regression of mean GST concentration in hepatopancreas against time (a) 23 days and (b) 28 days in each treatment (0, 3.75, 7.5, 15 and 30 μg/L) including baseline 58

Figure 29. Regression of mean GST concentration in gill against time 28 days in each treatment (0, 3.75, 7.5, 15 and 30 μg/L) including baseline 59

Figure 30. Regression of mean LPO concentration in gill against time 28 days in each treatment (0, 3.75, 7.5, 15 and 30 μg/L) including baseline 59

Figure 31. Regression of mean GSH concentration in (a) gills and GST in (b) hepatopancreas against oyster Cu concentrations after 23 days in each treatment (0, 3.75, 7.5, 15 and 30 μg/L) including baseline. 61

Figure 32. Regression of mean GSH concentration in gills against oyster Cu concentrations after 28 days in each treatment (0, 3.75, 7.5, 15 and 30 μg/L) including baseline 62

List of tables

Table 1. Mean ±1 SE physicochemical properties of water sampled at Site 1 and 2 on each collection over the deployment period. 19

Table 2. Summary of two-way ANOVAs on concentrations of each metal in oysters by site (Site 1 and Site 2) and time (baseline to collection eight) 20

Table 3. Mean ±1 SE concentration (µg/g dry wgt) of metals in oysters at Sites 1 and 2 throughout the deployment period 21

Table 4. One-way ANOVA comparing biomarker concentrations in oysters (a) within hours of collection from the lease, (b) baseline oysters prior to deployment or allocation to acclimation facilities and (c) at seven days post-acclimation prior to the beginning the bioassay 26

Table 5. Concentrations (µmol/g) of antioxidant enzymes in oysters including residents at Sites 1 and 2 throughout the deployment period 27

Table 6. Summary of two-way ANOVAs on concentrations of each enzyme in oyster tissues (gill and hepatopancreas) by site (Site 1 and Site 2) and time (baseline to collection eight) 29

Table 7. Pearson product moment correlations between metal concentrations and enzyme concentrations in gills and hepatopancreas of oysters in Sites 1 and 2 35

Table 8. Oyster copper concentrations in copper spiked treatments over the bioassay period. 45

Table 9. Summary of two-way ANOVAs on concentrations of copper in oysters by treatment (1= control to 5 = 30 µg/L) and time (baseline to 28 days includes depuration) 45

Table 10. Concentration of biomarkers in gill and hepatopancreas of copper exposed oysters in the five spiked treatments (0, 3.75, 7.5, 15 and 30 µg/L) including baseline concentrations. 48

Table 11. Summary of two-way ANOVAs on concentrations of each enzyme in oyster tissues (gill and hepatopancreas) by treatment (1= control to 5 = 30 µg/L) and time (baseline to collection seven includes depuration) 50

Table 12. Correlations between copper concentrations and biomarker concentrations in gills and hepatopancreas of oysters after 23 days of exposure and 28 days which included the depuration phase 60

List of photographs

Photograph 1. Unopened oyster Saccostrea glomerata (inset) and shell removed (main picture). 10

Photograph 2. Individual bags of oysters attached to buoys ready for deployment. 10

Photograph 3. Oysters in treatment tanks in copper bioassay. 12

1. Introduction

1.1. Background

Port Curtis is located just south of the tropic of Capricorn on the east coast of Queensland, Australia. The port is part of a composite estuarine system comprising the Calliope and Boyne Rivers, which merge with deeper waters to form a deep estuarine embayment, protected by Curtis and Facing Islands (Figure 1). The area is adjacent to the World Heritage listed Great Barrier Reef Marine Park and is home to extensive mining and chemical industry as well as supporting a large commercial and recreational fishing industry. Port Curtis is Queensland's largest port, shipping over 8% of Australia's exports, including coal, alumina, aluminium, cement, woodchip and chemicals (Central Queensland Ports Authority, 2005). Industry in the area includes the world's largest alumina refinery (Queensland Alumina Ltd), Australia's largest aluminium smelter (Boyne Smelters Ltd), a coal-fired power station (NRG), the largest cement kiln in Australia (Queensland Cement) and a chemical plant producing sodium cyanide, ammonium nitrate and chlorine (Orica) (Central Queensland Ports Authority, 2005).

The Port Curtis estuary is one of the three key areas studied by the Coastal CRC. In a screening-level risk assessment of contaminants in Port Curtis, Apte *et al.* (2005) found that concentrations of metals in sediments and dissolved metals in the waters were generally below levels of regulatory concern, but that concentrations of a variety of metals were significantly enriched in marine biota in comparison with organisms sampled at reference sites. Studies prior to this had flagged concentrations of some metals; in particular copper and zinc in mud crabs (Andersen & Norton, 2001) and copper in seagrass (Prange, 1999) and fiddler crabs (Andersen *et al.*, 2002), as potentially anomalous in Port Curtis relative to background levels. A subsequent study by the Coastal CRC of metal bioaccumulation through foodweb pathways (Andersen *et al.*, 2005a) confirmed enrichment of metals in a variety of inner harbour organisms, which was most likely related to the retention time of water in the inner harbour area.

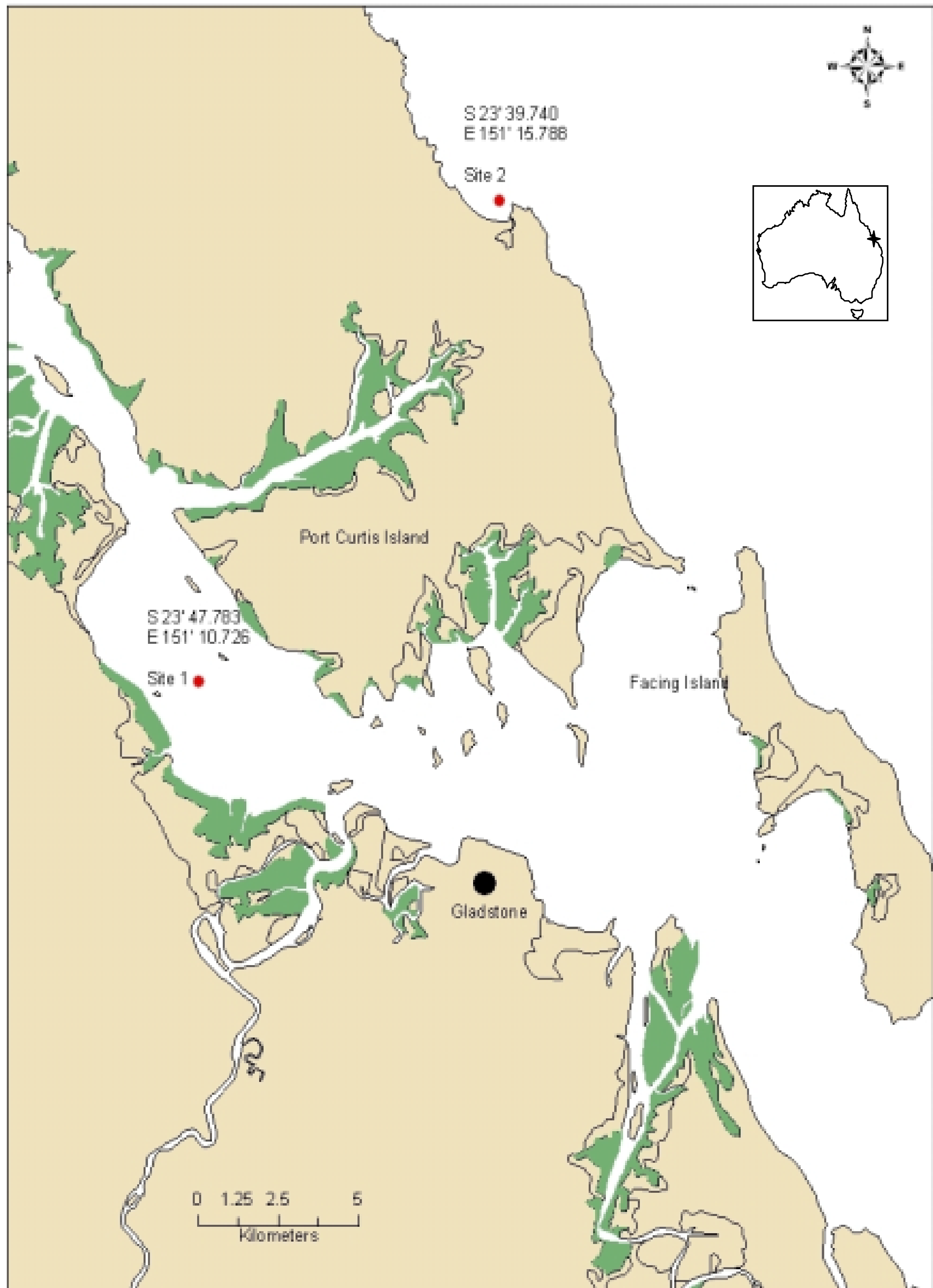


Figure 1. Location of Sites 1 and 2 for field experiments in Port Curtis harbour

However, the demonstration of bioaccumulation of a contaminant does not necessarily mean that environmental harm has occurred. There is a need to demonstrate a link between exposure to and/or accumulation of a contaminant and an adverse, sublethal, biological response. One of the recommendations made by Apte *et al.* (2005) was that the ecological health of metal-enriched organisms should be further studied. Suggestions for assessments included measuring the incidence of imposex in gastropods as a bioindicator of tributyltin (TBT) contamination and the analysis of sublethal stress indicators such as enzyme biomarkers as a response to metal exposure. Subsequently, an imposex survey of over 1000 mulberry whelks (*Morula marginalba*) in Port Curtis was conducted, which determined that the prevalence of imposex was related to shipping intensity, with a decreasing gradient of the number of affected snails from inner to outer harbour (Andersen, 2004). The study therefore demonstrated a relationship between exposure to a contaminant and the production of a sublethal response.

The objective of the current study was to examine the use and suitability of antioxidant enzymes and enzyme systems as biomarkers of metal stress in the Sydney rock oyster (*Saccostrea glomerata*) in order to link metal bioaccumulation with a biological response in Port Curtis. Other contaminants such as fluoride, cyanide, polycyclic aromatic hydrocarbons (PAHs) and tributyltin (TBT) were flagged by Apte *et al.* (2005) as potential chemical stressors in the harbour and therefore could also exert a biomarker response in the field. However, the report highlighted metal enrichment in biota and recommended that the ecological health of organisms be investigated. The research examined biomarkers both in a field environment (Port Curtis) and in a controlled laboratory bioassay in response to metal exposure. A repeated sampling over time approach was adopted, which allowed the detection of immediate or intermittent short term responses that may not persist over the entire exposure period.

1.2. Oysters in environmental monitoring

Within any aquatic system, both water and sediment can be analysed to quantify contaminant concentrations. However, there are inherent problems associated with the analysis of both media (Rainbow, 1995). Contaminant concentrations in water are typically low, often below detection limits, and can vary greatly over time and space (Villares *et al.*, 2001). Contaminants accumulate in sediments, and so are easy to measure and can provide a degree of time integration not found in water analysis (Rainbow & Phillips, 1993). However, both in sediments

and in water, contaminant concentrations determined by chemical analysis cannot be reliably used to assess the likely toxicity of contaminants to biota (Rainbow, 1995). Aquatic organisms have, therefore, become increasingly used in the assessment of contamination (Melville, 2005).

Oysters can accumulate many contaminants in their tissues, concentrations of which can then be measured to provide a time-integrated estimate of bioavailable contaminant concentrations (Cruz-Rodriguez & Chu, 2002). Oysters are suspension feeders and take up metals both directly from sea water and from suspended particles collected during feeding (Rainbow, 1995). Dissolved metals (dissolved ions and colloidal particles) are taken up through the gills (Laodong *et al.*, 2002); however, dietary sources (which can include phytoplankton and resuspended sediment particles) tend to account for a large proportion of the metal intake by oysters (Olivier *et al.*, 2002; Andersen *et al.*, 2005a).

Due to their ability to accumulate contaminants, oysters have been successfully used as biomonitors in many pollution assessment studies in Port Curtis (Andersen *et al.*, 2003; Andersen *et al.*, 2004; Andersen *et al.*, 2005b) and elsewhere (Odzak *et al.*, 2001). The field study component of this project involved the deployment of oysters transplanted from a non-contaminated area. The use of transplanted oysters has several advantages and has been used successfully in several previous studies (Curran *et al.*, 1986; Chan *et al.*, 1999) including those in Port Curtis mentioned previously. Oysters can be introduced into an area where they may not have been previously abundant, serving to control site selection and increase the number of sampling sites. The number of samples available for analysis can also be increased, thereby placing no limitations on the proposed scope of analyses. Confounding variables such as size and age can be eliminated, and background exposure levels can be assured, through the use of oysters of an even age from leases in non-impacted areas (Andersen *et al.*, 2005b).

1.3. Use of biomarkers in environmental monitoring

The response of an organism to pollution can be measured at several different levels (Figure 2). At a community level, adverse effects of pollution may result in a loss of species richness or evenness (Courtney & Clements, 2002; Melville, 2005), while at a population level, pollution may result in the loss of sensitive organisms, resulting in a restricted gene pool (Muyssen & Janssen, 2001; Melville & Burchett, 2002; Melville *et al.*, 2004). Many studies examine the responses of organisms to contamination, using the endpoints of reduced growth or

reproduction (Wright & Welbourne, 2002). Biomarkers are biochemical, physiological or histological changes that measure effects of, or exposure to, toxic chemicals (Weeks, 1995; Luebke *et al.*, 1997), and generally but not exclusively pertain to a response at a specific organ, cellular or subcellular level of organisation (O'Halloran *et al.*, 1998), measuring biochemical endpoints (Bresler *et al.*, 1999; Cruz-Rodriguez & Chu, 2002). These cellular and molecular responses can be used as early warning signals of environmental stress, before whole-organism effects become apparent (Regoli *et al.*, 1998).

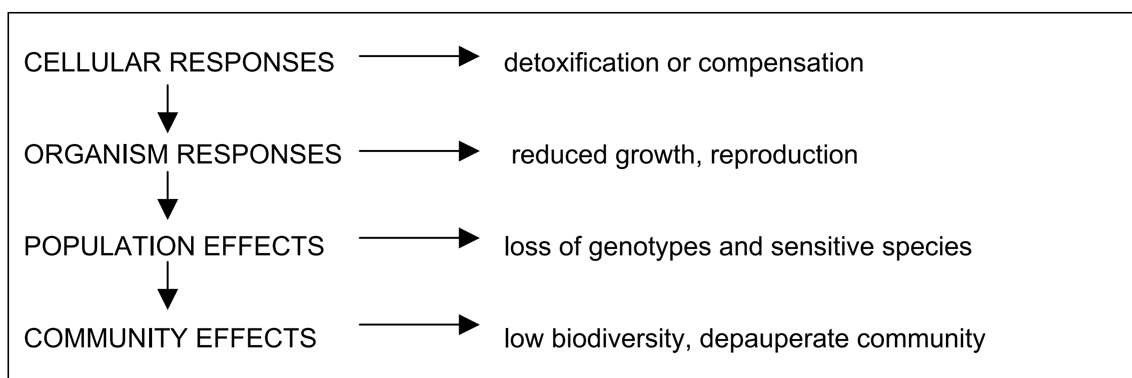


Figure 2. Generalised scheme depicting relationships between cellular responses and higher level effects (adapted from Ringwood *et al.*, 1999)

The exposure of bivalves to high environmental levels of metals can induce synthesis of biomarker responses (Irato *et al.*, 2003). Biomarkers are being increasingly recognised as accurate and cost-effective methods for identifying the *in situ* toxic effects of pollutants on biota (Winston & Giulio, 1991; Brown *et al.*, 2004). Bivalves have also been successfully used in biomarkers studies, showing significant variation in a range of biochemical markers, in both gill and digestive gland tissues (Cheung *et al.*, 2001; Cheung *et al.*, 2002; Irato *et al.*, 2003).

1.4. Common biomarkers used in environmental monitoring

Environmental pollutants generally cause an increase in peroxidative processes within cells, causing oxidative stress (Winston & Giulio, 1991; Cheung *et al.*, 2001; Nusetti *et al.*, 2001). Hydroxyl radicals are produced in electron transfer reactions, and are potent oxidants capable of damaging important cell components, such as proteins and DNA (Doyotte *et al.*, 1997; Cheung *et al.*, 2001). Lipid peroxidation (LPO) has often been used as a biomarker of environmental stress, reflecting damage to cell membranes from free radicals (Ringwood *et al.*, 1999) and is an important feature in cellular injury (Reddy, 1997). The extent of damage caused by oxyradical production is dependent on antioxidant defences, which include antioxidant enzymes and free radical

scavengers, such as glutathione (Doyotte *et al.*, 1997). Therefore, antioxidant enzymes are some of the most common biomarkers used in environmental monitoring (Regoli *et al.*, 1998). The enzymes usually respond rapidly and sensitively to biologically active pollutants (Fitzpatrick *et al.*, 1997).

Some of the most commonly used antioxidant enzyme biomarkers include catalase and glutathione-s-transferase. Catalase (CAT, EC 1.11.1.6) is induced by the production of hydrogen peroxide in the cells and catalyses the reaction, which reduces this compound to water and oxygen (Winston & Giulio, 1991; Regoli & Principato, 1995; Regoli *et al.*, 1998). Glutathione-s-transferase (GST, EC 2.5.1.18) catalyses the conjugation of a large variety of xenobiotics containing electrophilic centres to reduced glutathione (Regoli & Principato, 1995; Sharma *et al.*, 1997). Concentrations of this enzyme have been found to increase with exposure to contaminants (Fitzpatrick *et al.*, 1997).

Glutathione (GSH) is often used in biomarker studies, as it is an overall modulator of cellular homeostasis (Ringwood *et al.*, 1999). Glutathione (GSH) is a low molecular weight scavenger of oxygen radicals (Regoli *et al.*, 1998). The reduced form conjugates with electrophilic xenobiotics transforming them into water-soluble and thus easily excretable products (Nusetti *et al.*, 2001). Often, GSH concentrations have been found to be depleted in contaminant-exposed organisms (Regoli *et al.*, 1998).

Using a combination of biomarkers as this study does, including antioxidant enzymes (CAT and GST), free radical scavengers (GSH) and measurements of peroxidative processes (LPO) in both a field and laboratory situation, ensures that all aspects of the biochemical effects of metal exposure are being assessed.

1.5. Objectives of study

The major objective of the study was to determine whether selected biomarkers can be used as bioindicators of metal-induced stress in oysters, both in the field and in the laboratory environment. Copper was selected for the laboratory exposures because, in addition to being identified as a contaminant of concern in Port Curtis (Andersen & Norton, 2001; Andersen *et al.*, 2005a), the metal had been shown to induce strong biomarker responses in other studies (Regoli & Principato, 1995; Doyotte *et al.*, 1997; Regoli *et al.*, 1998; Brown *et al.*, 2004). Since the gills are the first point of contact for metal exposure and digestive gland is an important organ for which metals are known to sequester, these tissues were chosen to measure biomarker responses (Andersen, 2003). The specific aims of this research were to:

- Determine whether biomarkers in oysters deployed in two sites (an impact and a reference site) in Port Curtis can be correlated to bioaccumulated metal concentrations
- Investigate the response of selected biomarkers to sublethal concentrations of copper in dose-response laboratory bioassays
- Determine the temporal response of biomarkers to metal exposure through an intensive sub sampling program in both the field and the laboratory in order to identify induction, inhibition or adaptation of biomarker responses
- Evaluate the potential of selected biomarkers as bioindicators of environmental stress in oysters
- Assess the health of Port Curtis harbour through biomarker studies.

The use of both field and laboratory experiments in this study followed the recommended approach used in environmental risk assessments (Pascoe *et al.*, 1994). The laboratory experiment was used to establish clear cause-effect relationships without any confounding variables often found in the field environment, and without the presence of unknown mixtures of contaminants. The field experiment was used to establish whether the selected biomarkers could be used successfully in realistic environmental monitoring situations.

2. Methodology

2.1. Field studies

The field component of the research involved the examination of concentrations of biomarkers (CAT, LPO, GSH and GST) and metal concentrations in oysters deployed at two sites; one in the inner harbour area and the other outside of Port Curtis. Both sites have been monitored previously or are currently monitored for other research in Port Curtis. Site 1 is considered an impacted site located adjacent to the Fisherman's Landing trade waste effluent outfall (refer to Figure 1) where metal bioaccumulation has been demonstrated (Andersen *et al.*, 2005b). Site 2 is relatively pristine, located on the oceanic side of Curtis Island. Previous studies indicate metal bioaccumulation in this area to be low and dissolved metal concentrations are likely to be similar to background oceanic levels (Andersen *et al.*, 2005a).

The oysters used in the experiments (*Saccostrea glomerata*) (Photograph 1) were obtained from a commercial lease located in Moreton Bay, Queensland. Baseline metal concentrations of oysters from the lease are considered relatively low (Andersen *et al.*, 2003; Andersen *et al.*, 2004; Andersen *et al.*, 2005b). Oysters were deployed in a series of mesh bags (18 oysters per bag), with seven bags deployed per site. The bags were attached approximately 0.5 m below the water surface, to anchored buoys (Photograph 2). One bag was collected from each site twice weekly for two weeks, and then weekly for the following two weeks (collection days at 3, 5, 8, 12, 15, 22 and 29 days) in a similar sampling strategy to the laboratory bioassay, with inclement weather preventing the strategies from being exactly the same. Ten of the retrieved oysters were used for biomarker analysis [gill (n=10) and hepatopancreas (n=10) tissues], six oysters (two oysters pooled to form one composite) underwent metal analysis [whole soft tissue (n=3)] to quantify metal concentrations in oyster tissues and two oysters were kept in reserve.

Photograph 1. Unopened oyster *Saccostrea glomerata* (inset) and shell removed (main picture)

Photograph 2. Individual bags of oysters attached to buoys ready for deployment

The same number of oysters from the lease were analysed for enzyme and metal concentrations prior to deployment in order to determine baseline concentrations prior to deployment. On one occasion the same number of resident oysters from both sites were collected from adjacent rocks for both biomarker and metal concentrations. Resident oysters from Site 1 were identified as the same species of oysters as those from the lease (*Saccostrea glomerata*); however the dominant oyster sampled at Site 2 was a different, but closely related, species (*Saccostrea cucullata*). Oysters were processed as soon as practicable after retrieval. Physicochemical parameters were also measured at each site at the time of each collection.

2.2. Laboratory bioassay

The laboratory bioassay was undertaken in order to determine the effects of serial diluted Cu concentrations on biomarker responses (CAT, LPO, GSH and GST), without the confounding variables found in the field environment and consisted of an exposure phase (21 days) and depuration phase (7 days).

Prior to exposure, oysters from the same source as the transplanted field oysters were scrubbed to remove epiphytes and then randomly distributed to aerated treatment tanks containing 10 L filtered sea water for a seven-day acclimation period (Photograph 3). Oysters were maintained at 25°C, with a 12:12 light:dark cycle and fed three times a week with 200 mL of cultured marine algae, *Nanochloropsis occulata.* Subsamples of ten oysters were used for biomarker analysis [gill (n=10) and hepatopancreas (n=10) tissues] and six oysters (two oysters pooled to form one composite) underwent metal analysis [whole soft tissue (n=3)] to provide post-acclimation baseline biomarker concentrations as per the field study.

The bioassay used 10 L filtered sea water (background Cu concentrations: ~3 µg/L) spiked with the addition of Cu (stock 20 mg/L prepared from copper sulphate, $CuSO_4.5H_2O$, Merck Pty Ltd, Victoria, Australia and Milli-RO® deionised water) at concentrations of 3.75, 7.5, 15 and 30 µg/L, in addition to a filtered seawater control. Concentrations were chosen to represent environmentally realistic concentrations (concentrations 2 and 3) as well as concentrations large enough to induce a biomarker response (concentrations 4 and 5). Therefore the designated concentrations were desired or nominal concentrations of Cu and were in addition to background Cu concentrations in the filtered sea water containing the algal food. Water total Cu concentrations were measured weekly from two randomly selected tanks within each treatment on three occasions during the exposure phase and on two occasions during the depuration phase (no added Cu) in order to determine actual concentrations of Cu in each treatment over the experimental period. Results of analyses were extremely variable, however, with large deviations from nominal in some tanks. There were some questions arising as to the validity of the inconsistent results, with the laboratory having difficulty in verifying accuracy below 5 µg/L. Therefore the results are not reported here.

Photograph 3. Oysters in treatment tanks in copper bioassay

Standard toxicity test protocols (Stauber *et al.*, 1994; USEPA, 2000) were followed, with physicochemical parameters measured in each treatment tank weekly, during both the exposure and depuration phases. Treatment water was renewed three times weekly, and 200 mL of cultured marine algae, *Nanochloropsis occulata*, was added at each water change to feed oysters. Copper spiking ceased on day 21, in order to allow a period of depuration. The bioassay was run at 25°C, with a 12:12 light: dark cycle and each tank aerated. Within each of the five replicate tanks for each control and copper treatment (25 tanks in total), a minimum of 26 oysters were deployed.

At each of the sampling days (2, 5, 8, 12, 15, 23 and 28), two oysters collected from each of three replicate tanks in each Cu treatment or control were pooled to form one replicate providing three replicate samples per treatment, per occasion for metal analysis. The replicates of each copper treatment chosen for each sampling day cycled throughout the experiment in order to sample oysters from each tank. In addition to the collection of oysters for metals, two oysters from each of the five replicate tanks in each treatment [total 10 oysters (n=5) per treatment] were collected for biomarker analysis in both gills and hepatopancreas tissues as per the post-acclimation baseline oysters.

2.3. Oyster analysis

2.3.1. Biomarker analysis

After removal from the field or laboratory treatment tanks oysters were dissected and gills and hepatopancreas removed then placed into centrifuge tubes and immediately frozen on dry ice. Samples were then stored frozen in liquid nitrogen (-80ºC) before transportation on dry ice to City University, Hong Kong, for biomarker analysis.

Biomarker analysis was carried out using an overall method adapted from Cheung *et al.* (2001). Tissues were thawed on ice and homogenised in a solution that contained 20% glycerol (v/v), 1 mM EDTA and 0.1 M sodium phosphate buffer (pH 7.4) using a tissue blender (Ultra Turrax T8 homogeniser). The tissue homogenate was centrifuged at 10,000 *g* at 4ºC for 20 min. The supernatant was collected and transferred to five 1.7 mL Eppendorf tubes, immediately frozen in liquid nitrogen, and stored at -80ºC for further biochemical analyses.

The protein content of each sample was measured using a Bio-Rad™ micro assay kit with bovine serum albumin as the standard. The absorbance of each reaction mixture was measured using a microplate reader (SpectraMAX 340, Molecular Device™) at 595 nm after 30-min incubation at room temperature.

Activities of the following enzymes in the extracts were determined spectrophotometrically using a SpectraMAX 340 microplate reader. GST activity was determined using a modified method of Jakoby (1985) following the conjugation of reduced glutathione with 1-chloro-2, 4-dinitrobenzene (CDNB) at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). GSH was measured using the DTNB-GSSG Reductase Recycling Assay (Anderson, 1985). CAT activity was measured using the method of Cohen *et al.* (1996) using a H_2O_2 substrate. CAT consumes H_2O_2 which was measured colourimetrically using ferrous sulphate and potassium thiocyanate. The extent of LPO was measured as thiobarbituric acid reactive substances, using a modified method of Miller and Aust (1989).

2.3.2. Metal analysis

Oysters collected for metal analysis were frozen whole until processing. Frozen oysters were defrosted overnight in a refrigerator, then the soft tissue was extracted from the shell and blotted dry. The tissues of the two replicate oysters from each treatment tank were pooled to form one composite sample, placed in polyethylene jars, and frozen until analysis at Griffith University, Queensland.

Preparation and digestion of oyster tissues followed a method similar to that used by Andersen *et al*. (1996). The pooled oysters were oven-dried overnight at 105°C, weighed, ground and homogenised with a mortar and pestle. A 200 µg (dry wt) subsample was taken from each pooled replicate and microwave digested with 2 ml of HNO_3 and 0.5 ml of H_2O_2. The samples were diluted 1:50 and analysed using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent-7500). Standard reference material NIST 2976 (freeze-dried mussel powder) was used to calculate the recovery efficiency for each of the trace metals. The precision of the analysis was usually less than 10% relative standard deviation (RSD).

2.4. Statistical analysis

2.4.1. General

Prior to analyses homogeneity of sample variances were tested with Levene's tests. Data were either square root or $\log_{10}(x+1)$ transformed to achieve equality of variances where required. Where homogeneity could not be achieved untransformed data is presented. ANOVAs were performed using SPSS (Version 13.1, 2004) and data were plotted and correlation/regression analyses determined using SigmaPlot (Version 9.01, 2004).

2.4.2. Field oyster metal concentrations

One replicate at Site 2 at the day 29 collection was lost during processing therefore giving n=2 for the sampling occasion.

Two-way ANOVA was used to compare metal concentrations across sites and times to determine if there were differences in metal concentrations; two sites: impact (Site 1) and reference (Site 2), with eight collection times including baseline over the 29-day deployment period. Where a significant main effect was detected, Tukey's HSD multiple range test was used to locate differences between levels of the significant main effect. Results were tabulated (including data from resident oysters from both sites) and plotted (arithmetic means ±1 SE).

In the first instance, the two-way ANOVA compares Site 1 with Site 2 and then compares each time period to determine if there are significant differences. The analysis also assesses how the metal concentrations at the two sites altered in comparison to each other over the eight time periods (including baseline) using

an interaction term. If both sites varied similarly to each other there would be a non-significant interaction term.

Arithmetic means ±1 SE for each collection period were tabulated and also plotted over time. Data from resident oysters from Site 1 and 2 were not included in ANOVA comparisons but were tabulated for comparison.

2.4.3. Field oyster biomarker concentrations

A comparison of biomarker concentrations of oysters within hours of collection from the oyster lease, baseline oysters prior to deployment or allocation to acclimation facilities and at seven days post-acclimation prior to the beginning the experimental procedure was performed using one-way ANOVA. The comparison would determine if handling procedures or acclimation conditions affected biomarker concentrations prior to experimental procedures.

Two-way ANOVA was also used to compare biomarker concentrations in oyster gills and hepatopancreas between two sites and times as above to determine if there were differences in biomarker concentrations at the two sites and if the patterns of biomarker response at the two sites were similar over time. Where a significant main effect was detected over time, Tukey's HSD multiple range test was used to locate differences between levels of the significant main effect. Results were tabulated (including data from resident oysters from both sites) and plotted (arithmetic means ±1 SE). Results for the Tukey's HSD multiple range test for time are tabulated in Appendix 2.

Regressions of biomarker concentrations against time were also performed to determine how the biomarker responded over the deployment period. Results were plotted and the line of best fit, that is, linear, second-order polynomial, was applied and significant results plotted for each biomarker in each tissue at both sites.

2.4.4. Field oyster metal and enzyme concentration comparisons

Pearson product moment correlations were performed between biomarker and metal concentrations at both sites in both tissues to determine whether there were significant linear associations between these two parameters, plotted and screened for two-point correlations. Biomarker concentrations were then plotted against metal concentrations and various regression equations tested (including sigmoidal, logarithmic, exponential decay and exponential rise to a maximum) to determine whether any significant nonlinear associations between metal and biomarker concentrations were apparent. Regressions were plotted and significant relationships highlighted.

2.4.5. Laboratory oyster copper concentrations

Two-way ANOVA was performed to test for differences in mean Cu concentrations in oysters between treatments (controls, 3.75, 7.5, 15 and 30 µg/L) and across time with eight collection times including baseline over the 28-day treatment period, including the depuration period. Where a significant main effect was detected, *a posteriori* Tukey's HSD multiple range test was used to locate differences between levels of the significant main effect. Arithmetic means ±1 SE for Cu in oysters for each collection period in each treatment were tabulated and plotted.

2.4.6. Laboratory oyster biomarker concentrations

Results for each biomarker in each tissue in each treatment for each collection were tabulated and plotted (arithmetic means ±1 SE).

Two-way ANOVA was performed to test for differences in mean biomarker concentrations in oysters between treatments (controls, 3.75, 7.5, 15 and 30 µg/L) and across time with eight collection times including baseline over the 28-day treatment period, including the depuration period. Where a significant main effect was detected, *a posteriori* Tukey's HSD multiple range test was used to locate differences between levels of the significant main effect. Arithmetic means ±1 SE for biomarkers in oysters for each collection period in each treatment were tabulated and plotted.

Regressions of biomarker concentrations against time were also performed at 15 days exposure, 23 days exposure (48 hr after copper spiking had ceased) and at 28 days, which included all data to determine how the biomarkers responded over the exposure and depuration periods respectively. The line of best fit, that is, linear, second-order polynomial, was applied and results plotted for each

biomarker in each tissue in the five treatments including the post-acclimation baseline, where significant relationships were determined. Significant regressions were reported.

2.4.7. Laboratory oyster metal and enzyme concentration comparison

Pearson product moment correlations were performed between biomarker and Cu concentrations among treatments to determine whether there were significant linear associations between these two parameters and plotted to screen for two-point correlations. The relationship between the biomarker concentrations in oysters and the accumulated copper concentrations in oysters were deemed more relevant than between biomarker concentration and concentration of copper in the exposure water. Biomarker concentrations were then plotted against oyster Cu concentrations and various regression equations tested (including sigmoidal, logarithmic, exponential decay and exponential rise to a maximum) over 15, 23 and 28 days respectively, to determine whether any significant nonlinear associations between Cu and biomarker concentrations were apparent. Significant regressions were reported.

3. Results

3.1. Field results

3.1.1. Physicochemical properties

Physiochemical parameters remained fairly consistent over the sampling period and did not vary greatly between the two sites (Table 1).

Table 1. Mean ±1 SE physicochemical properties of water sampled at Sites 1 and 2 on each collection over the deployment period

Site	pH	Temperature (°C)	DO (%)	Conductivity (mS/cm)	Turbidity (NTU)
1	7.9 ± 0.0	25.6 ± 0.3	85 ± 15	54.5 ± 0.4	7.5 ± 0.7
2	8.0 ± 0.0	25.1 ± 0.4	96 ± 17	52.0 ± 0.1	4.1 ± 0.5

3.1.2. Oyster metal concentrations

Oysters at Site 1 accumulated significantly greater concentrations of Al, Cu, Zn and Cr than those at Site 2 over the 29 days of deployment (Table 2, Appendix 1). Although the concentration of Al declined at both sites from baseline this was not significant. Conversely, concentrations of As and Ni were significantly more elevated in oysters from Site 2 for the same deployment period, but there was no difference in accumulation of Pb or Cd between the two sites, with Pb rapidly depurating from baseline at both sites (Table 2), which was significant. A summary indicating the significant site, time and interaction terms is presented (Table 2) with mean concentrations (±1 SE) tabulated (Table 3) and plotted in subsequent figures (Figures 3–6). Tukey's results for time are tabulated in Appendix 1.

Concentration of Cr and Al also tended to show a decline over time in comparison to initial baseline concentrations. Cu and Zn followed similar uptake patterns at both sites over the deployment period. Although statistically different, the biological significance of the differences in Cr and Ni at Sites 1 and 2 may not be relevant considering the low concentrations of metals accumulated or depurated. Although accumulation of metals generally followed a linear pattern, uptake was variable. As oysters at both locations had the same baseline concentrations for some metals, there was a variable lag period before the two sites became significantly separated from each other in terms of accumulated metal concentrations.

Resident oysters within each site exhibited similar trends in accumulation to the deployed oysters (Table 3) indicating that the difference in oyster metal accumulation between the sites is due to the ambient metal conditions at each site. Deployed oysters, however, did not accumulate the same magnitude of metal concentrations as the resident oysters (Table 3). At Site 1, deployed oysters on day 29 contained only one-quarter of the Cu, and approximately one-third of the Zn of the resident oysters. At Site 2, a similar pattern was observed in Cu and As accumulation, although Zn concentrations were similar in deployed oysters compared to the residential oysters.

Table 2. Summary of two-way ANOVAs on concentrations of each metal in oysters by site (Sites 1 and 2) and time (baseline to collection eight)

Tukey's multiple comparison test was used to locate between-level differences for significant main effects. Nonsignificant interaction terms are indicated = ns. *Where equality of variances could not be achieved through transformation of data, untransformed data is used. Sites are in descending order and arithmetic means are in parenthesis. Results of *a posteriori* Tukey's test for time are located in Appendix 1.

Metal	Site significance	Site			Time significance	Interaction term significance
*Al	0.011	Site 1	>	Site 2	0.004	ns
		(87.38)		(59.67)		
*As	<0.0001	Site 2	>	Site 1	ns	ns
		(13.88)		(10.35)		
Cd	ns	Site 2	=	Site 1	ns	0.042
Sqrt		(3.16)		(2.78)		
*Cr	0.024	Site 1	>	Site 2	0.002	ns
		(0.72)		(0.63)		
*Cu	0.002	Site 1	>	Site 2	ns	0.039
		(74.58)		(50.78)		
*Ni	<0.0001	Site 2	>	Site 1	0.003	0.002
		(1.12)		(0.89)		
*Pb	ns	Site 1	=	Site 2	<0.0001	ns
		(0.22)		(0.22)		
*Zn	<0.0001	Site 1	>	Site 2	ns	ns
		(691.55)		(450.27)		

Table 3. Mean ±1 SE concentration (µg/g dry wt) of metals in oysters at Sites 1 and 2 throughout the 29-day deployment period, including one collection of resident oysters from each site

N=3 except where * (n=2) due to samples lost in processing.

Site	Day	Cu	Zn	As	Cd	Pb	Al	Cr	Ni
1	0	53 ± 7	623 ± 52	11 ± 0	3 ± 0	0.4 ± 0.0	131 ± 44	0.8 ± 0.1	0.8 ± 0.0
	3	51 ± 12	520 ± 151	10 ± 1	3 ± 1	0.2 ± 0.0	108 ± 35	0.8 ± 0.1	0.8 ± 0.1
	5	95 ± 19	881 ± 190	11 ± 1	4 ± 1	0.2 ± 0.0	73 ± 48	0.8 ± 0.1	0.9 ± 0.1
	8	60 ± 4	568 ± 77	11 ± 1	2 ± 0	0.2 ± 0.0	93 ± 5	0.7 ± 0.1	0.8 ± 0.1
	12	58 ± 2	665 ± 70	10 ± 0	2 ± 0	0.2 ± 0.0	88 ± 3	0.6 ± 0.1	0.9 ± 0.0
	15	68 ± 10	706 ± 124	10 ± 2	2 ± 0	0.2 ± 0.0	86 ± 3	0.6 ± 0.0	0.9 ± 0.1
	22	74 ± 12	602 ± 37	10 ± 1	2 ± 1	0.1 ± 0.0	51 ± 14	0.5 ± 0.1	0.8 ± 0.1
	29	138 ± 48	967 ± 162	9 ± 1	4 ± 0	0.2 ± 0.1	61 ± 2	0.8 ± 0.2	1.2 ± 0.1
Resident		583 ± 91	2563 ± 182	8 ± 0	1 ± 0	0.1 ± 0.0	28 ± 5	0.6 ± 0	1 ± 0
2	0	53 ± 7	623 ± 52	11 ± 0	3 ± 0	0.4 ± 0.0	131 ± 44	0.8 ± 0.1	0.8 ± 0.0
	3	51 ± 4	485 ± 70	15 ± 1	4 ± 1	0.2 ± 0.1	91 ± 17	0.8 ± 0.0	1.1 ± 0.1
	5	51 ± 2	386 ± 37	15 ± 2	3 ± 1	0.2 ± 0.0	69 ± 20	0.7 ± 0.0	0.8 ± 0.0
	8	49 ± 8	373 ± 43	16 ± 1	3 ± 0	0.2 ± 0.0	30 ± 7	0.6 ± 0.1	1.2 ± 0.2
	12	58 ± 30	549 ± 311	11 ± 2	3 ± 1	0.2 ± 0.0	25 ± 4	0.5 ± 0.1	1.3 ± 0.2
	15	49 ± 4	374 ± 77	14 ± 2	3 ± 1	0.2 ± 0.0	43 ± 13	0.6 ± 0.1	1.2 ± 0.0
	*22	53 ± 18	429 ± 231	16 ± 5	4 ± 1	0.2 ± 0.0	50 ± 25	0.6 ± 0.0	1.5 ± 0.1
	*29	40 ± 7	339 ± 78	14 ± 0	2 ± 1	0.2 ± 0.1	23 ± 6	0.5 ± 0.1	1.2 ± 0.2
Resident		256 ± 16	490 ± 71	31 ± 2	1 ± 0	0.1 ± 0.1	38 ± 21	0.7 ± 0.1	1.7 ± 0.2

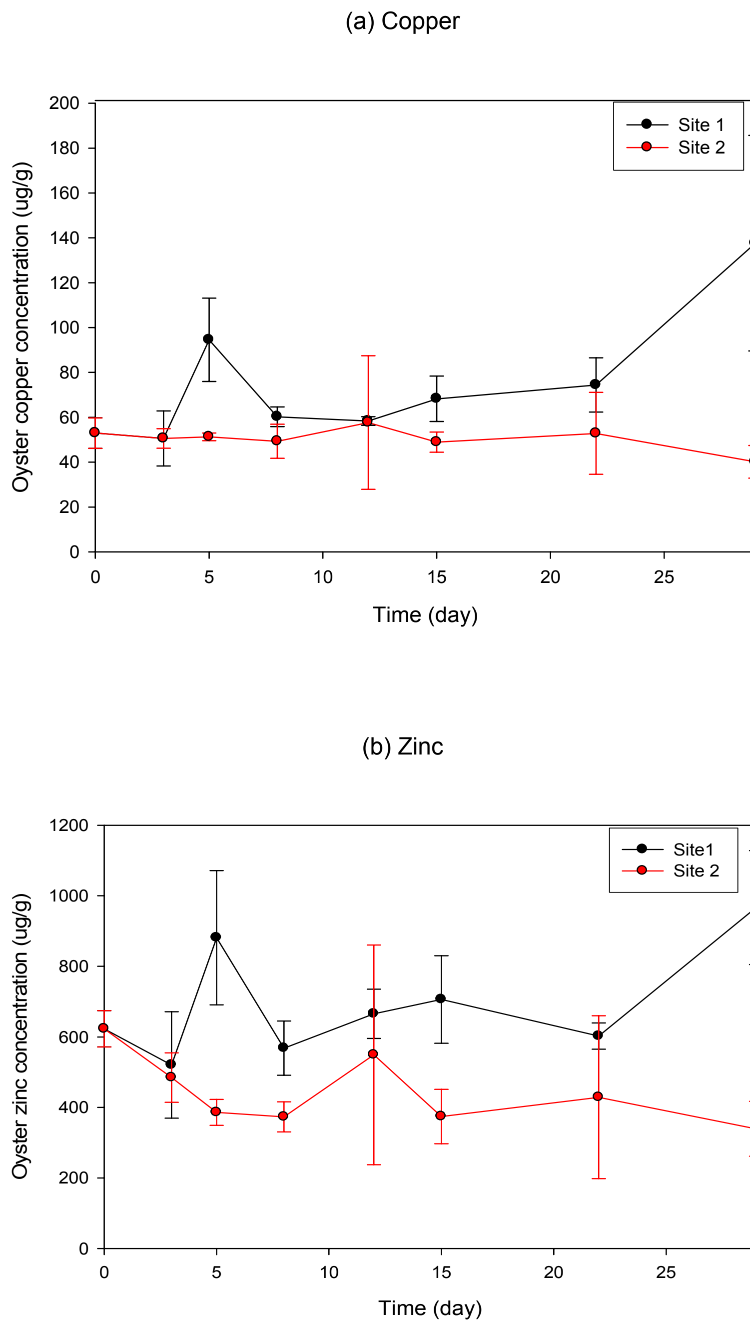
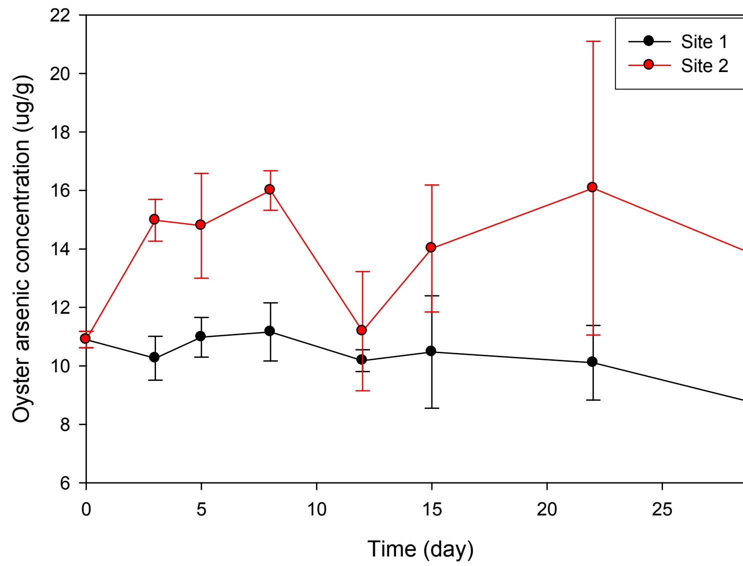


Figure 3. Mean ±1 SE concentration of (a) copper and (b) zinc in oysters from Sites 1 and 2 over time (29 days deployment) including baseline concentrations

(a) Arsenic



(b) Cadmium

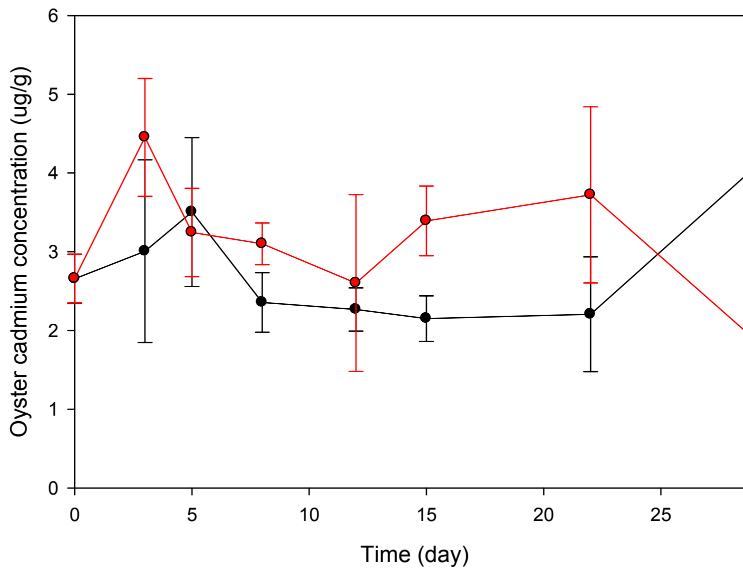
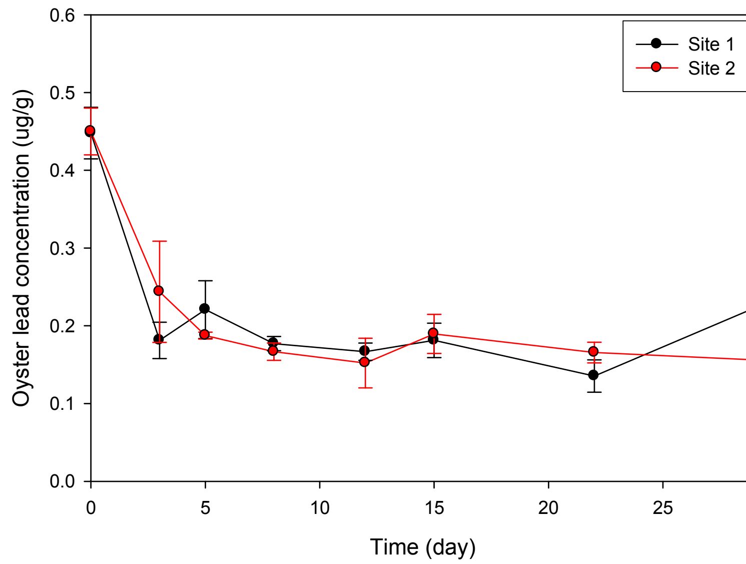


Figure 4. Mean ±1 SE concentration of (a) arsenic and (b) cadmium in oysters from Sites 1 and 2 over time (29 days deployment) including baseline concentrations

(a) Lead



(b) Aluminium

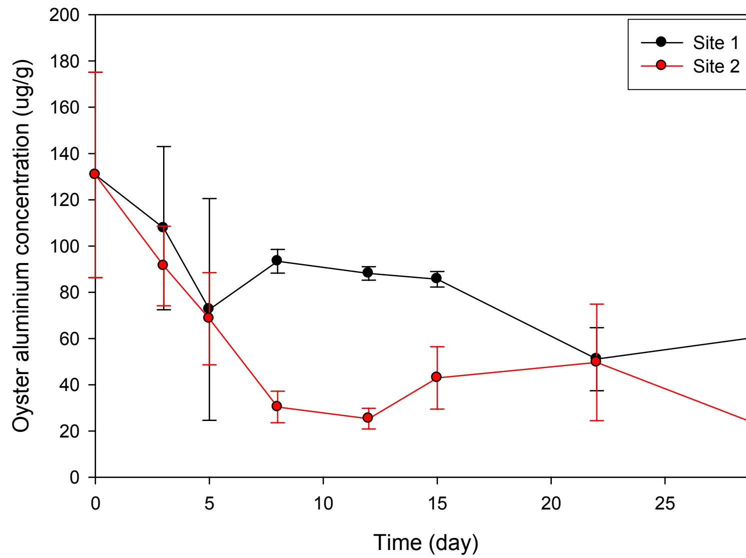
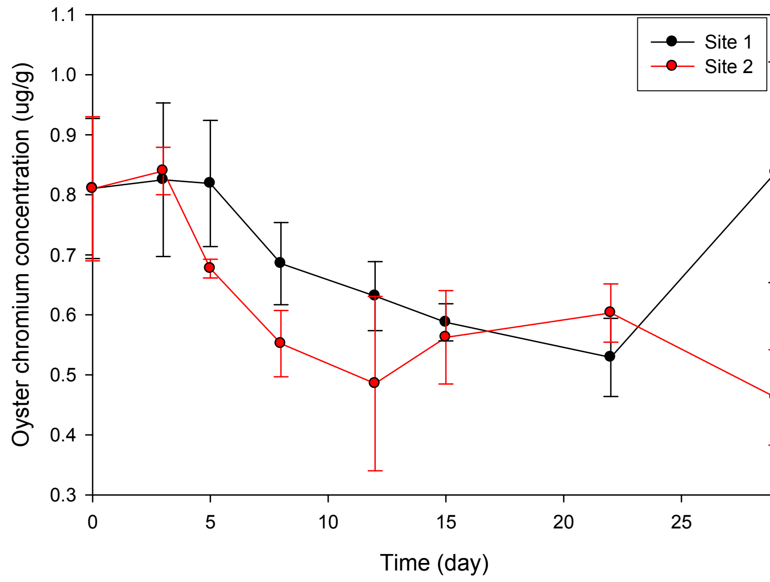


Figure 5. Mean ±1 SE concentration of (a) lead and (b) aluminium in oysters from Sites 1 and 2 over time (29 days deployment) including baseline concentrations

(a) Chromium



(b) Nickel

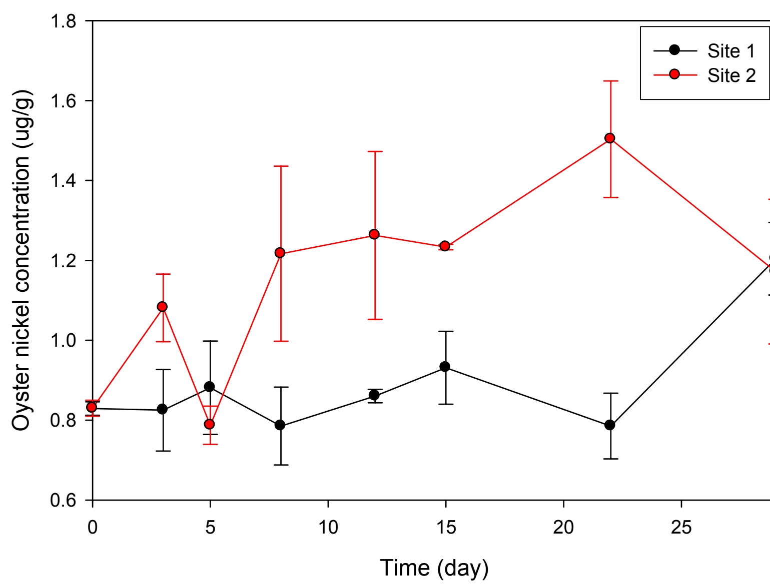


Figure 6. Mean ±1 SE concentration of (a) chromium and (b) nickel in oysters from Sites 1 and 2 over time (29 days deployment) including baseline concentrations

3.1.3. Oyster biomarker concentrations

Baseline comparisons

A comparison of biomarker concentrations of oysters (a) within hours of collection from the lease, (b) baseline oysters prior to deployment or allocation to acclimation facilities and (c) at seven days post-acclimation prior to beginning the bioassay, determined that there were some significant differences between groups for CAT in hepatopancreas and LPO and GST in both tissues (Table 4). Generally, for LPO and GST in both gills and hepatopancreas, there was no distinct pattern to the significant changes in concentrations from collection at the lease to post-acclimation. For CAT in hepatopancreas, however, there was a large decline in concentrations from when oysters were sampled at the lease to their arrival two days later and prior to deployment in the field or allocation to the bioassay. The concentrations recovered to some degree after the seven days of acclimation in natural sea water and also after deployment to both field locations, suggesting transportation had an effect on hepatopancreas CAT. This pattern to a lesser extent was observed in gill GST (Table 4).

Table 4. One-way ANOVA comparing biomarker concentrations in oysters (a) within hours of collection from the lease, (b) baseline oysters prior to deployment or allocation to acclimation facilities and (c) at seven days post-acclimation prior to the beginning the bioassay

An *a posteriori* Tukey's range test was applied to locate differences between groups; groups not significantly different from each other are joined by a common line and are arranged in ascending order of arithmetic mean concentration (shown above).

Enzyme	df	F	p	Tukey's multiple range test		
Gills						
CAT	2,26	1.625	ns			
				(53.85)	(61.64)	(86.71)
LPO	2,27	8.162	0.002	b	a	c
				(48.02)	(54.87)	(66.54)
GST	2,26	4.403	0.023	b	c	a
GSH	2,27	1.987	ns			
Hepatopancreas						
				(739.29)	(3064.39)	(6334.5)
CAT	2,27	21.96	<0.001	b	c	a
				(58.88)	(64.19)	(81.37)
LPO	2,27	4.86	0.16	a	c	b
				(103.54)	(125.47)	(160.14)
GST	2,27	5.754	0.008	c	b	a
GSH	2,27	1.760	ns			

The concentration of biomarkers varied across the tissue types. Both CAT and GST were generally more elevated in the hepatopancreas, whereas GSH was at slightly lower concentrations in the hepatopancreas than in the gills. LPO was found at similar concentrations in both the gills and hepatopancreas. The tissue differences were found consistently at both sites (Table 5).

Table 5. Concentrations (μmol/g) of antioxidant enzymes in oysters including residents at Sites 1 and 2 throughout the deployment period

N=10 except where * (n=9) due to insufficient protein in the sample for analyses.

Site	Day	Catalase		Lipid peroxidase		Glutathione-s-transferase		Glutathione	
		Gills	Hepato	Gills	Hepato	Gills	Hepato	Gills	Hepato
1	0	*1475 ± 138	739 ± 103	54 ± 4	81 ± 5	*48 ± 4	125 ± 10	*16 ± 3	10 ± 3
	3	1455 ± 187	4784 ± 392	87 ± 2	75 ± 6	48 ± 2	121 ± 15	14 ± 2	6 ± 0
	5	1541 ± 78	5347 ± 672	85 ± 5	74 ± 9	23 ± 5	123 ± 8	15 ± 1	4 ± 1
	8	*1510 ± 116	*6095 ± 933	*74 ± 3	69 ± 14	*47 ± 3	*129 ± 13	14 ± 2	4 ± 1
	12	1423 ± 162	6210 ± 919	62 ± 3	65 ± 4	41 ± 3	95 ± 7	14 ± 2	3 ± 1
	15	1419 ± 134	4769 ± 363	65 ± 5	69 ± 5	49 ± 5	133 ± 7	10 ± 1	9 ± 1
	22	1420 ± 149	5895 ± 756	80 ± 4	63 ± 7	50 ± 4	135 ± 12	14 ± 2	10 ± 1
	29	1573 ± 139	3130 ± 379	70 ± 4	78 ± 4	49 ± 4	109 ± 11	16 ± 2	5 ± 1
Resident		1515 ± 178	4347 ± 959	183 ± 31	121 ± 11	31 ± 4	200 ± 33	21 ± 6	12 ± 2
2	0	*1475 ± 138	739 ± 103	54 ± 5	81 ± 5	*48 ± 4	125 ± 10	*16 ± 3	10 ± 3
	3	1277 ± 157	5348 ± 498	56 ± 8	95 ± 5	24 ± 2	112 ± 12	14 ± 1	8 ± 1
	5	1496 ± 175	4636 ± 697	72 ± 7	90 ± 9	81 ± 9	138 ± 9	23 ± 6	10 ± 2
	8	1705 ± 170	6356 ± 968	50 ± 4	64 ± 4	52 ± 4	112 ± 10	14 ± 2	7 ± 1
	12	1902 ± 108	6074 ± 568	63 ± 9	66 ± 4	61 ± 5	101 ± 7	16 ± 2	10 ± 1
	15	1857 ± 190	*4879 ± 667	63 ± 10	78 ± 10	70 ± 4	117 ± 7	17 ± 2	10 ± 2
	22	1704 ± 264	*5549 ± 744	68 ± 10	61 ± 6	76 ± 5	138 ± 13	16 ± 2	10 ± 1
	29	1438 ± 169	3361 ± 231	53 ± 4	70 ± 5	34 ± 2	93 ± 7	19 ± 2	8 ± 1
Resident		2922 ± 413	*15468 ± 3372	163 ± 20	142 ± 21	50 ± 13	234 ± 27	25 ± 4	14 ± 2

Concentrations of biomarkers at both sites were variable over the deployment period with responses in both tissues not necessarily following the same patterns. Generally there appeared to be a greater initial positive response in biomarkers at the reference site (Site 2) compared to Site 1 in both tissues.

Gills

In gill tissue there tended to be more elevated concentrations of GST, CAT and GSH at Site 2 compared to Site 1 over the deployment period, the difference being significant for GST and GSH (Table 6, Appendix 2), whereas LPO was significantly more elevated overall at Site 1. There appeared to be a small initial peak in biomarker concentrations at Site 2 after deployment. The peak was also observed in hepatopancreas GSH, GST and LPO (at three and five days) but was not significant on two-way ANOVA. Apart from LPO, biomarkers appeared to remain fairly stable at Site 1 across time. LPO at both sites tended to follow a similar response pattern although overall concentrations of LPO at Site 1 were significantly higher than at Site 2. For CAT and GST at Site 2 there was an initial increase followed by a decrease toward the end of the deployment period (Table 6, Figures 7–10), which was significant for GST.

Hepatopancreas

CAT tended to follow the same pattern with similar concentrations at both sites and with a substantial initial significant increase in concentrations from baseline to three days, which continued to be maintained. The abnormally low baseline concentration of CAT in hepatopancreas tissue may be due to transportation stress and may be considered an anomaly rather than a true baseline reference point. LPO also followed a similar pattern (variable but stable over time) at both sites except for the initial peak at Site 2, which was also seen in gill tissue. GST also followed a variable pattern which was again similar at both sites except for the peak at Site 2 at five days deployment. There was no significant difference in concentrations between sites for all three biomarkers. GSH remained fairly stable at Site 2; however, at Site 1 there was a significant rapid decline in concentrations followed by an increase after 15 days (Table 6, Figures 7–10). Results of *a posteriori* Tukey's tests for time are tabulated in Appendix 2.

Table 6. Summary of two-way ANOVAs on concentrations of each enzyme in oyster tissues (gill and hepatopancreas) by site (Site 1 and Site 2) and time (baseline to collection eight)

Tukey's multiple comparison test was used to locate between-level differences for significant main effects. Non-significant interaction terms are indicated = ns. Arithmetic mean concentrations (µmol/g) are shown in parenthesis. Results of *a posteriori* Tukey's test for time are located in Appendix 2.

Enzyme	Site significance	Site			Time significance	Interaction term significance
Gill						
CAT	ns	Site 2	=	Site 1	ns	ns
		(1608)		(1476)		
LPO	0.001	Site 1	>	Site 2	0.023	ns
		(71.94)		(59.84)		
GST	<0.0001	Site 2	>	Site 1	<0.0001	<0.0001
		(55.92)		(44.33)		
GSH	0.012	Site 2	>	Site 1	ns	ns
		(16.96)		(14.07)		
Hepatopancreas						
CAT	ns	Site 1	=	Site 2	<0.0001	ns
		(4602)		(4602)		
LPO	ns	Site 1	=	Site 2	0.005	ns
		(72.78)		(76.61)		
GST	ns	Site 1	=	Site 2	0.001	ns
		(121.24)		(117.26)		
GSH	0.001	Site 2	>	Site 1	0.002	ns
		(9.10)		(6.56)		

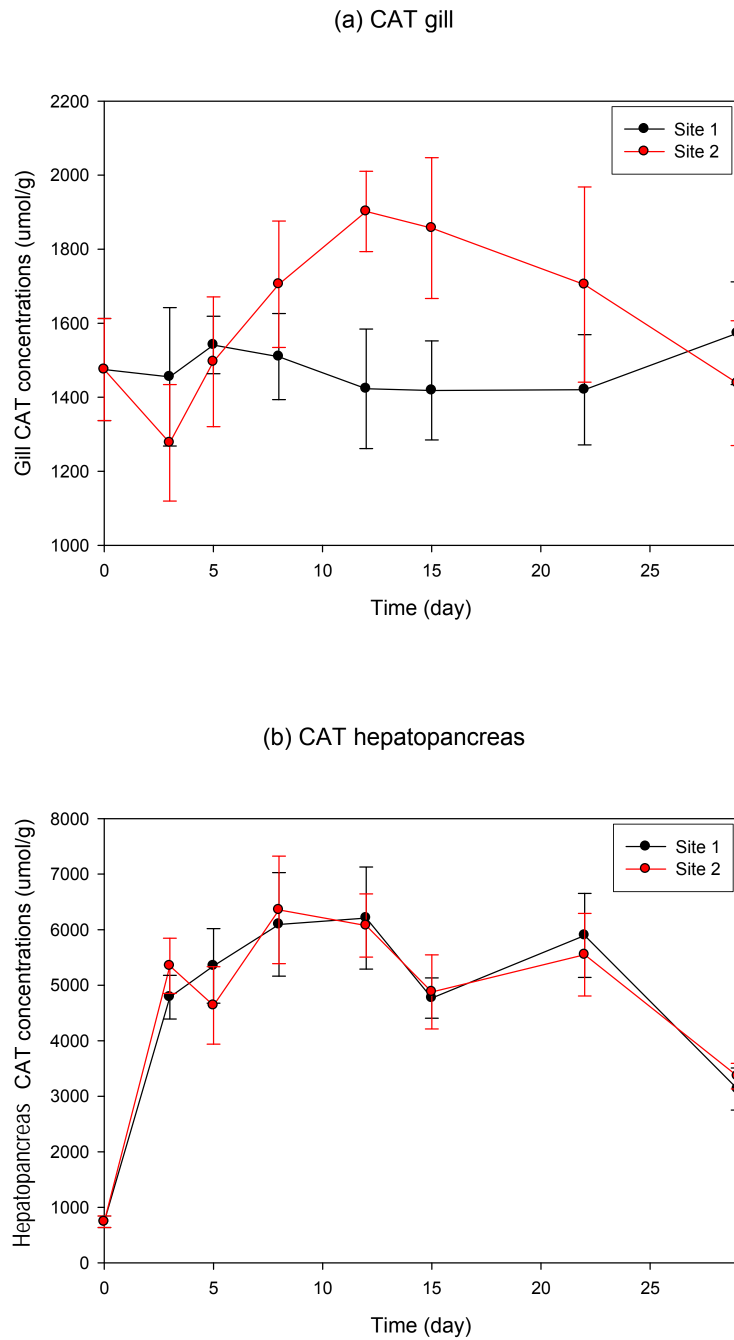


Figure 7. Mean ±1 SE concentration (μmol/g) of CAT in (a) gill and)b) hepatopancreas in oysters from Sites 1 and 2 over time (29 days deployment) including baseline concentrations

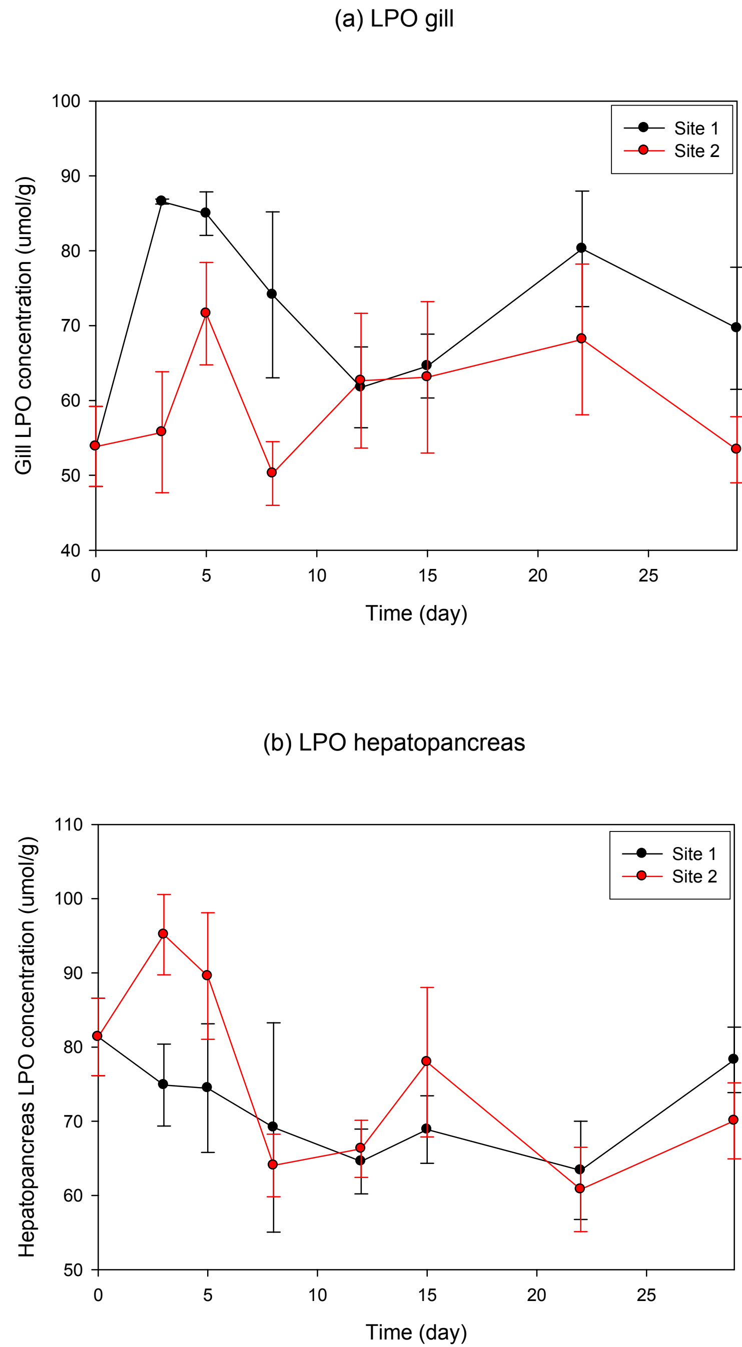
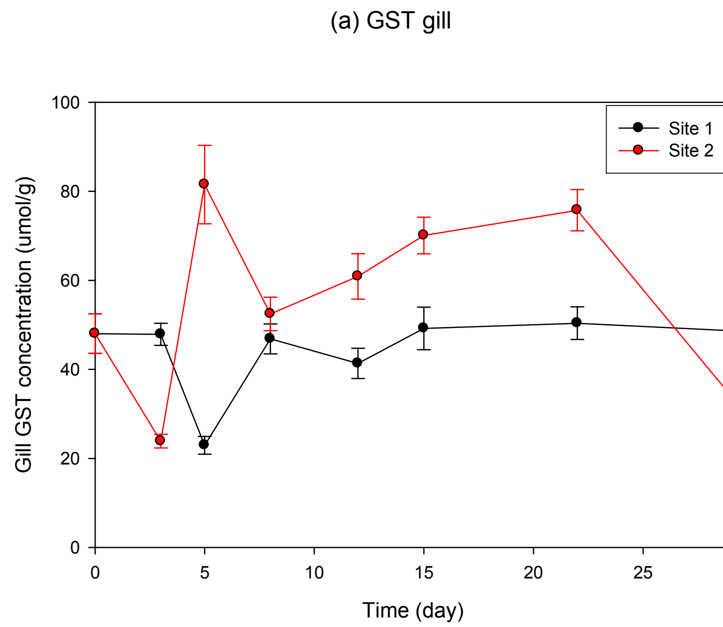


Figure 8. Mean ±1 SE concentration (µmol/g) of LPO in (a) gill and)b) hepatopancreas in oysters from Sites 1 and 2 over time (29 days deployment) including baseline concentrations



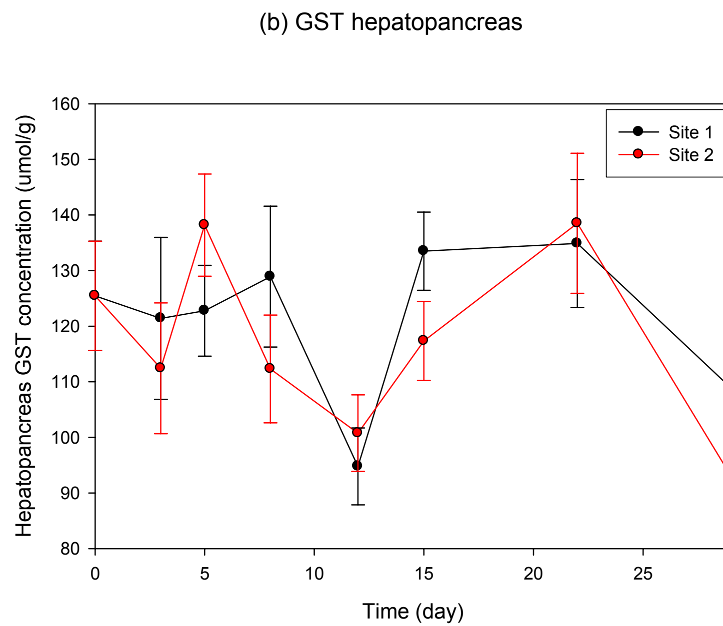
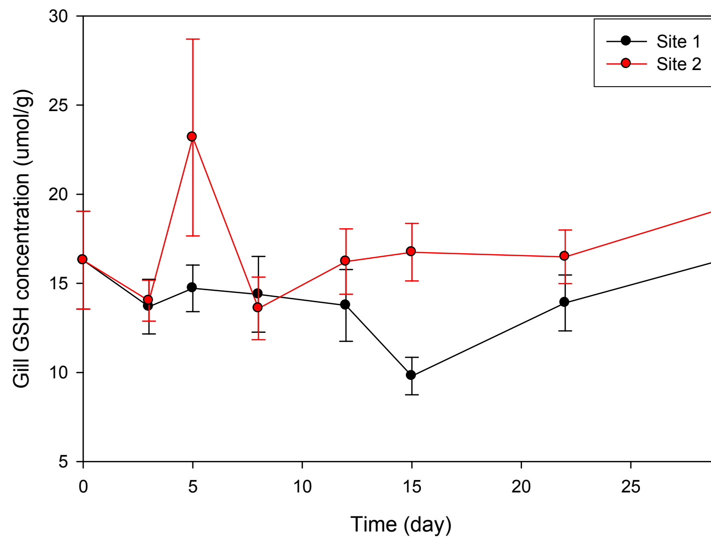


Figure 9. Mean ±1 SE concentration (μmol/g) of GST in (a) gill and (b) hepatopancreas in oysters from Sites 1 and 2 over time (29 days deployment) including baseline concentrations

(a) GSH gill



(b) GSH hepatopancreas

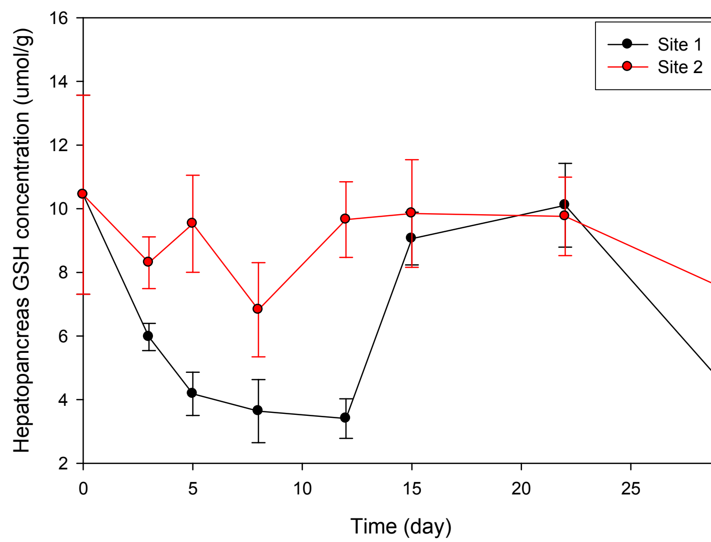


Figure 10. Mean ±1 SE concentration (μmol/g) of GSH in (a) gill and(b) hepatopancreas in oysters from Sites 1 and 2 over time (29 days deployment) including baseline concentrations

Regression over time

The only enzyme to demonstrate a significant relationship with time was CAT in hepatopancreas at Site 1 and CAT in both tissues at Site 2. CAT in hepatopancreas followed the same response at both sites over the deployment period whereas in gill tissue the relationship between CAT and time was best described using a polynomial regression (Figure 11).

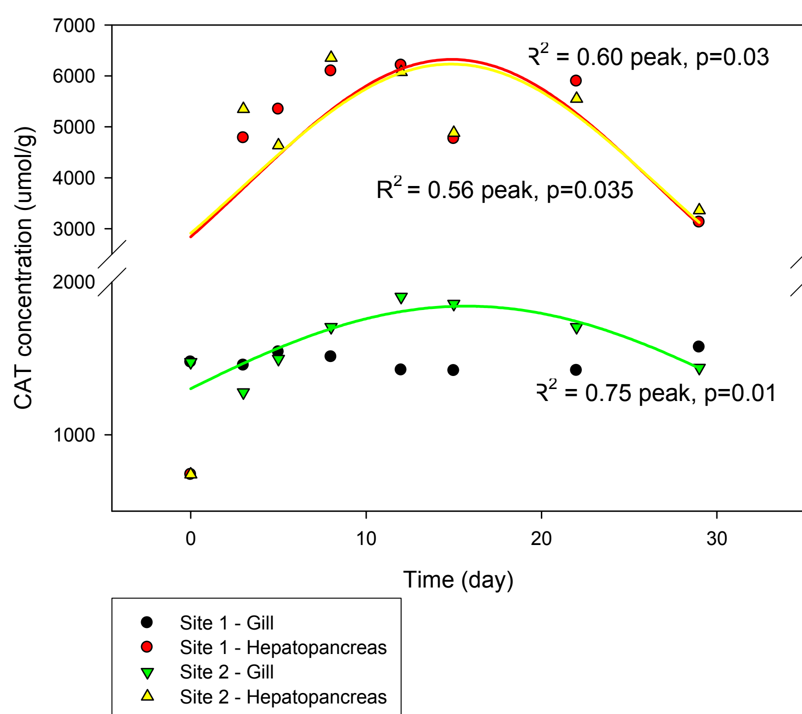


Figure 11. Regression of mean CAT activity over time at Sites 1 and 2

Regressions were significant for CAT gill activity over time in Site 2 (peak, $r^2 = 0.75$, p=0.010), and CAT hepatopancreas activity in Sites 1 and 2 (peak, $r^2 = 0.60$, p=0.030 and $r^2 = 0.56$, p=0.035 respectively).

3.1.4. Comparison of metal and biomarker concentrations

Correlations

Several significant correlations were found between enzyme concentrations and metal concentrations in deployed oysters at Sites 1 and 2 (Table 7). CAT and LPO concentrations correlated with metal concentrations, but there were no significant correlations found for either GST or GSH in either tissue, at either site (Table 6). At both sites, Pb was negatively correlated with CAT, and Cr was positively correlated with LPO in the hepatopancreas. A general trend shown by the correlations was that as metal concentrations increased, CAT increased in the gills, while decreasing in the hepatopancreas, while LPO increased in both

the gills and hepatopancreas. Although significant, the relationships between Pb and CAT in hepatopancreas at both sites were strongly influenced by a few high values and therefore could be considered as two-point correlations. The same applied to Pb and LPO in hepatopancreas at Site 1, and the results therefore should be treated with caution as to the strength of the relationships. The majority of relationships were strong with r^2 values above 0.72 (Table 7).

Table 7. Pearson product moment correlations between metal concentrations and enzyme concentrations in gills and hepatopancreas of oysters in Sites 1 and 2

Only significant correlations shown (α = 0.05). *Two-point correlations are noted.

Site	Metal	Tissue	Enzyme	R value	P value
1	Copper	Gill	CAT	0.722	0.043
	*Lead	Hepatopancreas	LPO	0.777	0.023
	*Lead	Hepatopancreas	CAT	-0.910	0.002
	Aluminium	Hepatopancreas	CAT	-0.736	0.038
	Aluminium	Hepatopancreas	LPO	0.788	0.020
	Chromium	Hepatopancreas	LPO	0.895	0.003
	Cadmium	Gill	CAT	0.860	0.006
	Chromium	Gill	CAT	0.735	0.038
2	*Lead	Hepatopancreas	CAT	-0.833	0.005
	Chromium	Hepatopancreas	LPO	0.766	0.016
	Nickel	Hepatopancreas	LPO	0.744	0.036

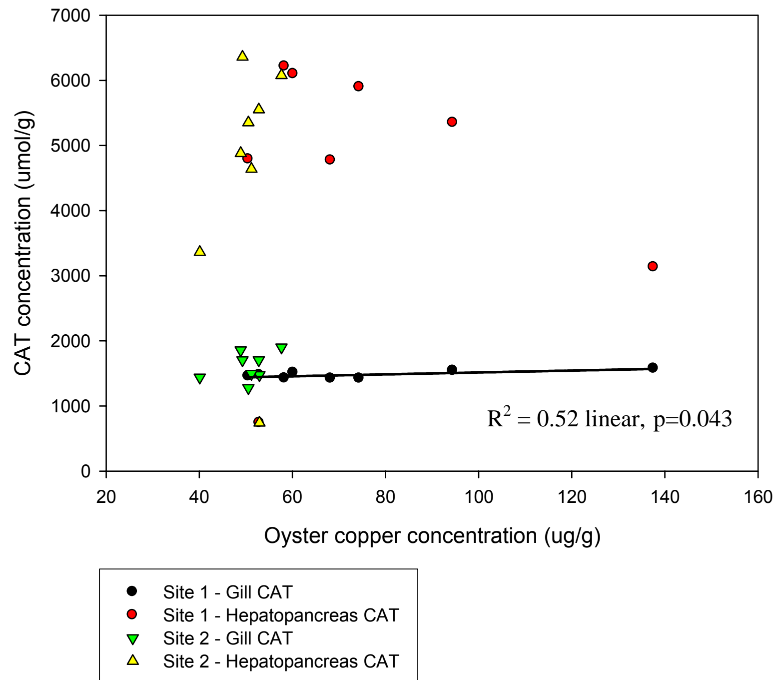
Regressions

Oyster metal concentrations were plotted against biomarker concentrations in each tissue, and regression analyses performed in order to determine whether there were any additional nonlinear associations and significant regressions plotted. While no significant regressions between gill CAT and metal concentrations were found at Site 2, at Site 1 this enzyme displayed weak linear associations with Cu, Zn, and Cr and a moderate relationship with Cd, increasing as metal concentrations increased (Figures 12–20). At both sites, hepatopancreas CAT activity decreased as Pb and Al concentrations increased similar to the results gained by the correlation analyses (Table 7). CAT activity increased with increasing Al initially then decreased significantly. However, as two-point relationships occurred for hepatopancreas and Pb at both sites and Al

at Site 2, results should be interpreted carefully. Hepatopancreas CAT concentrations at Site 2 increased to a maximum as Ni concentrations increased in a weak relationship (Figures 12–20).

Hepatopancreas LPO concentrations increased linearly as Al and Cr increased at both sites, and as Ni increased at Site 2 and Pb increased in Site 1 (Figures 12–20). However all relationships were moderate and the latter was a two-point relationship. At Site 1, hepatopancreas LPO activity increased exponentially to a maximum as cadmium concentrations increased. Correlation analyses indicated that there were no significant linear relationships between tissue GST activity and oyster metal concentrations (Table 7). However, several nonlinear associations were found between these parameters. Site 2 gill GST activity increased as cadmium and chromium concentrations increased, then decreased after a certain metal concentration was reached. Similar patterns were seen in hepatopancreas GST concentration with chromium in Site 1, and cadmium, aluminium and chromium in site 2, although for aluminium the GST concentrations were maintained after the critical concentration was reached. No linear or nonlinear associations were exhibited between GSH concentrations in gills or hepatopancreas with any of the metals analysed (Figures 12–20).

(a) CAT copper



(b) CAT zinc

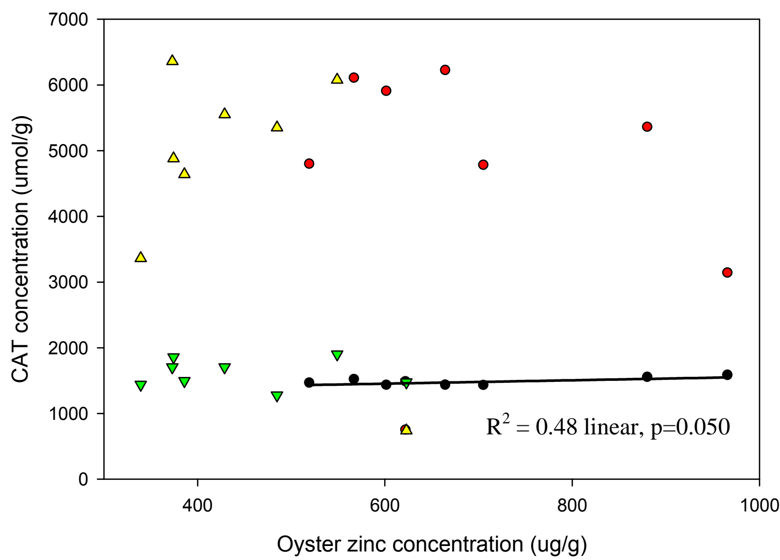
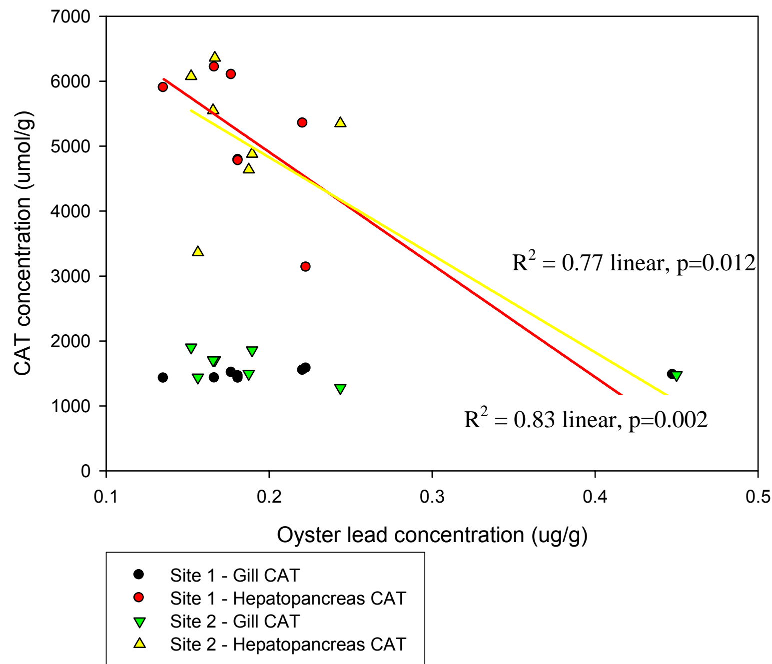


Figure 12. Regression of mean CAT concentration against mean oyster (a) copper and (b) zinc concentrations at Sites 1 and 2

The r^2 values of significant regressions are demonstrated.

(a) CAT lead



(b) CAT aluminium

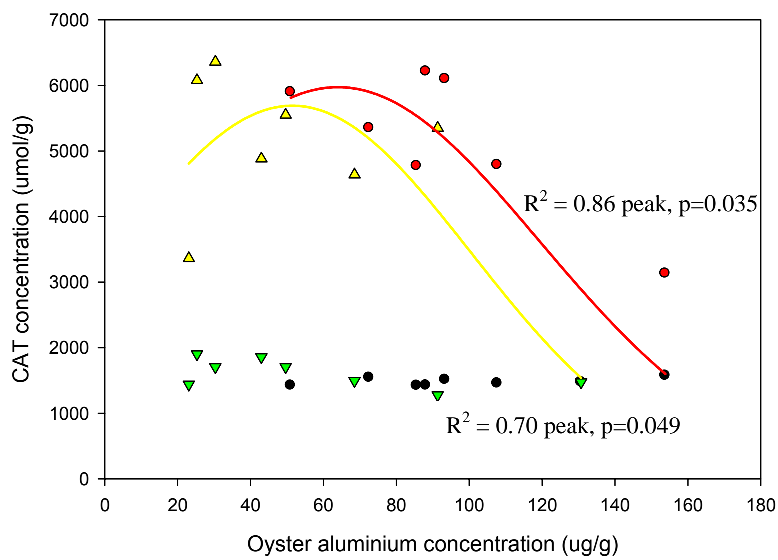
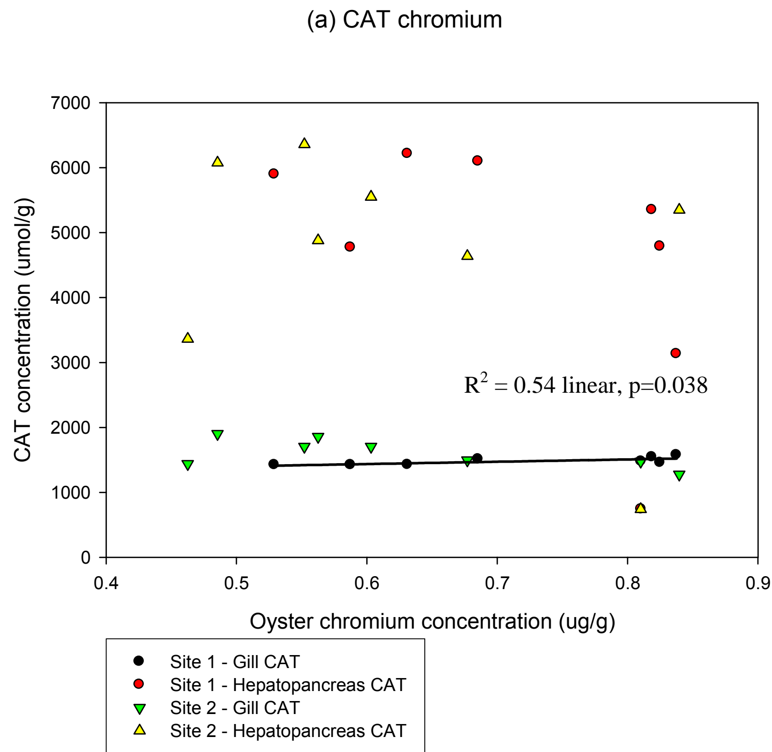


Figure 13. Regression of mean CAT concentration against mean oyster (a) lead and (b) aluminium concentrations at Sites 1 and 2

The r^2 values of significant regressions are demonstrated.



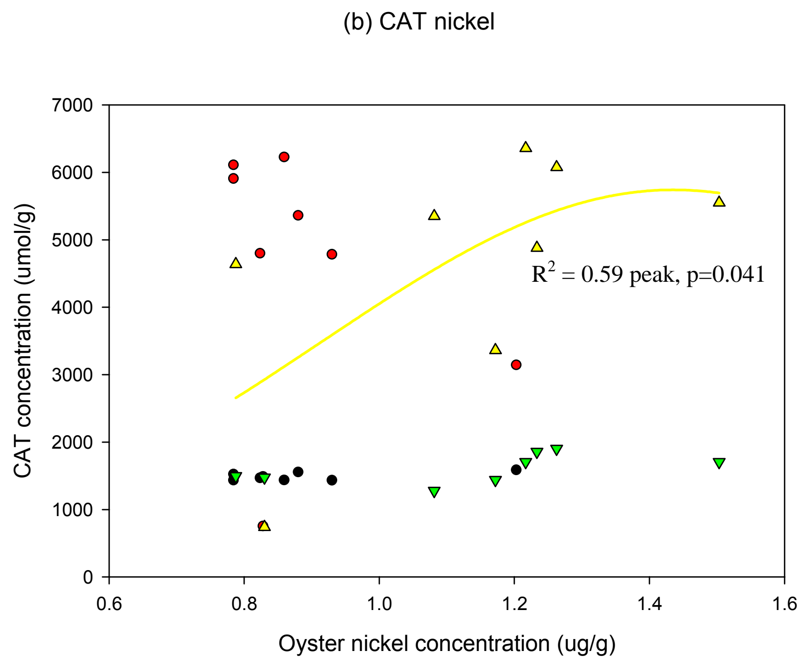


Figure 14. Regression of mean CAT concentration against mean oyster (a) chromium and (b) nickel concentrations at Sites 1 and 2

The r^2 values of significant regressions are demonstrated.

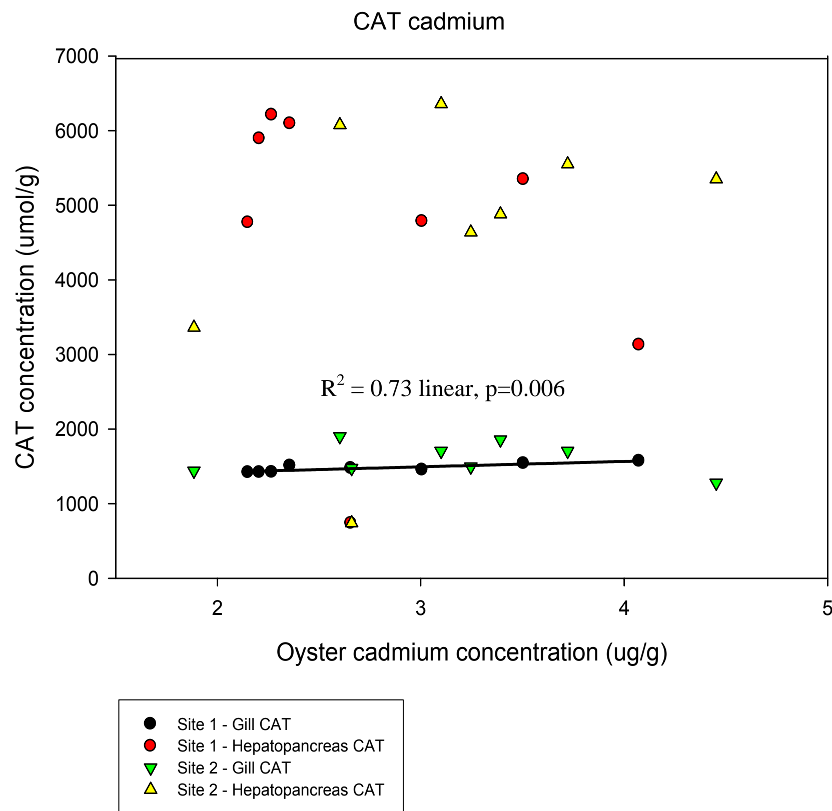


Figure 15. Regression of mean CAT concentration against mean oyster cadmium concentrations at Sites 1 and 2

The r^2 values of significant regressions are demonstrated.

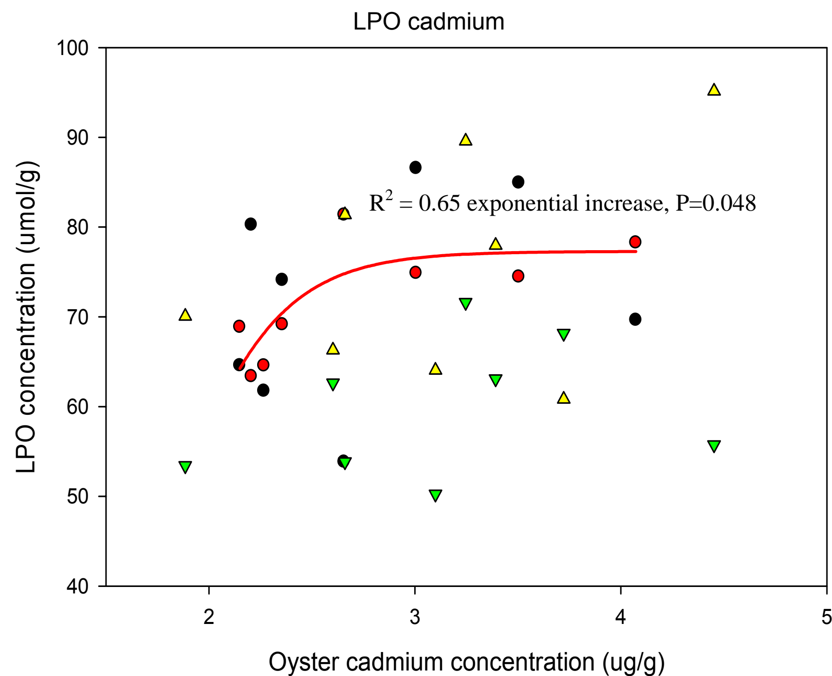
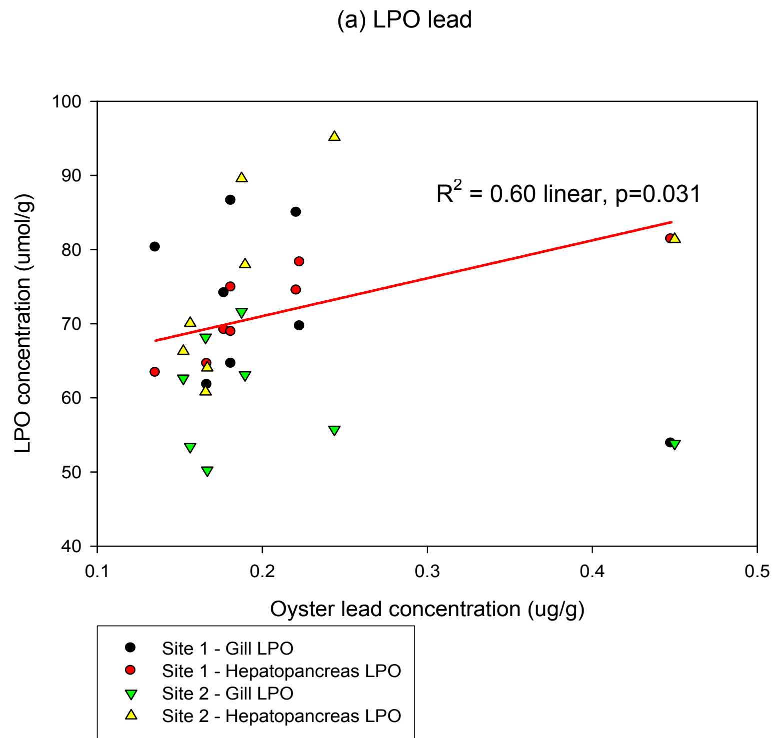


Figure 16. Regression of mean LPO concentration against mean oyster cadmium concentrations at Sites 1 and 2

The r^2 values of significant regressions are demonstrated.



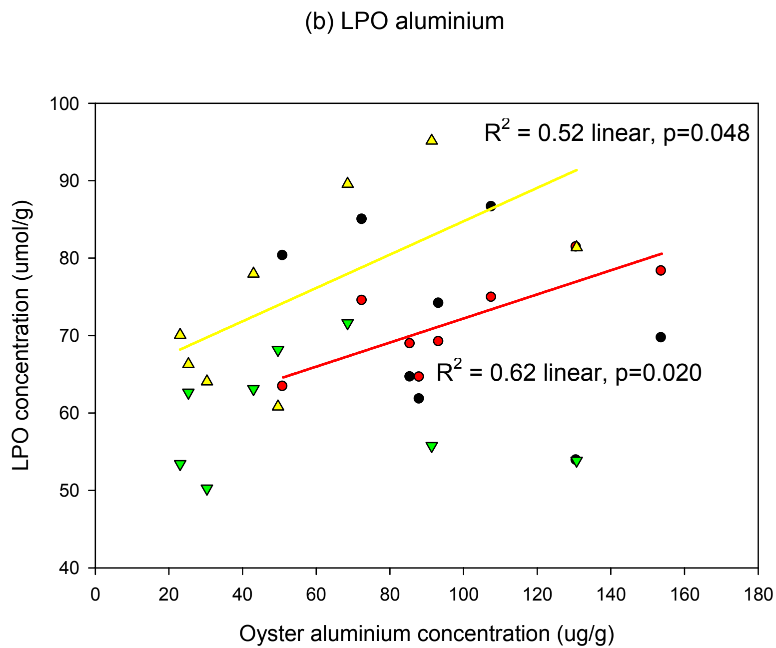
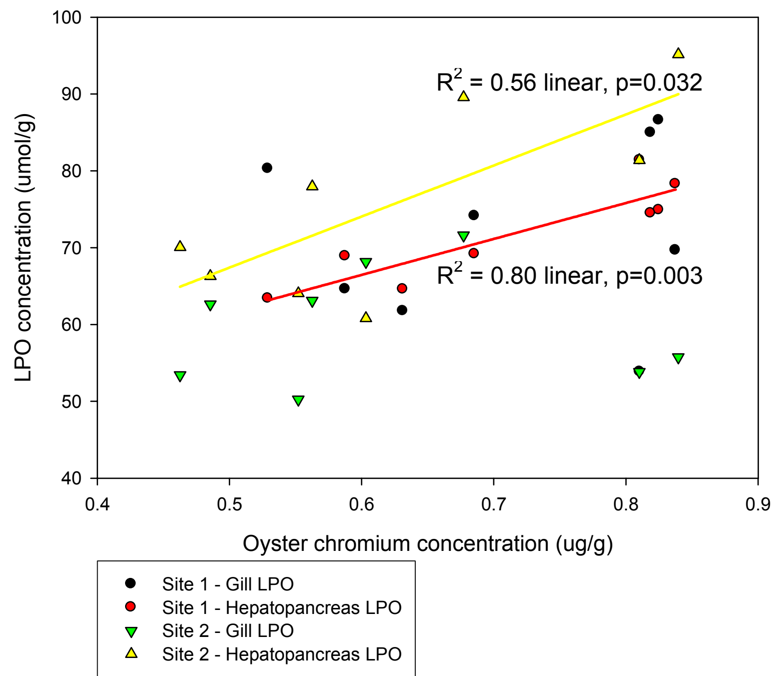


Figure 17. Regression of mean LPO concentration against mean oyster (a) lead and (b) aluminium concentrations at Sites 1 and 2

The r^2 values of significant regressions are demonstrated.



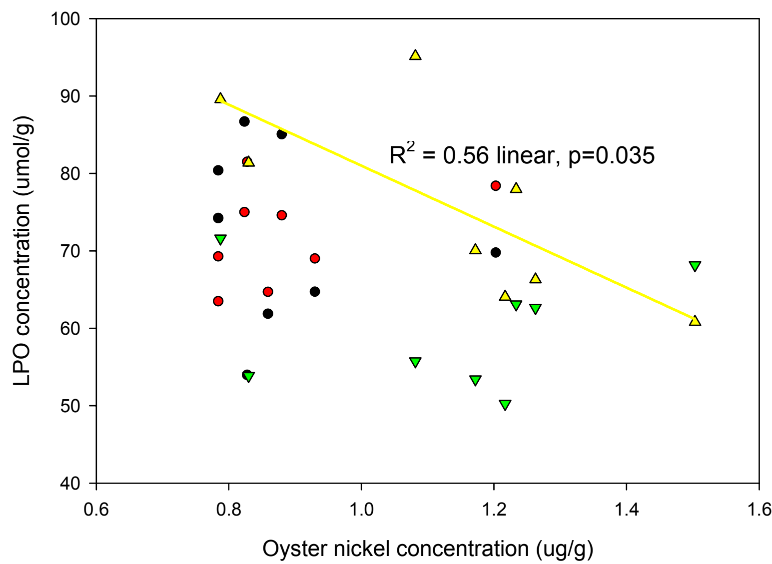
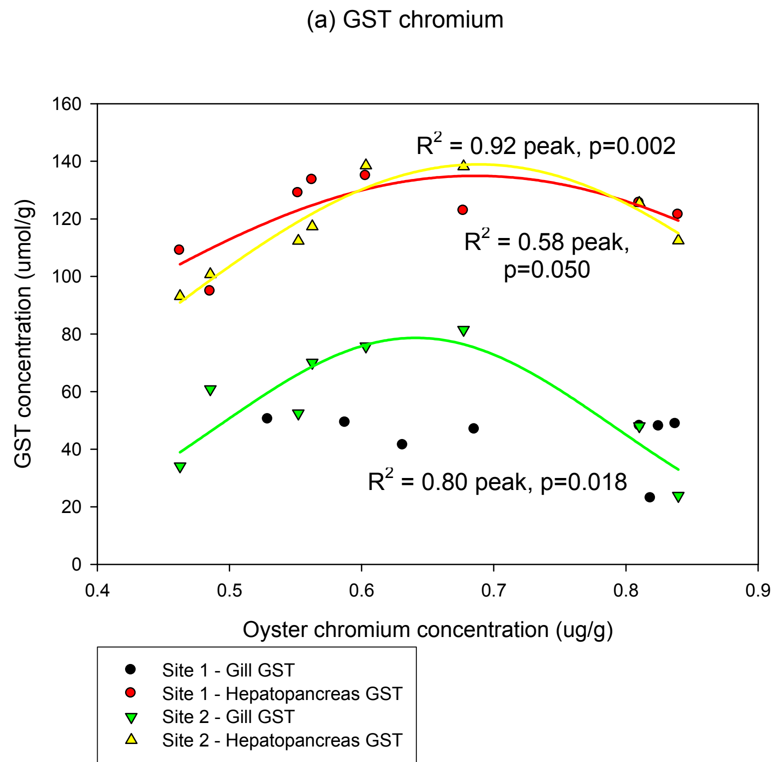


Figure 18. Regression of mean LPO concentration against mean oyster (a) chromium and (b) nickel concentrations at Sites 1 and 2

The r^2 values of significant regressions are demonstrated.

(a) GST chromium



(b) GST aluminium

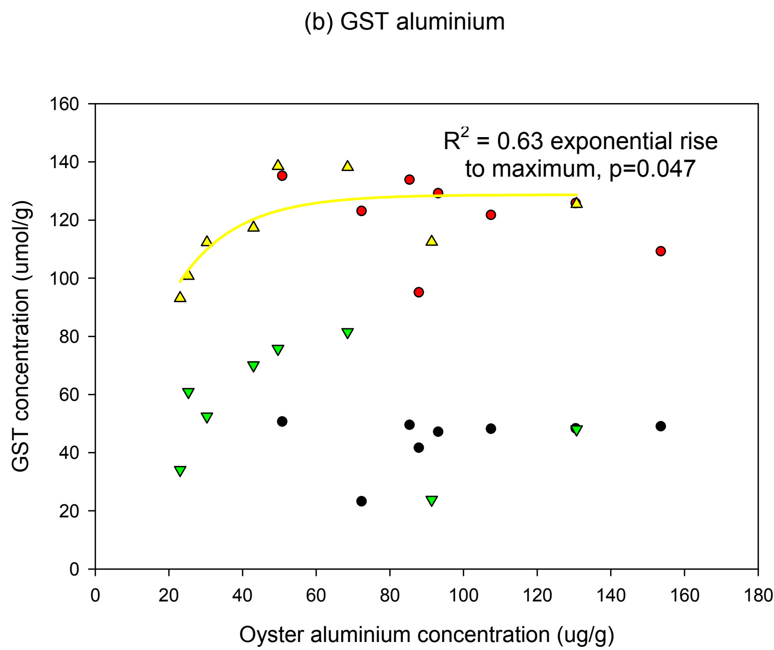


Figure 19. Regression of mean GST concentration against mean oyster (a) chromium and (b) aluminium concentrations at Sites 1 and 2

The r^2 values of significant regressions are demonstrated.

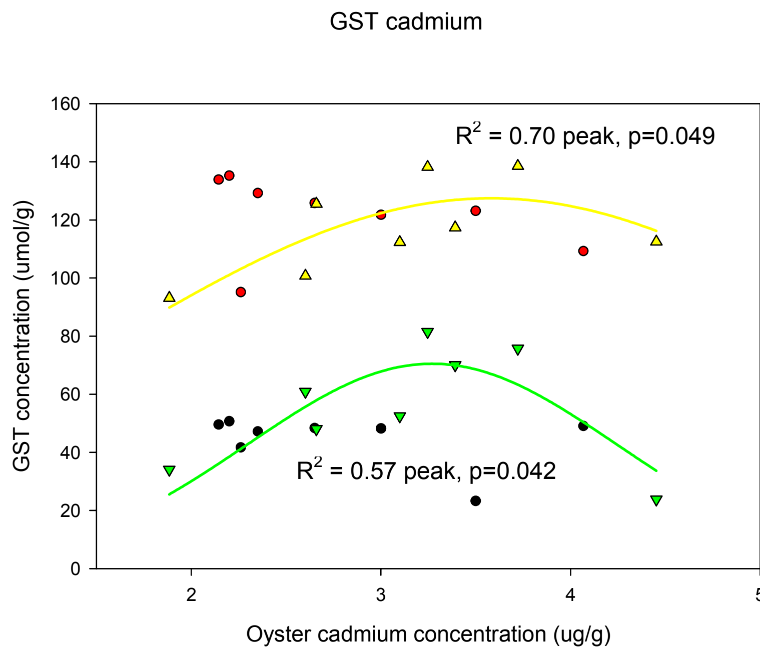


Figure 20. Regression of mean GST concentration against mean oyster cadmium concentrations at Sites 1 and 2

The r^2 values of significant regressions are demonstrated.

3.2. Laboratory bioassay

In the original experimental design the exposure phase was to cover a longer period, but this was later revised to 21 days to allow for a longer depuration phase. Copper spiking ceased on day 21; however, due to a timing oversight, sampling for metal and biomarker analyses did not occur until 23 days as per the original schedule. This meant that the final Cu and biomarker concentrations at 21 days when spiking ceased were not determined. It was postulated that it was unlikely that Cu concentrations would decrease appreciably over 48 hours but that in contrast the biomarker response was likely to be more immediate. Therefore data for both Cu and biomarkers were reviewed at 15, 23 and 28 days respectively and interpreted and reported accordingly.

3.2.1. Oyster copper concentrations

Cu concentrations in oysters varied among the spiked treatment concentrations (Table 8, Figure 21, Table 9), although at the majority of sampling times, the highest Cu treatments (15 and 30 µg/L) produced oysters with greater Cu accumulation than the lower treatments. Overall, oysters accumulated Cu for the 21-day exposure period with depuration of the accumulated Cu, which was

significant, demonstrated between days 23 and 28 after spiking had ceased. Results of two-way ANOVA of Cu concentrations in treatments across all time periods including exposure and depuration determined that there was a significant difference between treatment concentrations and time periods (Table 9). Although there was some overlap of concentrations, Cu concentrations in the control group were not significantly different to those in the next two highest treatments but were significantly lower than concentrations in the two highest treatment groups (15 and 30 µg/L) (Table 9).

Table 8. Oyster copper concentrations in copper spiked treatments over the bioassay period

N=3 except where * (n=2) due to samples being lost during processing.

Day	Copper Treatment (µg/L)				
	0	3.75	7.5	15	30
*0	66 ± 8	66 ± 8	66 ± 8	66 ± 8	66 ± 8
2	77 ± 12	66 ± 2	106 ± 9	*79 ± 18	83 ± 10
5	91 ± 10	82 ± 13	*115 ± 6	83 ± 6	71 ± 8
8	122 ± 3	*137 ± 21	*77 ± 3	141 ± 24	121 ± 7
12	99 ± 15	128 ± 23	*92 ± 23	104 ± 12	*121 ± 26
15	89 ± 10	93 ± 10	104 ± 16	150 ± 20	*130 ± 14
23	*126 ± 4	141 ± 19	160 ± 23	170 ± 30	222 ± 28
28	76 ± 5	132 ± 4	110 ± 5	138 ± 29	171 ± 15

Table 9. Summary of two-way ANOVAs on concentrations of copper in oysters by treatment (1= control to 5 = 30 µg/L) and time (baseline to 28 days includes depuration)

Tukey's multiple comparison test was used to locate between-level differences for significant main effects. Non- significant interaction terms are indicated = ns. *Where equality of variances could not be achieved through transformation of data, untransformed data is used. Sites are in descending order and arithmetic means are in parentheses.

Treat-ment sign.	Tukey's HSD multiple range test for treatment	Time sign.	Inter-action term sign.	Tukey's HSD multiple range test for time (days)
0.005	1 2 3 4 5	<0.0001	0.012	0 2 5 12 15 8 28 23
	1 2 3 (underlined)			0 2 5 (underlined)
	2 3 4 5 (underlined)			2 5 12 15 (underlined)
				12 15 8 28 (underlined)

Because oysters in all treatments had the same baseline Cu concentrations there was a lag period (up to 15 days) before oysters in each treatment began to separate in terms of the concentration of Cu accumulated. Concentrations of Cu were significantly higher at 23 days than at any other time period (Table 9). A longer exposure period or greater nominal concentrations may have produced more significant results. Although results were variable over time, the control group did not appear to be a true control with some accumulation of Cu. The value at 23 days for the control group may be an anomaly. Again a longer depuration period and a greater number of replicates (some were destroyed in processing) may have produced more significant results.

Physicochemical properties did not vary among treatments over the acclimation, exposure or depuration periods, with mean pH, conductivity (µs/cm), dissolved oxygen (mg/L) and temperature (ºC) (±1 SE) at 8.0 ± 0.0, 55.86 ± 0.14, 10 ± 0 and 22.4 ± 0.0, respectively.

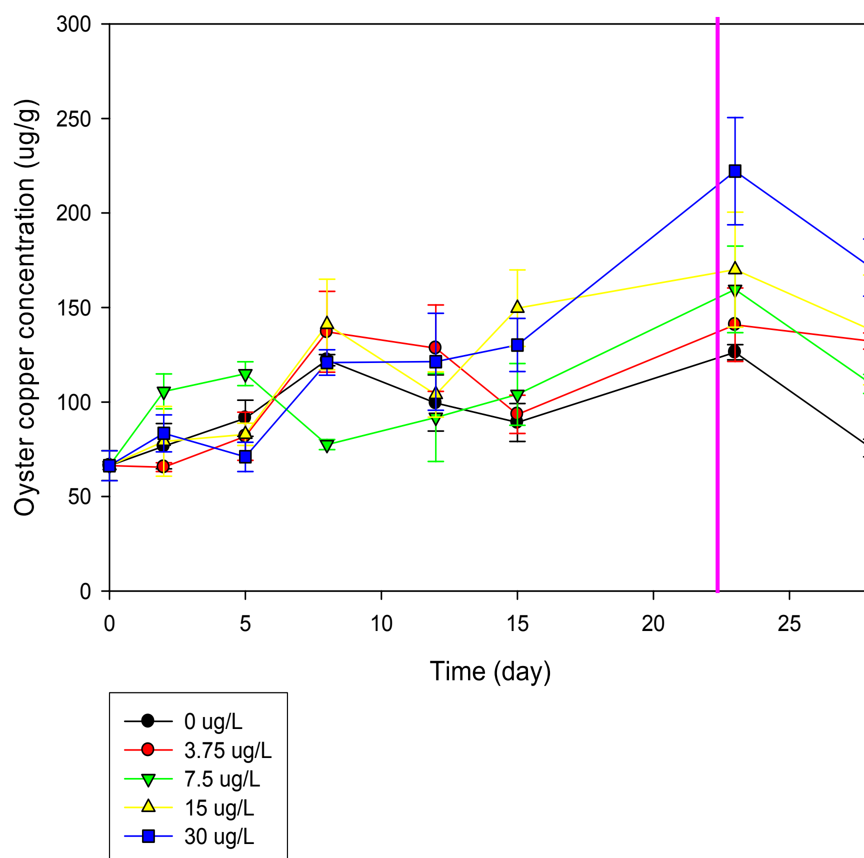


Figure 21. Accumulation in copper-exposed oysters from the five treatment concentrations
The vertical pink line indicates the start of the depuration period at 21 days, where all treatments used unspiked filtered sea water.

3.2.2. Oyster enzyme concentrations

A comparison of biomarker concentrations across the different tissues (Table 10) showed a similar pattern to the field-deployed oysters (Table 5). CAT and GST concentrations were higher in the hepatopancreas than the gills, while GSH concentrations were slightly higher in the gills than the hepatopancreas and LPO concentrations were similar across the two tissues (Table 10). Patterns of response for all biomarkers in both tissues were similar in the control group to the treatment, which indicated that a true control for the experiment may not have existed due to elevated background Cu concentrations in the filtered sea water to which green algae was added.

Gills

Two-way ANOVA demonstrated that there was no significant difference between treatment groups for any of the biomarkers; however there were some significant time differences and interaction terms (Table 11). All four biomarkers in gill tissue demonstrated a similar response to some degree in all treatments. This included controls, where there was an initial small decrease in enzyme concentrations at three days followed by a stimulatory response which then declined to less than baseline concentrations at 15 days (Table 10, Figures 22–25). The decline at 15 days was significant for LPO, GST and GSH (Table 11). There was little change in response for LPO or GSH during the depuration phase (23 to 28 days) except for the LPO control group which continued to decline up to 28 days whereas in the treatment groups LPO increased. For CAT and GST concentrations there appeared to be a stimulation of response back to baseline concentrations during the depuration phase (Figures 22–25), which was significant (Table 11).

Table 10. Concentration of biomarkers in gill and hepatopancreas of copper-exposed oysters in the five spiked treatments (0, 3.75, 7.5, 15 and 30 µg/L) including baseline concentrations

N=5 except where * (n=4) due to removal of outliers and ^ (n=3 or 4) due to insufficient protein in the sample.

Copper (µg/L)	Day	Catalase		Lipid peroxidase		Glutathione-S-transferase		Glutathione	
		Gills	Hepato	Gills	Hepato	Gills	Hepato	Gills	Hepato
0	0	1750 ± 112	3064 ± 378	87 ± 8	64 ± 6	55 ± 4	104 ± 9	15 ± 2	5 ± 2
	3	1695 ± 168	4798 ± 1009	65 ± 8	101 ± 10	39 ± 3	195 ± 13	15 ± 1	6 ± 1
	5	1818 ± 195	5475 ± 456	95 ± 12	106 ± 9	90 ± 9	230 ± 24	25 ± 5	7 ± 1
	8	2892 ± 406	2602 ± 341	102 ± 18	103 ± 5	73 ± 5	184 ± 18	17 ± 5	4 ± 1
	12	1338 ± 234	1601 ± 154	124 ± 12	123 ± 9	84 ± 9	198 ± 14	15 ± 2	4 ± 1
	15	1278 ± 86	1924 ± 223	60 ± 7	50 ± 4	17 ± 1	136 ± 13	5 ± 1	4 ± 1
	23	1953 ± 156	2518 ± 187	41± 4	57 ± 5	49 ± 2	286 ± 12	11 ± 1	5 ± 1
	28	1482 ± 148	3316 ± 401	26 ± 2	43 ± 6	69 ± 7	331 ± 34	3 ± 1	4 ± 1
3.75	0	1750 ± 112	3064 ± 378	87 ± 8	64 ± 6	55 ± 4	104 ± 9	15 ± 2	5 ± 2
	3	1905 ± 80	4137 ± 729	70 ± 6	91 ± 6	51 ± 6	177 ± 19	13 ± 1	8 ± 2
	5	3798 ± 683	3544 ± 590	101 ± 9	105 ± 4	81 ± 8	170 ± 17	21 ± 3	6 ± 2
	8	1925 ± 338	3238 ± 497	81 ± 8	117 ± 11	62 ± 6	168 ± 11	17 ± 1	5 ± 1
	12	1540 ± 186	1989 ± 211	113 ± 13	109 ± 5	80 ± 8	149 ± 17	18 ± 2	6 ± 1
	15	1048 ± 55	2355 ± 208	48 ± 4	44 ± 4	20 ± 3	178 ± 13	5 ± 1	4 ± 1
	23	1751 ± 223	2222 ± 336	36 ± 5	61 ± 8	56 ± 4	263 ± 22	9 ± 1	3 ± 1
	28	2371 ± 224	4359 ± 394	62 ± 12	71 ± 5	60 ± 4	289 ± 36	3 ± 0	4 ± 1
7.5	0	1750 ± 112	3064 ± 378	87 ± 8	64 ± 6	55 ± 4	104 ± 9	15 ± 2	5 ± 2
	3	1524 ± 114	3994 ± 476	75 ± 6	92 ± 3	44 ± 4	182 ± 15	16 ± 1	7 ± 2
	5	1843 ± 119	5035 ± 989	95 ± 8	99 ± 9	64 ± 6	126 ± 16	22 ± 3	5 ± 1
	8	2048 ± 204	2674 ± 256	92 ± 10	123 ± 10	75 ± 5	202 ± 28	18 ± 1	5 ± 1
	12	1626 ± 188	1491 ± 145	89 ± 10	88 ± 7	59 ± 6	125 ± 13	17 ± 3	4 ± 1
	15	920 ± 60	1616 ± 116	39 ± 3	48 ± 4	21 ± 1	179 ± 14	7 ± 1	3 ± 0
	23	1869 ± 184	2858 ± 196	48 ± 6	55 ± 6	50 ± 2	269 ± 20	11 ± 1	5 ± 1
	28	1859 ± 281	2578 ± 259	55 ± 7	53 ± 4	53 ± 4	303 ± 40	2 ± 0	4 ± 1
15	0	1750 ± 112	3064 ± 378	87 ± 8	64 ± 6	55 ± 4	104 ± 9	15 ± 2	5 ± 2
	3	1411 ± 165	3257 ± 527	79 ± 7	92 ± 8	53 ± 5	166 ± 16	15 ± 2	6 ± 1
	5	2355 ± 335	3676 ± 486	92 ± 10	85 ± 5	61 ± 4	142 ± 6	18 ± 2	5 ± 1
	8	1501 ± 153	2086 ± 320	110 ± 8	126 ± 10	82 ± 11	205 ± 24	18 ± 2	6 ± 2
	12	1586 ± 218	1579 ± 177	105 ± 15	112 ± 6	63 ± 4	168 ± 15	19 ± 2	5 ± 1
	15	812 ± 56	1831 ± 165	40 ± 4	41 ± 4	17 ± 2	122 ± 15	6 ± 1	4 ± 0
	*23	1810 ± 252	3067 ± 250	41 ± 7	48 ± 4	71 ± 13	323 ± 30	4 ± 1	3 ± 1
	*28	1698 ± 233	2870 ± 264	76 ± 23	73 ± 5	52 ± 5	297 ± 23	^2 ± 0	3 ± 1
30	0	1750 ± 112	3064 ± 378	87 ± 8	64 ± 6	55 ± 4	104 ± 9	15 ± 2	5 ± 2
	3	1374 ± 108	5401 ± 839	74 ± 9	110 ± 8	67 ± 3	186 ± 13	17 ± 2	7 ± 1
	5	2097 ± 264	4535 ± 414	103 ± 9	100 ± 6	73 ± 5	134 ± 17	19 ± 3	6 ± 1
	8	2403 ± 364	2025 ± 302	101 ± 11	131 ± 13	80 ± 6	213 ± 23	18 ± 2	8 ± 2
	12	1408 ± 165	2039 ± 511	105 ± 11	139 ± 13	52 ± 4	258 ± 36	17 ± 3	5 ± 1
	15	1289 ± 75	^1505 ± 229	63 ± 9	^51 ± 4	27 ± 4	^133 ± 15	5 ± 1	4 ± 1
	23	2044 ± 155	2695 ± 329	61 ± 9	59 ± 7	52 ± 3	294 ± 17	^2 ± 1	3 ± 0
	28	2881 ± 599	2693 ± 198	72 ± 10	74 ± 9	51 ± 4	289 ± 22	^2 ± 1	3 ± 1

Hepatopancreas

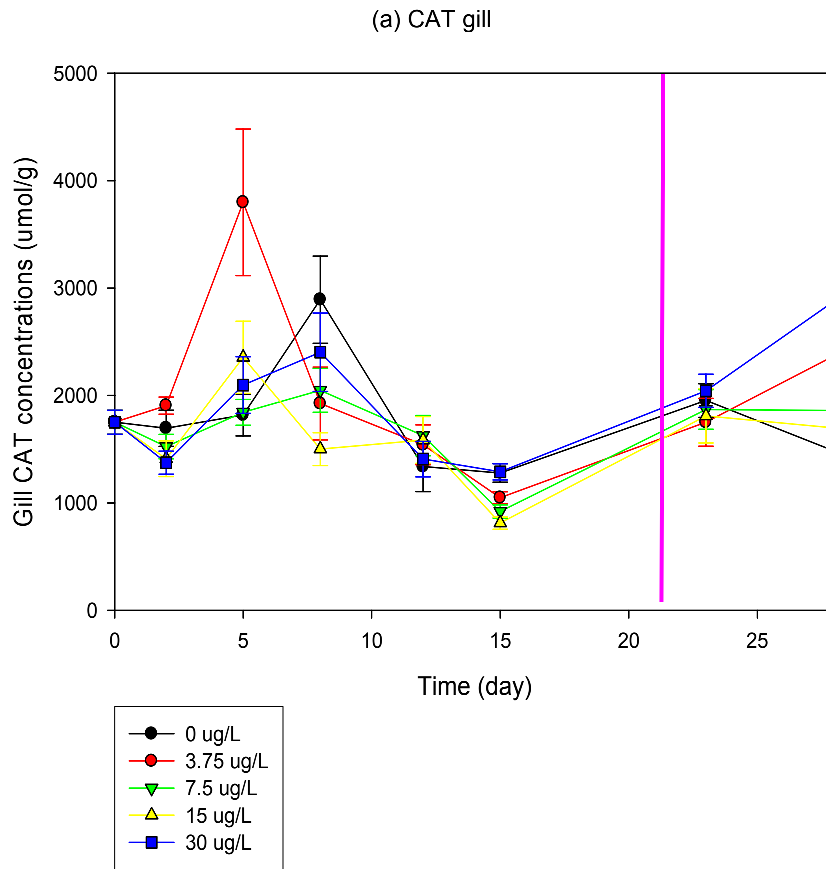
The only biomarker to show significant differences between treatments was LPO, with highest concentrations in the 30 µg/L treatment which was not significantly different to the first treatment group. Concentrations of LPO in the 30 µg/L treatment were, however, more elevated than in all other treatments (Tables 10 and 11). There were some significant time and interaction terms for all biomarkers. CAT and LPO in hepatopancreas followed a similar pattern to that in gill tissue but the response was immediate, without the initial delay or decline at three days as seen in gill tissue. For CAT in hepatopancreas the stimulatory response was much shorter, declining at 8 days rather than 15 days as seen for LPO in both tissues (Table 11, Figures 22–25). GST was variable but remained stable over the exposure phase apart from an initial significant increase from baseline, whereas GSH demonstrated a trend for a steady decline to 15 days.

As with CAT and GST in gills, there was a stimulation of enzyme response for CAT and GST in hepatopancreas in the depuration phase in all treatments, which was significant for GST. Concentrations returned to baseline for CAT but were significantly higher than any other time period for GST (Table 11). In contrast, for LPO and GSH in gill tissue there was no significant change in these biomarkers during the depuration phase, except for LPO which declined in the control group at 28 days whereas the other treatments increased or remained stable. This was a similar response as LPO in gill tissue (Tables 10 and 11, Figures 22–25).

Table 11. Summary of two-way ANOVAs on concentrations of each enzyme in oyster tissues (gill and hepatopancreas) by treatment (1= control to 5 = 30 µg/L) and time (baseline to collection seven, includes depuration)

Tukey's multiple comparison test was used to locate between-level differences for significant main effects for time. Significant main effects for treatment for LPO are described in the text. Nonsignificant interaction terms are indicated = ns. Results of *a posteriori* Tukey's test for treatment (one significant value) are described in the text.

Enzyme		Treatment sign.	Time sign.	Tukey's HSD multiple range test for time (days)								Inter-action term sign
Gill	CAT	ns	<0.0001	15	12	2	0	23	8	28	5	0.001
	LPO	ns	<0.0001	23	15	28	0	2	8	5	12	ns
	GST	ns	<0.0001	15	2	0	23	28	12	5	8	<0.0001
	GSH	ns	<0.0001	28	15	23	2	0	12	8	5	ns
Hep	CAT	ns	<0.0001	12	15	8	23	0	28	2	5	0.028
	LPO	0.004	<0.0001	15	23	28	0	2	5	12	8	0.013
	GST	ns	<0.0001	0	15	5	12	2	8	23	28	0.002
	GSH	ns	<0.0001	23	15	28	12	0	8	5	2	ns



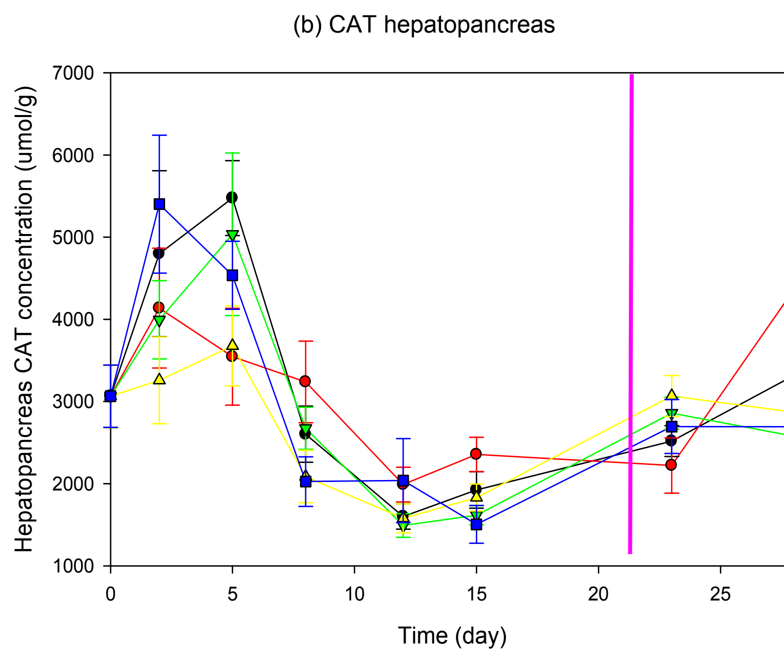
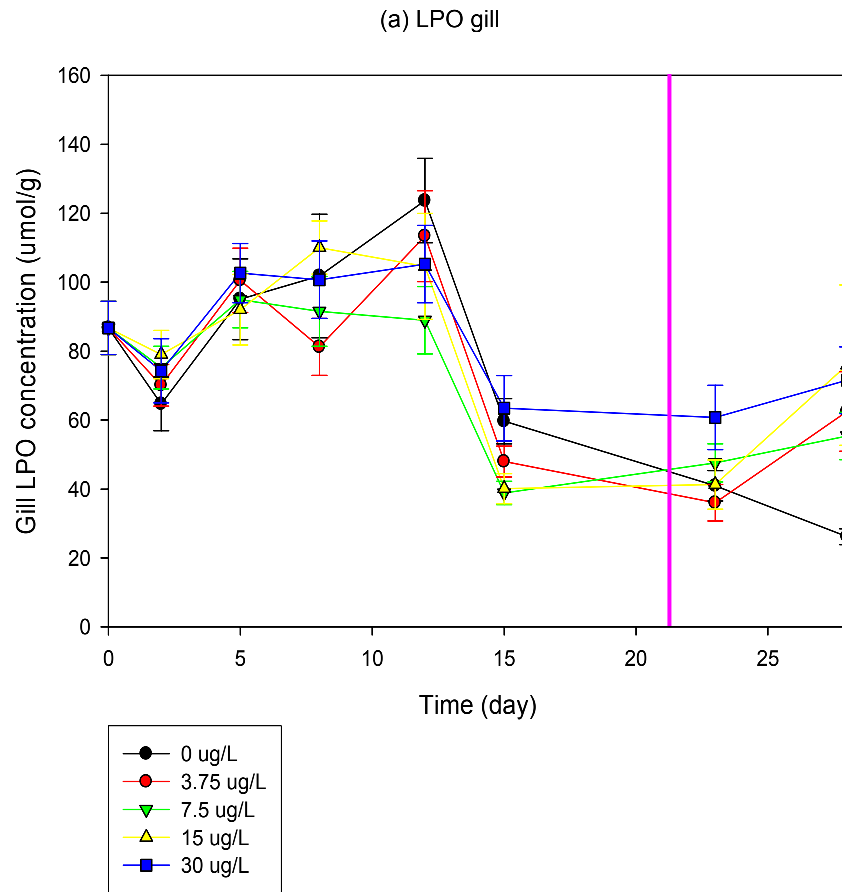


Figure 22. Mean ±1 SE concentration (μmol/g) of CAT in (a) gill and (b) hepatopancreas in oysters in the five spiked treatments (0, 3.75, 7.5, 15 and 30 μg/L) including baseline concentrations



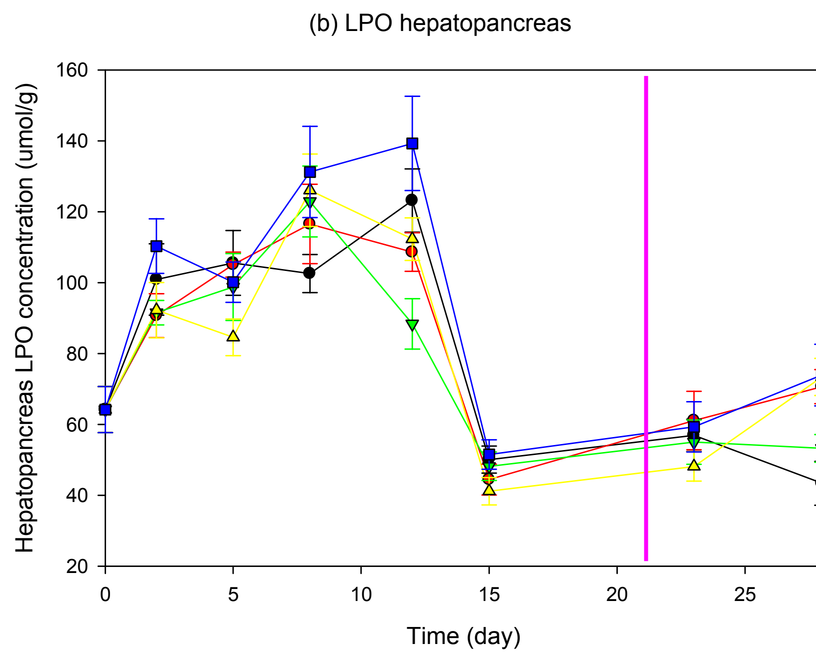
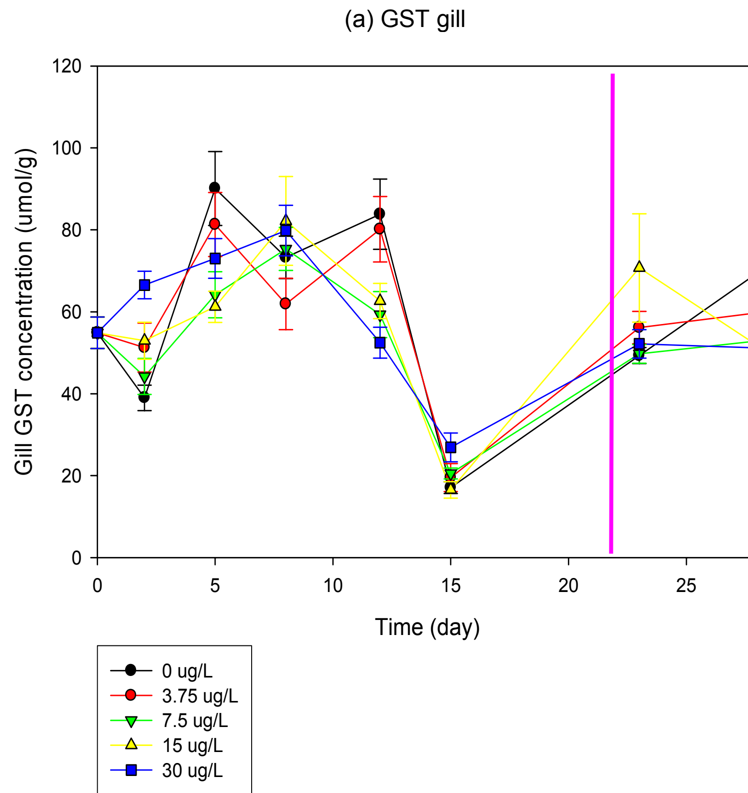


Figure 23. Mean ±1 SE concentration (µmol/g) of LPO in (a) gill and (b) hepatopancreas in oysters in the five spiked treatments (0, 3.75, 7.5, 15 and 30 µg/L) including baseline concentrations



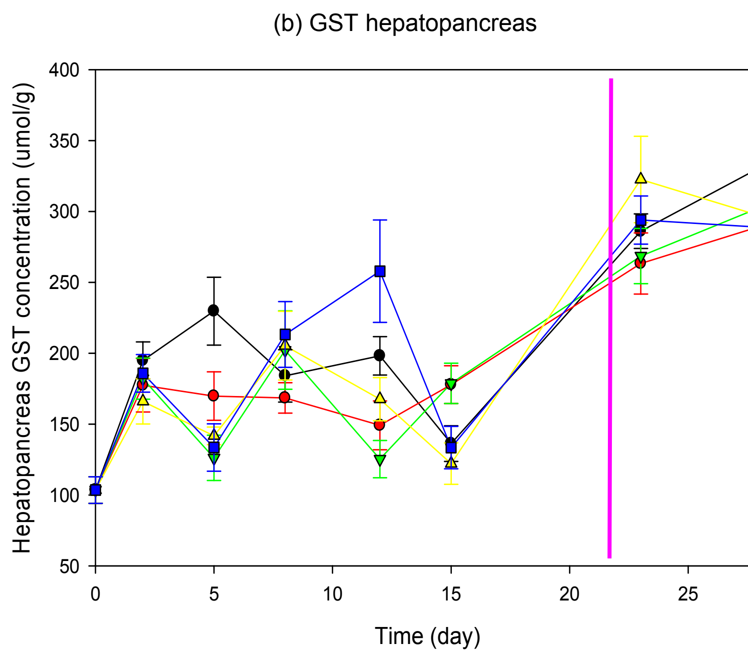
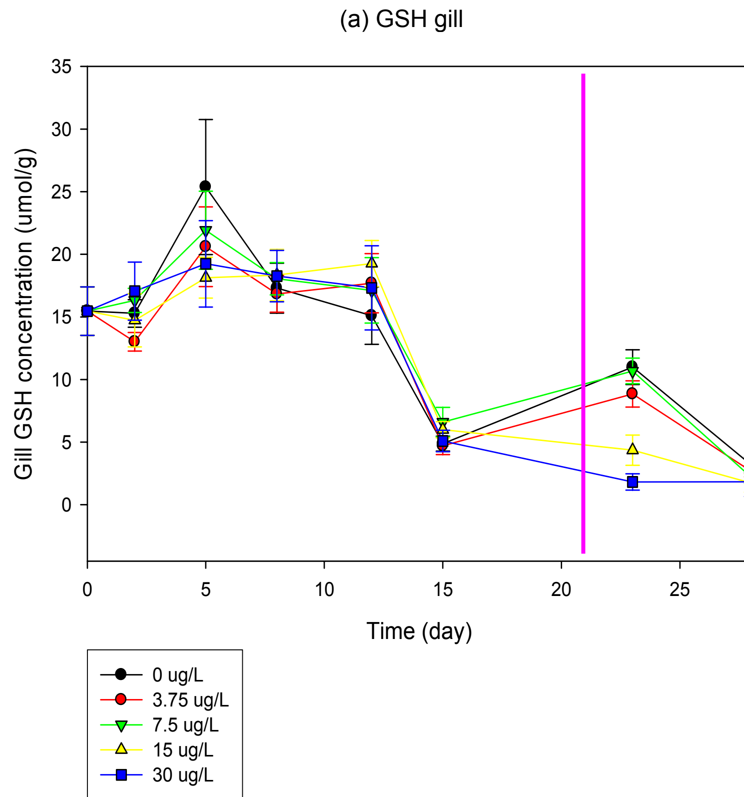


Figure 24. **Mean ±1 SE concentration (µmol/g) of GST in (a) gill and (b) hepatopancreas in oysters in the five spiked treatments (0, 3.75, 7.5, 15 and 30 µg/L) including baseline concentrations**



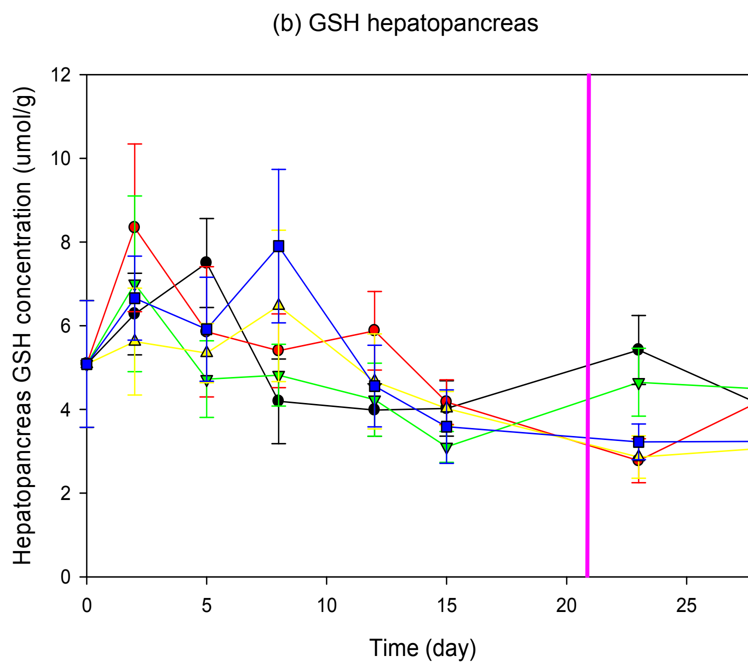


Figure 25. Mean ±1 SE concentration (µmol/g) of GSH in (a) gill and (b) hepatopancreas in oysters in the five spiked treatments (0, 3.75, 7.5, 15 and 30 µg/L) including baseline concentrations

Regression over time

There were a number of significant nonlinear relationships between Cu and biomarkers identified after 15 days, 23 days (two days after Cu spiking ceased) and at 28 days (which included depuration data) through regression analyses (Figures 26–30). Only LPO in hepatopancreas and GST in gill were significant at 15 days. Unlike the field experiment where the only significant regression over time was CAT in both tissues, in the Cu exposure experiment GSH and GST in both tissues were the only biomarkers to show significant regressions in spiked treatments.

For gill GSH there was a significant, strong relationship in the highest treatment group for 23 days [the second-highest treatment group was almost significant ($r^2 = 0.77$, $p = 0.053$)] and highest three treatment groups for 28 days [the first treatment group was almost significant ($r^2 = 0.65$, $p = 0.07$) (Figure 26)], whereas the relationship was not significant for controls. The relationships over both time periods became stronger and more significant as the concentrations of copper increased in successive treatments. In all groups there was an initial stimulation of GSH followed by a decline over time which was more pronounced in the higher concentration copper treatments. Gill GST followed a similar trend which was significant in the highest treatment group but at 15 days of exposure only ($r^2 = 0.93$, $p = 0.007$ therefore before copper spiking ceased) (Figure 29). This is likely due to the restimulation of GST production, which occurred during depuration.

Hepatopancreas GSH also demonstrated an initial response followed by a decrease in GSH over time which was significant in the 15 µg/L group but not the highest concentration group (30 µg/L) where there was a weak relationship ($r^2 = 0.58$, $p > 0.05$) at both 23 and 28 days (Figure 27). GST in hepatopancreas demonstrated a linear relationship across time at 23 and 28 days which was significant in all treatments including the controls at 28 days, although the relationship was stronger in the three middle treatments compared to the controls or highest treatment (Figure 28). The relationship was most likely influenced by the stimulation of GST production which occurred during depuration. Relationships were not significant at 15 days. LPO in gills at 28 days in the control group was the only significant regression over time for LPO and this may be due to the concentration of LPO in the control group decreasing at 28 days (depuration) whereas it increased in all treatment groups (Figure 30).

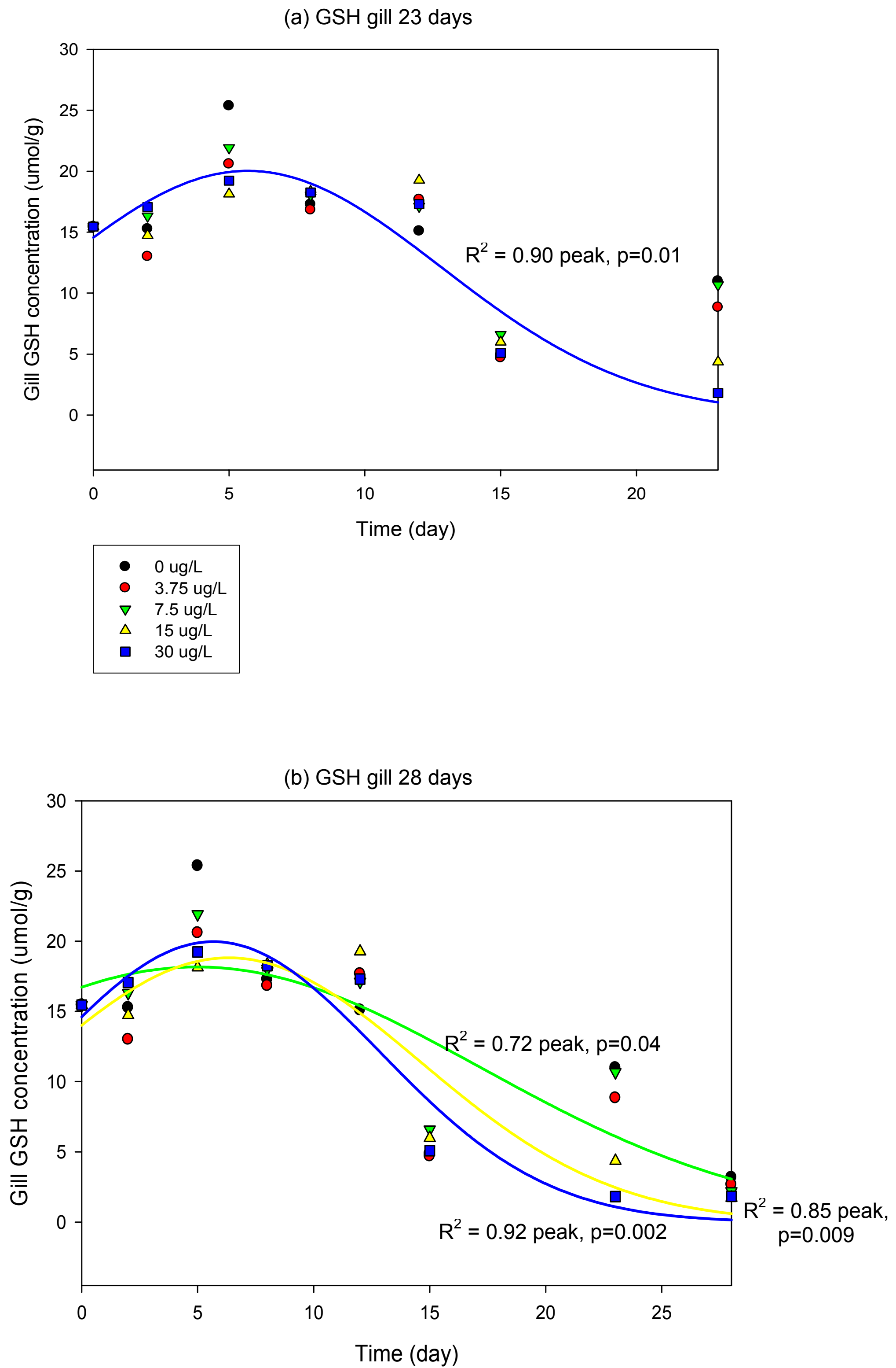
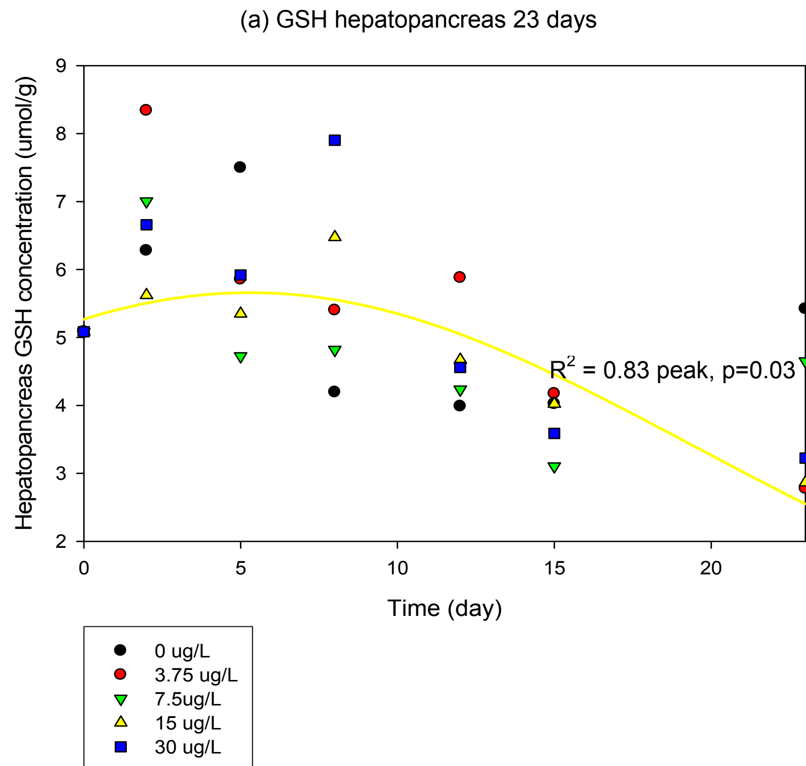


Figure 26. Regression of mean GSH concentration in gills against time (a) 23 days and (b) 28 days in each treatment (0, 3.75, 7.5, 15 and 30 µg/L) including baseline

The r^2 values of significant regressions are demonstrated. Regression was almost significant in 15 µg/L at 23 days **(r^2 = 0.77, p = 0.053).**



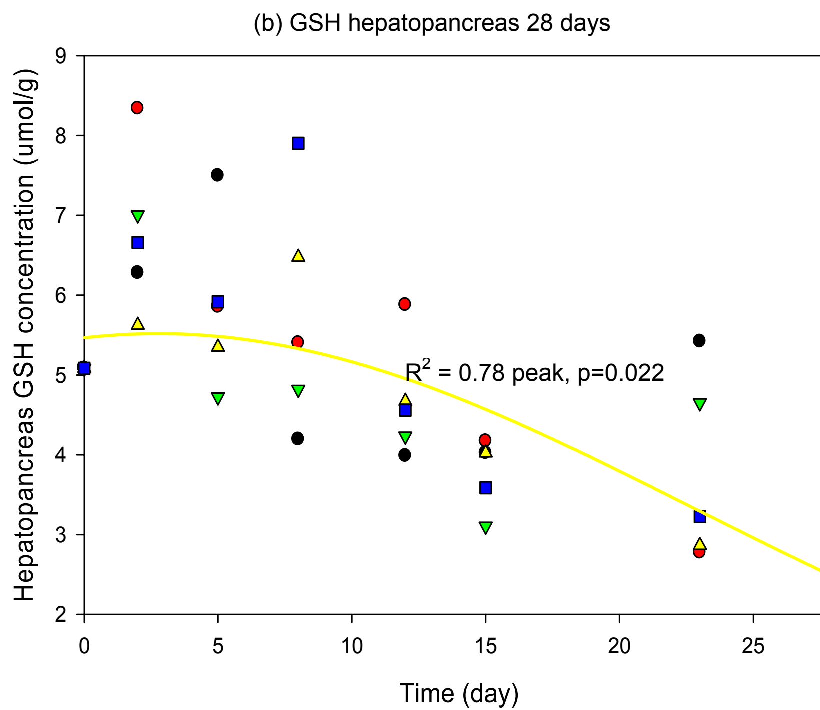
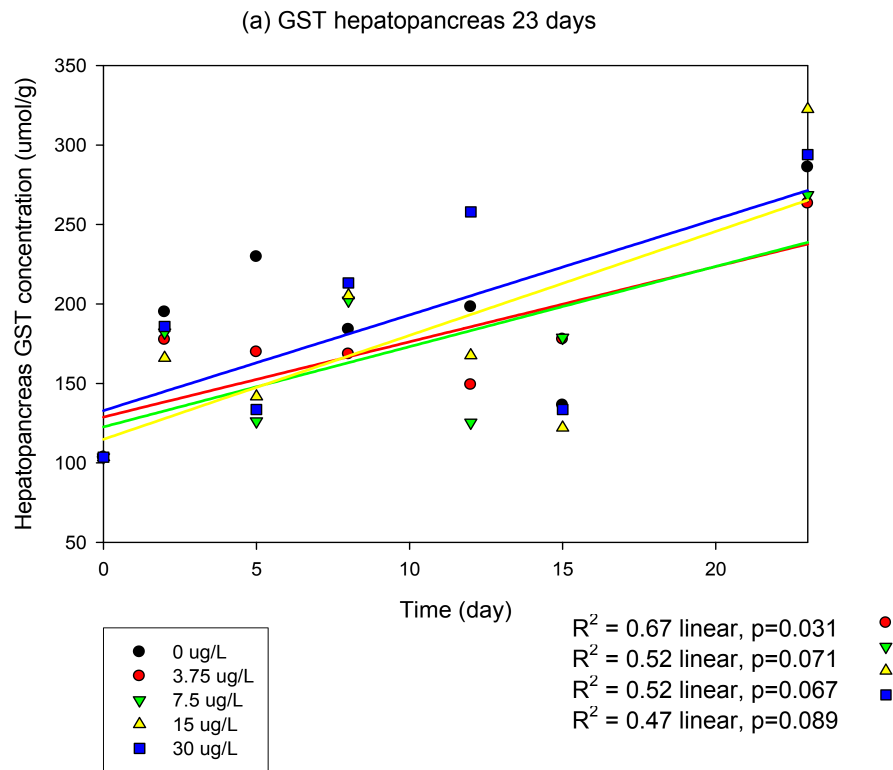


Figure 27. Regression of mean GSH concentration in hepatopancreas against time (a) 23 days and (b) 28 days in each treatment (0, 3.75, 7.5, 15 and 30 µg/L) including baseline

The r^2 values of significant regressions are demonstrated. Regressions were almost significant in 30 µg/L at 23 and 28 days **(r^2 = 0.58, p = 0.162 and r^2 = 0.58, p = 0.111, respectively).**



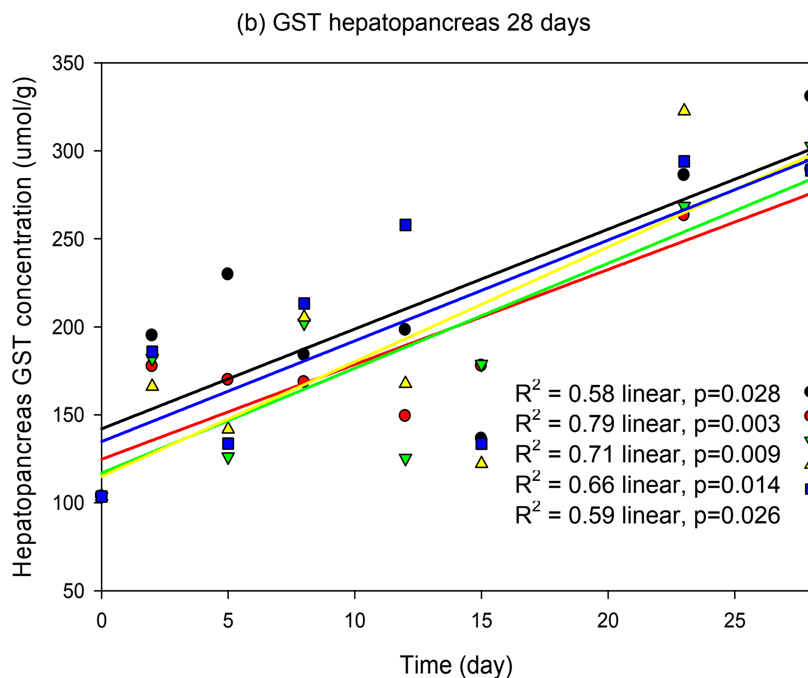


Figure 28. Regression of mean GST concentration in hepatopancreas against time (a) 23 days and (b) 28 days in each treatment (0, 3.75, 7.5, 15 and 30 µg/L) including baseline

The r^2 values of significant regressions are demonstrated.

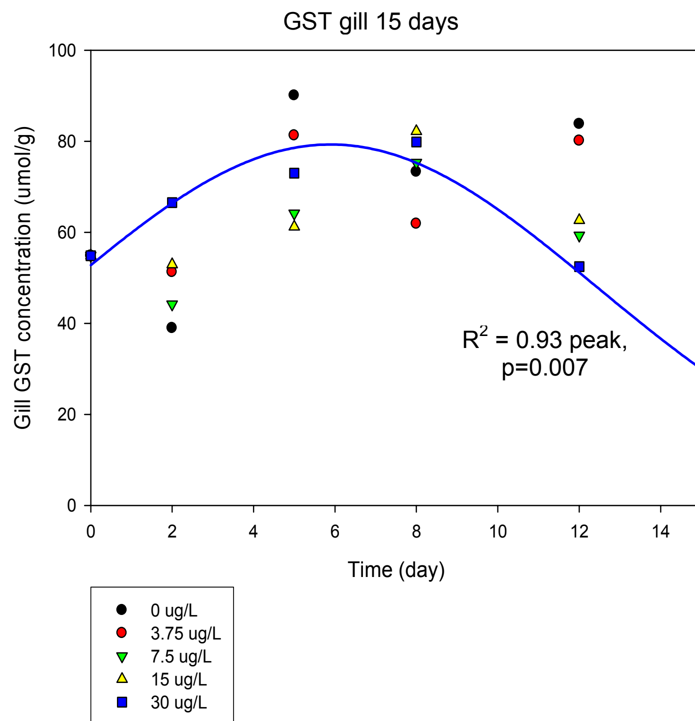


Figure 29. Regression of mean GST concentration in gill against time 28 days in each treatment (0, 3.75, 7.5, 15 and 30 µg/L) including baseline

The r^2 values of significant regressions are demonstrated.

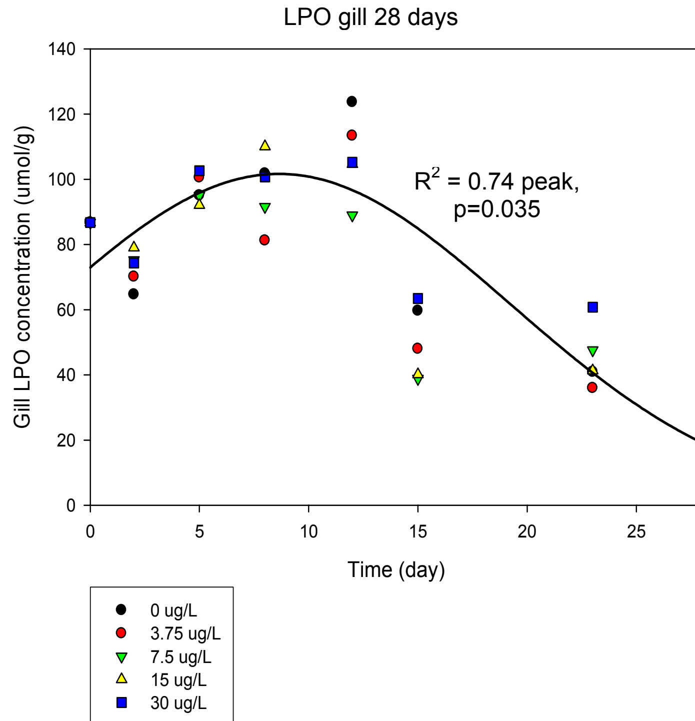


Figure 30. Regression of mean LPO concentration in gill against time 28 days in each treatment (0, 3.75, 7.5, 15 and 30 µg/L) including baseline

The r^2 values of significant regressions are demonstrated.

3.2.3. Comparison of copper and enzyme concentrations

Correlations

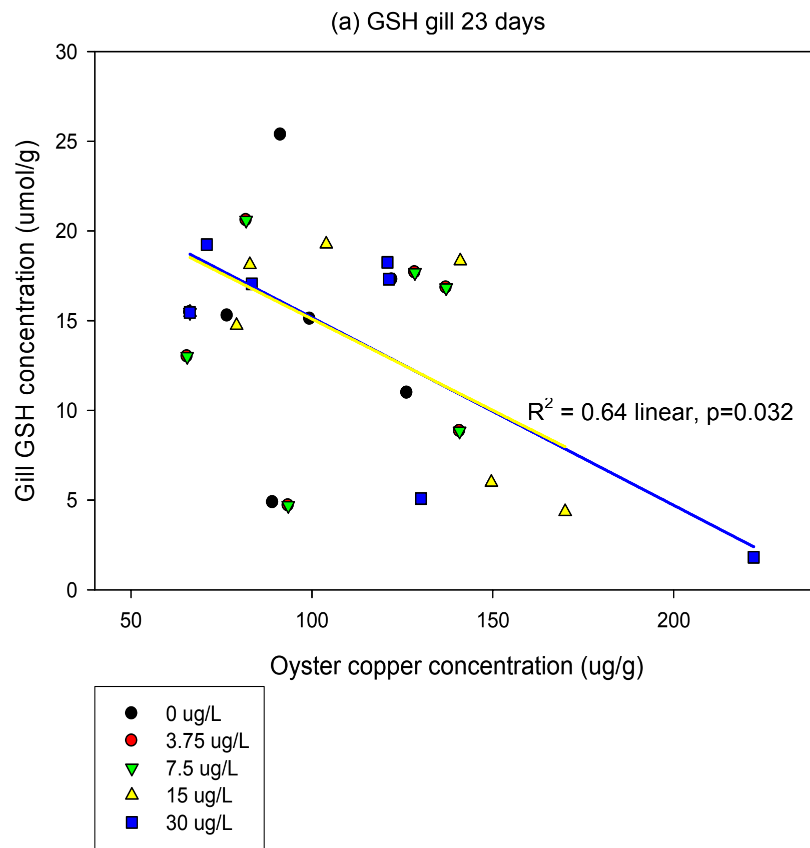
Significant correlations were found between biomarker and Cu concentrations in the laboratory oysters (Table 12). However, unlike the field-deployed oysters, where CAT and LPO were significantly correlated with metal concentrations, GST and GSH were the only biomarkers significantly correlated with oyster Cu concentration in the laboratory experiment, and only in the highest treatment (30 µg/L). Hepatopancreas GST increased significantly as oyster copper concentrations increased, while gill GSH concentrations decreased significantly over the exposure phase and combined exposure/depuration phases (Table 12).

Table 12. Correlations between copper concentrations and biomarker concentrations in gills and hepatopancreas of oysters after 23 days of exposure and 28 days which included the depuration phase. Only significant correlations shown (α = 0.05).

Tissue	Enzyme	Copper treatment (µg/L)	Correlation value	P value
23 days				
Gill	GSH	30	-0.798	0.032
Hepatopancreas	GST	30	0.784	0.037
28 days				
Gill	GSH	30	-0.823	0.012
Hepatopancreas	GST	30	0.815	0.014

Regressions

Oyster Cu concentrations were plotted against biomarker concentrations in each tissue in each treatment and regression analyses performed after 23 and 28 days in order to determine whether there were any additional nonlinear associations (Figures 31 and 32). GST in hepatopancreas and GSH in gills were the only enzymes to show significant linear relationships with oyster Cu concentrations after 23 days, similar to the correlation results. GSH decreased and GST increased with increasing Cu concentrations in oysters (Figure 31). For both biomarkers the moderate relationships were significant only in the highest treatment (30µg/L). However, for GSH the second treatment was almost significant (r^2 = 0.45, p = 0.099) and for GST the next highest two treatments (15 and 7.5 µg/L) were almost significant (r^2 = 0.47, p = 0.089 and r^2 = 0.49, p = 0.079), respectively. Interestingly, the best line of fit for gill GSH at 28 days which included depuration data was peak rather than the linear relationship demonstrated at 23 days (Figure 32).



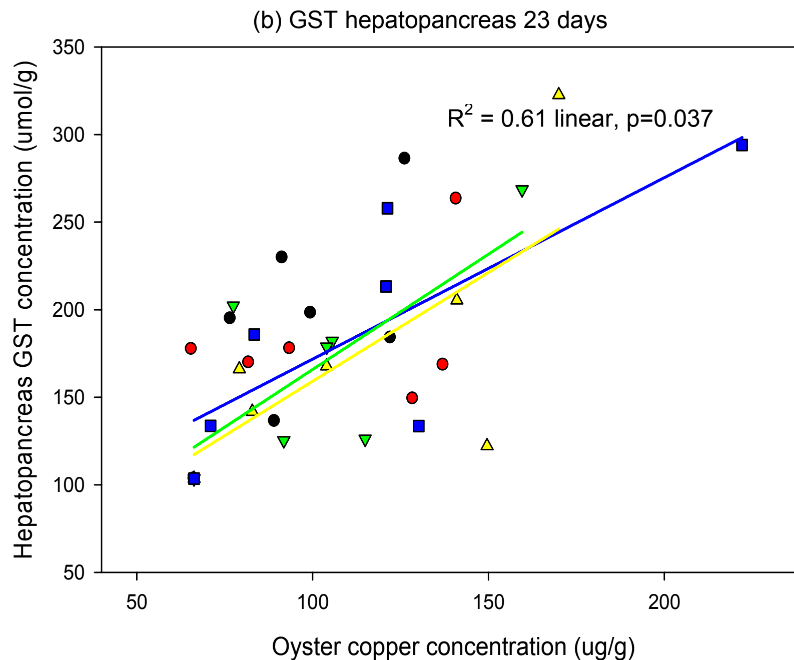


Figure 31. Regression of (a) mean GSH concentration in gills and (b) mean GST in hepatopancreas against oyster Cu concentrations after 23 days in each treatment (0, 3.75, 7.5, 15 and 30 µg/L) including baseline

The r^2 values of significant regressions are demonstrated. The regression for the 15 µg/L treatment for gills was almost significant at **r^2 = 0.45, p=0.099.** The regressions for the 15 and 7.5 µg/L treatments for hepatopancreas were almost significant at **r^2 = 0.47, p=0.089 and r^2 = 0.49, p=0.079 respectively.**

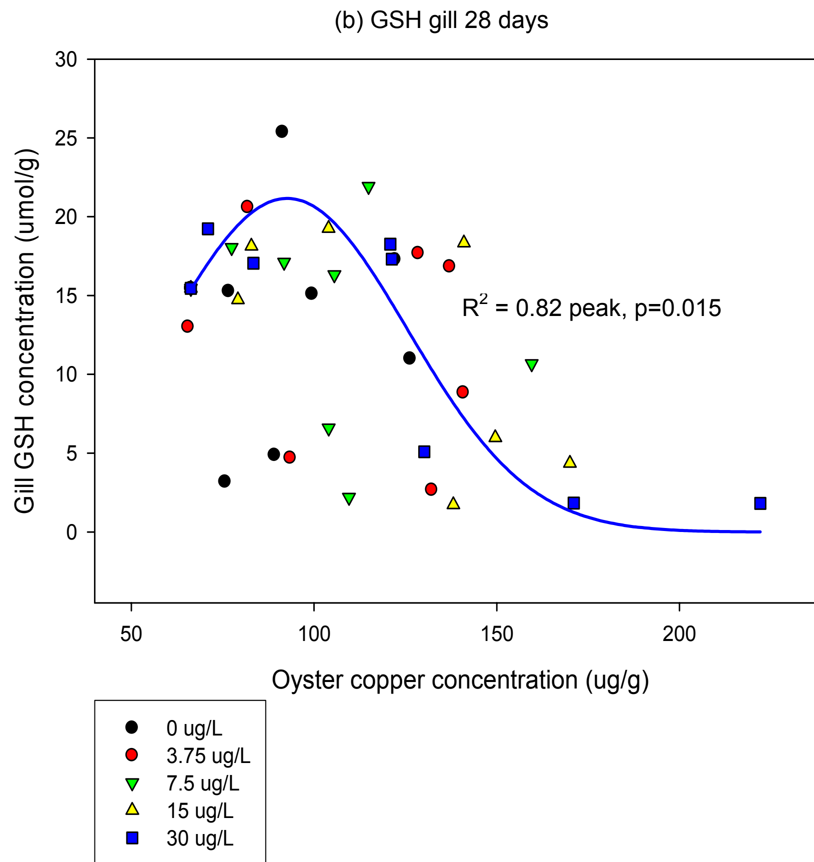


Figure 32. Regression of mean GSH concentration in gills against oyster Cu concentrations after 28 days in each treatment (0, 3.75, 7.5, 15 and 30 µg/L) including baseline

The r^2 values of significant regressions are demonstrated.

4. Discussion

4.1. Oyster metal accumulation

4.1.1. Field study

The patterns of metal accumulation in oysters deployed at two sites—an inner harbour impacted site and an outer harbour reference site—were similar to those observed in other deployment studies over longer time periods (Andersen *et al.*, 2004; Andersen *et al.*, 2005b). Copper, zinc (and to a lesser extent aluminium) tend to be the three common metals of concern bioaccumulated in biota in Port Curtis (Andersen *et al.*, 2005a), and these metals were accumulated at significantly greater amounts at Site 1 oysters in this study over the shorter deployment period. Due to oysters at both sites having started with the same baseline concentration, there was a lag period (5–8 days) before the deployed oysters at both sites began to reflect the different concentrations of metals. Although the uptake of metals in oysters is thought to be rapid and linear (Ritz *et al.*, 1982; Jones *et al.*, 2000), the deployment period may not have been sufficient to attain a significant difference in metal concentrations between the two sites for some metals. Concentrations of copper and zinc in oysters at Site 1 were 138 and 967 µ/g respectively after 29 days deployment in this study, compared to 272 and 1386 µg/g at the same site after 8 weeks of deployment (Andersen *et al.*, 2005b).

Although arsenic was more elevated in the reference site oysters this does not necessarily mean that there is contamination of arsenic at the reference site. Previous studies in the area have determined that arsenic tends to bioaccumulate in higher concentrations at oceanic sites compared with inner harbour sites in Port Curtis (Andersen & Norton, 2001; Andersen *et al.*; 2004, Andersen *et al.*, 2005a). Antagonistic interactions for uptake between metals have been demonstrated previously (Phillips, 1990) and the uptake of arsenic has been negatively correlated with the uptake of both copper and zinc in oysters (Mackay *et al.*, 1975). Therefore a competitive interaction may have caused a decrease in uptake of arsenic at the inner harbour site due to the presence of elevated copper and zinc.

It is difficult to compare concentrations of metals in resident oysters from Sites 1 and 2 with those in the transplanted oysters due to differences in exposure time, exposure history and physiology. However, similar patterns in accumulation occurred at both sites for the two different groups of oysters for many metals,

indicating that the transplanted oysters over the short term were a good indicator of ambient metal concentrations likely to occur over a longer period. Previous studies (Andersen *et al.*, 2003; Andersen *et al.*, 2004; Andersen *et al.*, 2005b) have used deployment periods of eight to ten weeks and have found this time period to be sufficient to allow metal accumulation representative of the environmental conditions in Port Curtis. However, the linear uptake of copper and zinc had not reached a threshold at 70 days, suggesting the acclimation to ambient had not yet occurred. This is substantially longer than the 28 day deployment in the current study.

4.1.2. Laboratory copper bioassay

Copper concentrations fluctuated over the laboratory bioassay period; however, net accumulation of copper from day 0 to day 23 (the last point recorded before depuration) was observed. Oysters in the laboratory had the same baseline concentrations as those in the field experiment, and there was a delay period of up to 15 days before there was obvious separation of treatments in terms of copper accumulation. Designated water concentrations were nominal rather than measured and it was suspected that there was some contamination of copper in the lower treatment groups through the introduced algal food which may have added to the lack of significance in copper concentrations between groups. Due to the fluctuating concentrations of copper in the control group a true control may not have existed.

Oysters from each copper treatment exhibited decreased copper concentrations following depuration. Studies determining the efflux rates for metals in oysters have found that they are slow for some metals in this species (Ke & Wang, 2001), with copper half-lives of up to 376 days in gills in other species (*Crassostrea gigas*) (Gefford *et al.*, 2002). Although copper spiking ceased on day 21 and concentrations in oysters were not measured until day 23, it is likely that due to the slow efflux rates very little depuration of copper would have occurred over the 48 hours.

4.2. Biomarker responses to metal concentrations

4.2.1. Field study

There appeared to be an initial stimulatory response of lipid peroxidation (LPO), glutathione-s-transferase (GST) and glutathione (GSH) in both tissues at Site 2, the oceanic site, which was unlikely to be related to metal concentrations. At the time of deployment there was a large blue-green algal bloom, later identified as *Trichodesmium erythraeum,* which dissipated after approximately two weeks. The oysters were located in an embayment where significant amounts of the algae accumulated. Cyanobacteria are known to produce antioxidant and peroxidative responses in bivalves due to their neurotoxic (Choi *et al.*, in press) or hepatotoxic (Davies *et al.*, 2005) properties. It is possible that the observed response at Site 2 was due to exposure to algal toxins rather than to metal contamination. Physiochemical properties were similar at the two sites which eliminates variations in results due to inherent changes in water parameters.

In addition to algal toxins it appears that handling stress in oysters may have an effect on some enzyme responses. Catalase (CAT) concentrations decreased appreciably between removal from water at the lease and deployment approximately three days later. Some recovery of CAT occurred within seven days once the oysters were returned to water. This may be a stress reaction by the oyster to changed conditions such as decreased oxygen and food supplies and retention of excretable products.

Aside from the initial response at Site 2, biomarker responses generally followed the same variable pattern at both sites, except for CAT and GST in gills and GSH in hepatopancreas. CAT was the only biomarker to demonstrate a significant relationship over time, having the same response in gills at Site 2 and hepatopancreas at both sites. CAT and LPO exhibited significant linear responses to increased concentrations of certain metals, namely aluminium, cadmium, chromium, copper, lead, and nickel with the majority of responses at Site 1, the more impacted site, although the relationships were not strong. This indicates that some biomarker responses were influenced by accumulated metal concentrations. As concentrations of these metals increased, CAT concentrations increased in the gills while decreasing in the hepatopancreas but LPO increased in both tissues. Elevated LPO concentrations indicate cell damage due to imbalance of the antioxidative mechanisms. Significantly elevated concentrations of LPO in gills at Site 1 indicate that some cell damage may be occurring.

Increased LPO during exposure to metals has been recorded in several organisms including oysters, mussels, polychaetes, fish and frogs (Winston &

Giulio, 1991; Regoli & Principato, 1995; Doyotte *et al*., 1997; Ringwood *et al*., 1999; Nusetti *et al*., 2001; Papadimitriou & Loumbourdis, 2002). An increase in peroxidative processes is a common pathway of toxicity induced by environmental pollutants, as reactive oxygen species are produced in electron transfer reactions (Regoli *et al*., 1998). LPO also increased exponentially to a maximum as cadmium concentrations increased at Site 1, indicating that at a threshold of cadmium concentration the LPO response levelled out. LPO levels were higher in the resident oysters of both sites than the deployed oysters. This could be due to the higher accumulated metal concentrations found in the resident oysters at each site, thus further confirming the increased incidence of LPO with increasing metal concentrations.

Exposure to contaminants tends to rapidly induce antioxidant enzymes, such as CAT (Fitzpatrick *et al*., 1997). Other studies have found varying responses of CAT to increased metal concentrations, with some organisms exhibiting increased activity, others exhibiting depressed activity, and still others showing no CAT response at all (Winston & Giulio, 1991; Doyotte *et al*., 1997; Regoli *et al*., 1998; Cheung *et al*., 2001). The hepatopancreas is the preferred organ for detoxification. However, many metal species may bind to GSH and inhibit the enzymes involved with GSH and CAT metabolism. The rapid initial decline of GSH at Site 1 which was not observed at Site 2 suggests that some metal species may have bound to this substrate. GSH may have been consumed during metal sequestration at Site 1. However, after 15 days there appeared to be recovery of GSH concentrations, again suggesting that there may be acclimation or stimulation of production to cope with the changed environmental conditions. Adaptation is commonly observed in biomarker responses in many species (Wu *et al*., 2005). It is also not uncommon to see inhibition rather than stimulation of some biomarker responses.

Several nonlinear associations were also identified between GST and aluminium and cadmium and chromium in both tissues. One of the objectives of this study was to determine if some biomarker responses were transient before some compensatory or adaptive mechanism occurred. Therefore, in both the laboratory and field studies, sampling occurred more intensively at the beginning of deployment in order to identify initial transitory responses and to record the overall changes over time. For the majority of GST responses there were initial increases in enzyme concentration with increasing metal concentrations until a threshold was reached, after which the response reversed. There may be an initial stimulatory response to metals after which time the oysters acclimates to its new ambient conditions; however, there may also be a point where breakdown of the enzyme response occurs. At the relatively low metal concentrations

experienced by the oysters in this study, it is more likely that the GST antioxidant response is a first line of defence and that other scavenging mechanisms may take over after a certain metal threshold is reached.

Conditions in Port Curtis could not be considered as contaminated *per se*, especially in comparison to other biomarker studies where bivalves have been transplanted into known polluted locations (Doyotte *et al*., 1997; Fitzpatrick *et al*., 1997; Regoli *et al*., 1998). Although bioaccumulation of metals in Port Curtis has been demonstrated (Andersen *et al*., 2002; Andersen *et al*., 2005a), dissolved metal concentrations in the water column are not above regulatory concern (Apte *et al*., 2005). However, the effects of pulse discharge events are unknown. The response of the biomarkers to exposure to algal toxins may have been more profound than the response to metal exposure. The results indicate that biomarker responses can be complicated and are dependent on the different metals and species. In addition there may be responses to other contaminants not measured in this study. Further investigation would be required to determine if these particular biomarkers are suitable for use in less contaminated situations.

4.2.2. Laboratory bioassay

In contrast to the field study where CAT and LPO responses featured, GST and GSH exhibited marked responses to increasing oyster copper accumulation in the laboratory exposure. While there were no significant relationships between GSH and metals in the field, there were significant responses to increasing copper exposure concentrations in the laboratory. GSH concentrations in hepatopancreas and gill tissue increased initially as copper accumulation increased which was maintained for approximately 12 days, after which time enzyme concentrations declined. The degree of both stimulation and decline was stronger with greater copper exposure concentrations and with significant relationships over time, only in the top three treatment concentrations for gill tissue. This indicates that a stress response could be induced at continual nominal copper exposure concentrations of at least 7.5 µg/L. However, as average dissolved copper concentrations in Port Curtis are less than half this value, it is not surprising that a similar response was not observed in the field. The effect of shorter, pulse discharges of greater concentrations of copper on biomarker responses is unknown.

The control group in most instances demonstrated the same temporal changes as the treatment groups. This suggests that either the observed temporal patterns were not due to copper exposure or that due to contamination a true control did not exist. Copper concentrations as low as 6.1 µg/L were observed to cause

biomarker responses in limpets (*Patella vulgata*) and therefore it is possible that there could be an observed response even in the lowest treatment group (3.75 µg/L). In addition, if the control water contained background copper concentrations above the ambient copper concentrations previously experienced by the lease oysters, then it is not inconceivable that a response would also be observed in the control group. For the majority of biomarkers the response correlated well with accumulated copper concentrations, with the stronger responses observed in the highest treatment groups indicating that higher copper exposure caused a more dramatic biomarker response.

Decrease in GSH in digestive gland appears a common response of molluscs to metal exposure, partly explained by the high affinity of these elements for the GSH molecule (Regoli & Principato, 1995). Copper-treated scallops (Regoli *et al*., 1998) and mussels (Doyotte *et al*., 1997) showed considerable depletion of GSH, however, none of the studies recorded an initial stimulation as demonstrated in this study. There appeared to be little change in GSH, however, during the depuration phase.

GSH is an oxygen radical scavenger that conjugates with electrophilic xenobiotics transforming them into water-soluble, excretable products (Nusetti *et al*., 2001). Decreases in concentrations of GSH during copper exposure are to be expected as GSH conjugates with Cu (II) producing oxidised GSH, which is often excreted from the cell more rapidly than it can be reconverted into the reduced form by the enzyme glutathione reductase (Irato *et al*., 2003). Further reductions of GSH were also likely to be caused by the increase in GST activity in the hepatopancreas. GSH is a cofactor of GST, which functions as a catalyst in conjugation reactions between GSH and xenobiotic compounds (Regoli & Principato, 1995) and therefore GSH may be 'used up' in the production of GST.

Unlike GSH, GST had a marked change in response during the depuration phase, as did CAT. Although biomarkers were not measured until 48 hours after spiking ceased, it is likely that the change in enzyme response was rapid, in contrast to changes in copper concentrations during depuration. GST was stimulated in the gill during depuration to greater concentrations than those observed during the exposure phase and in the digestive gland, to similar concentrations as initial stimulation. The second stimulation of GST during depuration may be a response by the oyster to assist in depuration of copper from the tissues. Initiation of antioxidant enzyme systems especially in the hepatopancreas where copper is known to sequester may allow detoxification processes to assist in purging the excess copper from the tissues.

Relationships over time at 15 days, which excluded the depuration phase, were not as significant, perhaps due to insufficient data points for this period. Therefore, the relationships for the significant regressions at 23 and 28 days for GST, which are likely to have included the depuration response, were observed to be linear. However, in reality GST had a similar response over time to GSH, particularly in gill, which was an initial stimulatory phase for approximately 12 days, followed by depletion during the copper exposure phase.

LPO demonstrated a similar pattern during exposure to GSH, in both gill and digestive gland, again with little change occurring to peroxidative processes during the depuration phase. CAT also demonstrated an initial stimulatory response followed by decline during exposure as did both LPO and the antioxidant enzymes, although the initial stimulation phase was not as sustaining, with declines in concentrations observed at around five to eight days in both tissues. The initial large increase in CAT may not be production as such but perhaps recovery from the oyster being 'stressed' during transport. The initial increase in LPO is indicative of oxidative damage of the tissues; however, the rapid decline in LPO at 15 days suggests an adaptive phase to the new ambient conditions, which was not observed in the field-deployed oysters. The finding highlights the use of repeated measures during the exposure phase.

Significant relationships between biomarkers and actual accumulated copper concentrations were only demonstrated for the antioxidant enzyme GST and the free radical scavenger GSH, but not for LPO or CAT. Although relationships for GST were linear in laboratory exposures to copper, the responses of oysters to accumulating aluminium, cadmium and chromium concentrations in the field oysters was an increase to a threshold before depletion or tapering of enzyme concentrations occurred. There was no significant response of any biomarker to increasing copper concentrations in the field. Concentrations of copper in the field oysters did not reach appreciable levels until the final sampling (day 29) in comparison to the copper bioassay, where appreciable concentrations were reached in half that time period for the higher treatment groups. Therefore there may have been insufficient copper exposure to generate a biomarker response in the field.

4.3. Use of biomarkers in oysters

Biochemical responses were apparent in both the field and laboratory experiments; however, the same responses were not observed in both studies. Although marked and logical results were gained in the laboratory experiment, the controlled laboratory environment does not simulate environmental realism. In the field, oysters were potentially exposed to a suite of unknown contaminants whereas laboratory oysters were exposed to just one known contaminant. Synergistic or antagonistic effects from different metals (or other contaminants) on biomarker response in the field would not be observed in the laboratory. Laboratory oysters were in controlled conditions being fed only one type of food on a regular basis in comparison to the 'natural' conditions in the field. The use of biomarker response as a measure of 'stress' in oysters in Port Curtis could not be determined from this study alone.

To be useful in environmental monitoring, the biomarker or biological response must reflect the environmental stress over time in a quantitative way (Wu *et al.*, 2005). In a review of over 900 papers of biomarker responses in the literature, Wu *et al*. (2005) highlighted the uses and limitations of biomarkers as well as the importance of understanding temporal changes in responses, especially in reference to the time for induction, adaptation or recovery of particular biomarker responses. Future experimentation should encompass longer deployment periods for oysters, both in the field and in the laboratory, in order for oysters to reach their full accumulation potential, although it appears that many of the biomarker responses are only transient. Field studies should encompass a higher number of sites, with a greater variation in contaminant loads using a wider range of bioindicator species. Repeated experiments will give an indication of whether these biomarkers are consistently useful monitoring tools.

5. References

Andersen, L.E. (2003) *A study into the epidemiology of mud crab shell disease. Master of Applied Science.* Arts, Health & Science, Centre for Environmental Management, Central Queensland University, Gladstone. 193 pp.

Andersen, L.E. (2004) Imposex: A biological effect of TBT contamination in Port Curtis, Queensland. *Australasian Journal of Ecotoxicology*, 10: 105–113.

Andersen, L.E., Boundy, K. and Melzer, A. (2002) *Intertidal crabs as potential biomonitors in Port Curtis.* Centre for Environmental Management, Central Queensland University and Cooperative Research Centre for Coastal Zone, Estuary and waterway Management, Gladstone. 23 pp.

Andersen, L E. and Norton, J.H. (2001) *Port Curtis mud crab shell disease – nature, distribution and management.* Central Queensland University, FRDC Project No. 98/210 Gladstone. 115 pp.

Andersen, L.E., Revill, A.T. and Storey, A.W. (2005a) *Metal bioaccumulation through foodweb pathways in Port Curtis.* Technical Report No. 31, CRC for Coastal Zone, Estuary and Waterway Management, Indooroopilly. 56 pp.

Andersen, L.E., Storey, A.W. and Fox, S. (2004) *Assessing the effects of harbour dredging using transplanted oysters as biomonitors*. Centre for Environmental Management, Central Queensland University, Gladstone. 214 pp.

Andersen, L.E., Storey, A.W., Sinkinson, A.W. and Dytlewski, N. (2003) *Transplanted oysters and resident mud crabs as biomonitors in Spillway Creek.* Centre for Environmental Management, Central Queensland University, Gladstone. 30 pp.

Andersen, L.E., Teasdale, P., Jordan, M. and Storey, A.W. (2005b) *Transplanted oysters and DGT devices to measure bioavailable metals: comparison of techniques.* Reports to Comalco Alumina Refinery and Institute of Sustainable Regional Development. Centre for Environmental Management, Central Queensland University, Gladstone.

Andersen, V., Maage, A. and Johannessen, P.J. (1996) Heavy metals in blue mussels (*Mytilus edulis*) in the Bergen harbour area, western Norway. *Bulletin of Environmental Contamination and Toxicology,* 57: 589–596.

Anderson, M.E. (1985) Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology,* 113: 548–555.

Apte, S.C., Duivenvoorden, L., Johnson, R., Jones, M., Revill, A., Simpson, S., Stauber, J. and Vicente-Beckett, V. (2005) *Contaminants in Port Curtis: screening level risk assessment.* Technical Report No. 25, CRC for Coastal Zone, Estuaries and Waterway Management, Indooroopilly. 146 pp.

Bresler, V., Bissinger, V., Abelson, A., Dizer, H., Sturm, A., Kratke, R., Fishelson, L. and Hansen, P.D. (1999) Marine molluscs and fish as biomarkers of pollution stress in littoral regions of the Red Sea, Mediterranean Sea and North Sea. *Helgol Marine Research*, 53: 219–243.

Brown, R.J., Galloway, T.S., Lowe, D.M., Browne, M.A., Dissanayake, A., Jones, M.B. and Depledge, M.H. (2004) Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. *Aquatic Toxicology*, 66: 267–278.

Central Queensland Ports Authority (2005) *Annual Report 2004–05.* Central Queensland Port Authority. 82 pp.

Chan, K.W., Cheung, R.Y.H., Leung, S.F. and Wong, M.H. (1999) Depuration of metals from soft tissues of oysters (*Crassostrea gigas*) transplanted from a contaminated site to clean sites. *Environmental Pollution*, 105: 299–310.

Cheung, C.C.C., Zheng, G.J., Lam, P.K.S. and Richardson, B.J. (2002) Relationships between tissue concentrations of chlorinated hydrocarbons (polychlorinated bipenyls and chlorinated pesticides) and antioxidative responses of marine mussels, *Perna viridis*. *Marine Pollution Bulletin,* 45: 181–191.

Cheung, C.C.C., Zheng, G.J., Li, A.M.Y., Richardson, B.J. and Lam, P.K.S. (2001) Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. *Aquatic Toxicology,* 52: 189–203.

Choi, N.M.C., Yeung, L.W.Y., Siu, W.H.L., So, I.M.K., Jack, R.W., Hsieh, D.P.H., Wu, R.S.S. and Lam, P.K.S. (in press) Relationships between tissue concentrations of paralytic shellfish toxins and antioxidant responses of clams, *Ruditapes philippinarum. Marine Pollution Bulletin.*

Cohen, M.B., Mimi, K. and Vivian, O. (1996) A modified catalase assay suitable for a plate reader and for the analysis of brain cell cultures. *Journal of Neurological Methods*, 67: 53–56.

Courtney, L.A. and Clements, W.H. (2002) Assessing the influence of water and substratum quality on benthic macroinvertebrate communities in a metal-polluted stream: An experimental approach. *Freshwater Biology*, 47: 1766–1778.

Cruz-Rodriguez, L.A. and Chu, F.L.E. (2002) Heat-shock protein (HSP70) response in the eastern oyster, *Crassostrea virginica,* exposed to PAHs sorbed to suspended artificial clay particles and to suspended field contaminated sediments. *Aquatic Toxicology*, 60: 157–168.

Curran, J.C., Holmes, P.J. and Yersin, J.E. (1986) Moored shellfish cages for pollution monitoring. *Marine Pollution Bulletin,* 17: 464–465.

Davies, W.R., Siu, W.H.L., Jack, R.W., Wu, R.S.S., Lam, P.K.S. and Nugegoda, D. (2005) Comparative effects of blue-green algae *Nodularia spumigena* and a lysed extract on detoxification and antioxidant enzymes in the green lipped mussel (*Perna viridis*). *Marine Pollution Bulletin,* 51: 1026–1033.

Doyotte, A., Cossu, C., Jacquin, M., Babut, M. and Vasseur, P. (1997) Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidis. Aquatic Toxicology,* 39: 93–110.

Fitzpatrick, P.J., O'Halloran, J., Sheehan, D. and Walsh, A.R. (1997) Assessment of a glutathione-s-transferase and related proteins in the gills and digestive gland of *Mytilus edulis* (L.), as potential organic pollution biomarkers. *Biomarkers*, 2: 51–56.

Gefford, A., Amiard, C. and Amiard-Triquet, C. (2002) Kinetics of metal elimination in oysters from a contaminated estuary. *Comparative Biochemistry and Physiology Part C*, 131: 281–293.

Irato, P., Santovito, G., Cassini, A., Piccinni, E. and Albergoni, V. (2003) Metal accumulation and binding protein induction in *Mytilus galloprovincialis*, *Scapharca inaequivalvis*, and *Tapes philippinarum* from the Lagoon of Venice. *Archives of Environmental Contamination and Toxicology,* 44: 476–484.

Jakoby, W.B. (1985) Glutathione transferase: an overview. In *Methods in enzymology*, S.P. Colowick and N.O. Kaplan (eds). Academic Press, New York, pp. 495–504.

Jones, G.B., Mercurio, P. and Olivier, F. (2000) Zinc in fish, crabs oysters and mangrove flora and fauna from Cleveland Bay. *Marine Pollution Bulletin*, 41: 345–352.

Ke, C. and Wang, W. (2001) Bioaccumulation of Cd, Se and Zn in an estuarine oyster (*Crassostrea rivularis*) and a coastal oyster (*Saccostrea glomerata*). *Aquatic Toxicology*, 56: 33–51.

Laodong, G., Santschi, P.H. and Sammy, M.R. (2002) Metal partitioning between colloidal and dissolved phases and its relation with bioavailability to American oysters. *Marine Environmental Research*, 54: 49–64.

Luebke, R.W., Hodson, P.V., Faisal, M., Ross, P.S., Grasman, K.A. and Zelikoff, J.T. (1997) Aquatic pollution-induced immunotoxicity in wildlife species. *Fundamental and Applied Toxicology*, 37: 1–15.

Mackay, N.J., Williams, R.J., Kacprzac, J.L., Kazacos, M.N., Collins, A.J. and Auty, E.H. (1975) Heavy metals in cultivated oysters (*Crassostrea commercialis* = *Saccostrea cucullata*) from the estuaries of New South Wales. *Australian Journal of Marine and Freshwater Research*, 26: 31–46.

Melville, F. (2005) *Mangrove algae in the assessment of estuarine pollution*. Ph.D. thesis. Department of Environmental Sciences, University of Technology, Sydney, Australia.

Melville, F. and Burchett, M.D. (2002) Genetic variation in Avicennia marina in three estuaries of Sydney (Australia) and implications for rehabilitation and management. *Marine Pollution Bulletin,* 44: 469–479.

Melville, F., Burchett, M.D. and Pulkownik, A. (2004) Genetic variation among age-classes of the mangrove *Avicennia marina* in clean and contaminated sediments. *Marine Pollution Bulletin*, 49: 695–703.

Miller, D.M. and Aust, S.D. (1989) Studies of ascorbate-dependent, iron-catalyzed lipid peroxidation. *Archives of Biochemistry and Biophysics*, 271: 113–119.

Muyssen, B.T.A. and Janssen, C.R. (2001) Zinc acclimation and its effect on the tolerance of *Raphidocelis subcapitata* and *Chlorella vulgaris* in laboratory experiments. *Chemosphere*, 45: 507–514.

Nusetti, O., Esclapes, M., Salazar, G., Nusetti, S. and Pulido, S. (2001) Biomarkers of oxidative stress in the polychaete *Eurythoe complanata* (Amphinomidae) under short-term copper exposure. *Bulletin of Environmental Contamination and Toxicology*, 66: 576–581.

Odzak, N., Zvonaric, Z., Kljakovic, G. and Barie, A. (2001) Biomonitoring of copper, cadmium, lead, zinc and chromium in the Kastela Bay using transplanted mussels. *Environmental Bulletin*, 10: 37–41.

O'Halloran, K., Ahokas, J. and Wright, P. (1998) The adverse effects of aquatic contaminants on fish immune responses. A*ustralasian Journal of Ecotoxicology*, 4: 9–28.

Olivier, F., Ridd, M. and Klumpp, D. (2002) The use of transplanted cultured tropical oysters (*Saccostrea commercialis*) to monitor Cd levels in North Queensland coastal waters (Australia). *Marine Pollution Bulletin,* 44: 1051–1062.

Papadimitriou, E. and Loumbourdis, N.S. (2002) Exposure of the frog *Rana ridibunda* to copper: impact on two biomarkers, lipid peroxidation and glutathione. *Bulletin of Environmental Contamination and Toxicology*, 69: 885–891.

Pascoe, G.A., Blanchet, R.J., Linder, G., Palawski, D., Brumbaugh, W.G., Canfield, T.J., Kemnle, N.E., Ingersoll, C.G., Farag, A. and Dalsoglio, J.A. (1994) Characterisation of ecological risks at the Milltown Reservoir–Clark Fork River Sediments Superfund site. *Environmental Toxicology and Chemistry*, 13: 2043–2058.

Phillips, D.J.H. (1990) Use of macroalgae and invertebrates as monitors of metal levels in estuaries and coastal waters. In *Heavy metals in the marine environment*, R.W. Furness and P.S. Rainbow (eds). CRC Press, Inc., Boca Raton, Florida, pp. 81–99.

Prange, J.A. (1999) *Physiological responses of five seagrass species to trace metals*. Bachelor of Science honours. Botany Department, University of Queensland, Brisbane. 52 pp.

Rainbow, P.S. (1995) Biomonitoring of heavy metal availability in the marine environment. *Marine Pollution Bulletin*, 31: 183–192.

Rainbow, P.S. and Phillips, D. (1993) Cosmopolitan biomonitors of trace metals. *Marine Pollution Bulletin*, 26: 593–601.

Reddy, P.S. (1997) Modulations in antioxidant enzymes in the gill and hepatopancreas of the edible crab *Scylla serrata* during exposure to cadmium and copper. *Environmental Bulletin*, 6: 589–597.

Regoli, F., Nigro, M. and Orlando, E. (1998) Lysosomal and antioxidant responses to metals in the Antarctic scallop *Adamussium colbecki. Aquatic Toxicology*, 40: 375–392.

Regoli, F. and Principato, G. (1995) Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. *Aquatic Toxicology*, 31: 143–164.

Ringwood, A.H., Conners, D.E., Keppler, C.J. and Dinovo, A. (1999) Biomarker studies with juvenile oysters (*Crassostrea virginica*) deployed *in situ*. *Biomarkers*, 4: 400–414.

Ritz, D.A., Swain, R. and Elliott, N.G. (1982) Use of mussel *Mytilus edulis planulatus* (Lamarck) in monitoring heavy metal levels in sea water. *Australian Journal of Marine and Freshwater Research*, 33: 491–506.

Sharma, S., Nemecz, S.K., Zhu, S. and Steele, V.E. (1997) Identification of chemoprotective agents by screening for induction of glutathione-s-transferase as a biomarker. *Methods in Cell Science*, 19: 49–52.

Stauber, J.L., Tsai, J., Vaughan, G., Peterson, S.M. and Brockbank, C.I. (1994) *Algae as indicators of toxicity of BEKM effluents*. Technical Report Series No. 3, CSIRO, Canberra.

USEPA (2000) *Method guidance and recommendations for whole effluent toxicity (WET) testing,* (40 CFR Part 136). U.S. Environmental Protection Agency, Office of Water, Cincinnati, OH. 60 pp.

Villares, R., Puente, X. and Carballeira, A. (2001) Ulva and Enteromorpha as indicators of heavy metal pollution. *Hydrobiologia*, 462: 221–232.

Weeks, J.M. (1995) The value of biomarkers for ecological risk assessment: Academic toys or legislative tools? *Applied Soil Ecology*, 2: 215–216.

Winston, G.W. and Giulio, R.T. (1991) Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology*, 19: 137–161.

Wright, D.A. and Welbourne, P. (2002) *Environmental toxicology*. Cambridge University Press, Cambridge.

Wu, R.S.S., Siu, W.H. L. and Shin, P.K.S. (2005) Induction, adaptation and recovery of biological responses: Implications for environmental monitoring. *Marine Pollution Bulletin*, 51: 623–624.

6. Appendices

Appendix 1. Results of *a posteriori* Tukey's test for time for field metals at both sites

Field copper

Tukey HSD

Time	N	Subset 1	Subset 2
1.00	6	50.5517	
.00	6	52.9233	52.9233
3.00	6	54.7383	54.7383
4.00	6	57.9767	57.9767
5.00	6	58.5633	58.5633
6.00	5	65.7500	65.7500
2.00	6	72.8600	72.8600
7.00	5		98.6020
Sig.		.756	.051

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = 566.723.
a Uses Harmonic Mean Sample Size = 5.714.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field zinc

Tukey HSD

Time	N	Subset 1
3.00	6	470.4800
1.00	6	502.4267
6.00	5	532.9540
5.00	6	540.1783
4.00	6	607.2083
.00	6	622.9200
2.00	6	633.4700
7.00	5	715.7280
Sig.		.326

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = 32 494.618.
a Uses Harmonic Mean Sample Size = 5.714.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field arsenic

Tukey HSD

Time	N	Subset
		1
4.00	6	10.6833
7.00	5	10.7580
.00	6	10.9000
5.00	6	12.2467
6.00	5	12.4980
1.00	6	12.6217
2.00	6	12.8867
3.00	6	13.5817
Sig.		.221

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = 3.745.
a Uses Harmonic Mean Sample Size = 5.714.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field cadmium

Tukey HSD

Time	N	Subset
		1
4.00	6	2.4367
.00	6	2.6567
3.00	6	2.7267
5.00	6	2.7717
6.00	5	2.8120
7.00	5	3.1980
2.00	6	3.3733
1.00	6	3.7317
Sig.		.280

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = .836.
a Uses Harmonic Mean Sample Size = 5.714.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field lead

Tukey HSD

Time	N	Subset	
		1	2
6.00	5	.1500	
4.00	6	.1600	
3.00	6	.1717	
5.00	6	.1850	
7.00	5	.1980	
2.00	6	.2033	
1.00	6	.2117	
.00	6		.4467
Sig.		.354	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = .002.
a Uses Harmonic Mean Sample Size = 5.714.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field aluminium

Tukey HSD

Time	N	Subset
		1
6.00	5	50.4740
4.00	6	56.6950
3.00	6	61.8433
5.00	6	64.2783
2.00	6	70.5317
1.00	6	99.5250
7.00	5	101.5100
.00	6	130.7100
Sig.		.225

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = 2890.594.
a Uses Harmonic Mean Sample Size = 5.714.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field chromium

Tukey HSD

Time	N	Subset	
		1	2
6.00	5	.5600	
4.00	6	.5600	
5.00	6	.5767	.5767
3.00	6	.6200	.6200
7.00	5	.6880	.6880
2.00	6	.7500	.7500
.00	6	.8100	.8100
1.00	6		.8333
Sig.		.063	.052

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = .018.
a Uses Harmonic Mean Sample Size = 5.714.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field nickel

Tukey HSD

Time	N	Subset	
		1	2
.00	6	.8300	
2.00	6	.8350	
1.00	6	.9533	.9533
3.00	6	1.0000	1.0000
4.00	6	1.0617	1.0617
6.00	5	1.0720	1.0720
5.00	6	1.0833	1.0833
7.00	5		1.1920
Sig.		.133	.182

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = .023.
a Uses Harmonic Mean Sample Size = 5.714.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Appendix 2. Results of *a posteriori* Tukey's test for time for field biomarker responses at both sites

Field gill GST

Tukey HSD

Time	N	Subset 1	2	3	4
1.00	20	35.8599			
7.00	20	41.4029	41.4029		
.00	18	48.0285	48.0285	48.0285	
3.00	19		49.7857	49.7857	49.7857
4.00	20		51.0986	51.0986	51.0986
2.00	20		52.2083	52.2083	52.2083
5.00	20			59.6370	59.6370
6.00	20				63.0513
Sig.		.118	.231	.157	.063

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = 192.336.
a Uses Harmonic Mean Sample Size = 19.599.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field gill CAT

Tukey HSD

Time	N	Subset 1
1.00	20	1366.0545
.00	18	1475.0740
7.00	20	1505.5054
2.00	20	1518.5788
6.00	20	1562.4628
3.00	19	1612.6800
5.00	20	1637.7489
4.00	20	1662.4447
Sig.		.594

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = 254 064.040.
a Uses Harmonic Mean Sample Size = 19.599.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field gill LPO

Tukey HSD

Time	N	Subset	
		1	2
.00	20	53.8473	
7.00	20	61.5283	61.5283
3.00	19	61.5482	61.5482
4.00	20	62.1898	62.1898
5.00	20	63.8458	63.8458
1.00	20	71.1590	71.1590
6.00	20	74.2076	74.2076
2.00	20		78.2791
Sig.		.105	.302

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = 528.100.
a Uses Harmonic Mean Sample Size = 19.869.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field gill GSH

Tukey HSD

Time	N	Subset
		1
5.00	20	13.2680
1.00	20	13.8540
3.00	20	13.9833
4.00	20	14.9871
6.00	20	15.1889
.00	18	16.2905
7.00	20	17.6891
2.00	20	18.9492
Sig.		.197

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = 50.283.
a Uses Harmonic Mean Sample Size = 19.726.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field hepatopancreas GST

Tukey HSD

Time	N	Subset		
		1	2	3
4.00	20	97.7665		
7.00	20	101.0005	101.0005	
1.00	20	116.9085	116.9085	116.9085
3.00	19	120.2347	120.2347	120.2347
.00	20	125.4490	125.4490	125.4490
5.00	19	125.8374	125.8374	125.8374
2.00	20		130.4785	130.4785
6.00	20			136.6845
Sig.		.103	.072	.503

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = 990.739.
a Uses Harmonic Mean Sample Size = 19.740.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field hepatopancreas CAT

Tukey HSD

Time	N	Subset		
		1	2	3
.00	20	739.2880		
7.00	20		3245.2880	
5.00	19		4820.7811	4820.7811
2.00	20		4991.7290	4991.7290
1.00	20		5066.3485	5066.3485
6.00	19			5731.4021
4.00	20			6142.2660
3.00	19			6232.6016
Sig.		1.000	.071	.309

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = 3 740 585.016.
a Uses Harmonic Mean Sample Size = 19.613.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field hepatopancreas LPO

Tukey HSD

Time	N	Subset		
		1	2	3
6.00	20	62.0895		
4.00	20	65.4320	65.4320	
3.00	19	70.1079	70.1079	70.1079
7.00	20	74.1670	74.1670	74.1670
5.00	19	77.2816	77.2816	77.2816
.00	20	81.3690	81.3690	81.3690
2.00	20		82.0110	82.0110
1.00	20			85.0110
Sig.		.054	.157	.272

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = 395.038.
a Uses Harmonic Mean Sample Size = 19.740.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.

Field hepatopancreas GSH

Tukey HSD

Time	N	Subset	
		1	2
3.00	19	5.5058	
7.00	20	6.1145	6.1145
4.00	20	6.5315	6.5315
1.00	20	7.1345	7.1345
2.00	19	7.2153	7.2153
6.00	20	9.9320	9.9320
5.00	19	9.9516	9.9516
.00	20		10.4410
Sig.		.071	.087

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square (Error) = 22.279.
a Uses Harmonic Mean Sample Size = 19.613.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c Alpha = .05.