

2000

MODELS OF OXIDATIVE STRESS INDUCED BY DISEASE OR POLLUTION IN INVERTEBRATES AND VERTEBRATES

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<http://hdl.handle.net/10026.1/2393>

<http://dx.doi.org/10.24382/4402>

University of Plymouth

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**MODELS OF OXIDATIVE STRESS
INDUCED BY DISEASE OR POLLUTION IN
INVERTEBRATES AND VERTEBRATES.**

by

HEATHER CLARE MEIGH

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

DOCTOR OF PHILOSOPHY

The Plymouth Postgraduate Medical School

and

The Plymouth Environmental Research Centre

June 2000

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Models of oxidative stress induced by disease or pollution in invertebrates and vertebrates.

Heather Clare Meigh

ABSTRACT

Glutathione and its related enzymes have a central role in the antioxidant mechanisms of both invertebrates and vertebrates. Evidence suggests that changes in antioxidant defence mechanisms are associated with the late complications of diabetes. In addition, invertebrates show changes in antioxidant mechanisms in response to contamination; these changes have the potential to be utilised in the environmental monitoring of pollution. The present study investigated the role of glutathione and its related enzymes with regard to complications of diabetes and toxicity exposure using the crab, *Carcinus maenas*.

A preliminary investigation showed that glutathione peroxidase and glutathione reductase activities are unaltered in the peripheral blood mononuclear cells from diabetic patients with long term complications of the disease. However, crabs were shown to have significantly reduced glutathione-s-transferase and glutathione peroxidase activities in response to cadmium exposure. The biochemical responses of crabs to pollution exposure were investigated in further experiments.

Crabs are exposed to a variety of influences within their natural environment that may affect their ability to tolerate oxidative stress. These stressors include climatic changes, age, sex, nutritional status, contamination, accumulation of toxins and adaptation to a polluted environment. Results showed that seasonality also affects the activities of glutathione related enzymes glutathione reductase and glutathione-s-transferase, as well as physiological parameters such as tissue protein composition. Seasonal changes of enzyme activities may be partly due to the altered nutritional status of the crab over the year. Nutritional status also reduced the total glutathione status and total antioxidant scavenging ability of crab haemolymph and gill tissues. The levels of these parameters were reinstated to normal when the starved crabs were exposed to a mixed affluent. The ability to control the production of antioxidant scavenging compounds during fasting may help to preserve the crabs energy reserves. The promptly reinstated glutathione and total antioxidant scavenging ability in response to contamination helps to prevent the oxidative damage caused by pollution exposure.

Several tissues were removed from the crab and the amount that each contributed to the crabs overall antioxidant scavenging ability was calculated. Haemolymph and muscle tissues were found to contribute the most to the crabs overall antioxidant scavenging ability. This is due to the large proportion of the crab that these tissues occupy. The level of glutathione within the crabs haemolymph, gill and muscle tissues did not contribute significantly to their overall antioxidant scavenging ability in normal conditions. However, when the stress was induced in the crabs in response to mixed effluent exposure or fasting, total glutathione levels became significantly correlated with total antioxidant scavenging ability. The results suggest that under these circumstances glutathione levels are maintained by the activity of glutathione reductase.

A field trial was performed in the highly industrialised area of the Tees Estuary to establish whether the biomarkers that had been successfully applied in laboratory experiments could be used within the field. The results were analysed using multi-dimensional scaling techniques. This allowed a suite of biomarkers to be analysed simultaneously. The biomarker responses measured at the different sites indicated a gradient of toxicity from the top of the estuary to its mouth. These results were consistent with water chemical analysis data. The study showed that more information could be gained from this type of analysis than by examining the biomarker results separately. The biomarkers measured and the method of data analysis have potential to be used in routine toxicity assessment.

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LIST OF ABBREVIATIONS

ABTS	2,2'-Azino-di-[3-ethylbenzthiazoline]
AGE	Advanced Glycation End Product
ANOSIM	Analysis of Similarities
ANOVA	Analysis of Variance
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
BSO	Buthionine Sulfoximine
CDNB	1-chloro-2,4-dinitrobenzene
DMSO	dimethyl sulphoxide
DTNB	dithionitrobenzene
DTT	dithiothreitol
EA	Environmental Agency
EDTA	ethylenediamine tetraacetate
EROD	ethoxyresorufin O-deethylase
GP	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GST	Glutathione-S-Transferase
HCl	hydrochloric acid
HPLC	High Pressure Liquid Chromatography
ICP-AES	Inductively Coupled Plasma / Atomic Emission Spectrometer.
LC ₅₀	Lethal Concentration to 50% of organisms
MDS	Multidimensional scaling x

MFO	Mixed Function Oxidase
MT	Metallothionein
PAH	Polycyclic Aromatic Hydrocarbon
PMSF	Phenylmethylsulfonylfluoride
PRIMER	Plymouth Routines In Multivariate Ecological Research
ROS	Reactive Oxygen Species
SIMPER	Similarity Percentages
SSA	5 Sulfo-salicylic acid

ACKNOWLEDGEMENTS

This research was made possible by the award of a Research Studentship from the University of Plymouth.

I would particular like to thank my supervisors; Prof Mike Depledge for his support and constant guidance; Dr Jim Braven for his patience and consistent help and encouragement and Dr Andy Demaine for his advice and help with the diabetes study. I am grateful to Mrs Sarah McMahon for her technical and practical help, which was above and beyond the call of duty. In addition I am thankful to Mr Stan McMahon for maintenance of stock animals and aquarium support.

I particularly would like to thank Katrina Astley for all her hard work and friendship during the field studies. Also Britt Cordi; Vikky Cheung; Shaw Bamber and all other friends and colleagues that created such an enjoyable atmosphere to work in.

Finally I would like to thank my mother for all her phone calls, enthusiasm and financial support without whom it would not have been possible to have completed this thesis. In addition, Keith Spencer for his support and numerous distractions.

AUTHORS DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

This study was financed with the aid of a studentship from the University of Plymouth.

A program of advanced study was undertaken, which included a course in “Statistical Methods in Biological Research”.

Relevant scientific seminars and conferences were regularly attended at which work was often presented; external institutions were visited for consultation purposes, and papers prepared for publication.

Publications:

Astley, K.N., Meigh,H.C., Glegg,G.A., Braven,J. and Depledge,M.H. (1999) Multi-variate analysis of biomarker responses in *Mytilus edulis* and *Carcinus maenas* from the Tees estuary. **Marine Pollution Bulletin.** 39; 1-2, pp.145-154

Meigh, H.C., Astley,K.N., Glegg,G. and Depledge,M.H. A field study using the crab (*Carcinus maenas*) as a potential biomarker of toxicity. **Interfaces in Environmental Chemistry and Toxicology: from the global to the molecular level.** 8th Annual Meeting of SETAC-Europe 14-18 April 1998. P219 (abstract).

Astley,K.N., Meigh,H.C., Glegg, G. and Depledge,M.H. Field and laboratory biomarker responses: are they related. **Interfaces in Environmental Chemistry and Toxicology: from the global to the molecular level.** 8th Annual Meeting of SETAC-Europe 14-18 April 1998. P209. (abstract).

Oral Presentations:

2nd International Conference on Marine Pollution and Ecotoxicology, Hong Kong. 10th-14th June, 1998. A Novel statistical Analysis of Biomarker Data from the Tees Estuary.

Signed *H. Meigh*.....
Date *29/10/00*.....

INTRODUCTION.

The initial aim of this study was to investigate two models of oxidative stress and then to develop the most effective model for further investigation of antioxidant mechanisms. Part I of the introduction describes the evolution of antioxidant metabolism. The mechanisms that organisms have developed to combat oxidative stress are then discussed with particular focus upon the pivotal role of glutathione metabolism. In addition free radicals that are not derived from oxygen but can also have detrimental effects on biological tissues are discussed.

Part II of the introduction describes the involvement of oxidative stress in the two models that were investigated. The two models chosen were complications of diabetes and toxicological responses of invertebrates to contamination. The processes by which oxidative stress is induced in these two model systems are explained. A thorough understanding of oxidative metabolism within these two systems could contribute to medical treatment for diabetics and to pollution monitoring around the coast of Britain, respectively.

Part I. An introduction to oxidative stress and free radicals.

1.1 A brief history of oxygen.

2.5 Billion years ago, the micro-organisms that existed on this planet inhabited an anaerobic environment. Geological evidence suggests that it was about this time that blue-green algae acquired the ability to split water into hydrogen and oxygen thereby releasing oxygen into the atmosphere. The increase in atmospheric oxygen was accompanied by the formation of the ozone layer, which protects the earth from intense, solar ultra-violet light (Gutteridge and Halliwell, 1996). The increase in atmospheric oxygen to the levels experienced today changed the atmosphere from a highly reducing state to an oxygen-rich state. Oxygen currently constitutes approximately 21% of the

atmosphere. The organisms, which existed during this changing atmospheric era, had to evolve to survive in an oxygen rich environment, or else retreat to habitats where they could lead an anaerobic existence.

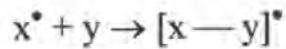
The organisms that adapted to an oxygen rich environment evolved mechanisms which utilised oxygen for energy producing purposes (e.g. the electron transport chain). They also required systems which could protect them from the damaging effects of reactive oxygen species (ROS). Oxygen is capable of oxidising organic molecules including vital structural components of cells. This process usually takes a long period of time, unless it is accelerated by other factors, for example, combustion (e.g. burning fuels) or by enzyme activity, (e.g. digestive enzymes in the gut). Organisms have evolved biological mechanisms to protect themselves from potential oxidative damage and other free radicals, either by (i) producing molecules which can be oxidised in preference to important cellular structures, (ii) by removing oxidative catalysts from the cell, or alternatively (iii) by repairing cell damage incurred by oxidation.

1.2 The chemical nature of oxygen.

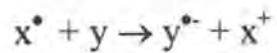
Oxygen in its ground state is not especially reactive, despite the fact that it possesses two un-paired electrons in its outer valence shell, (it is a bi-radical molecule). When two unpaired electrons are brought together in a molecule they generally spin-pair to form a stable atomic configuration. Oxygen is an exception to this rule. However, if a free radical is unable to pair with another free-radical it will most likely react with a non-radical thereby producing a second free-radical product. The reactions that free-radicals may undergo are shown in Figure 1.1.

Oxygen undergoes a variety of reactions that can produce very reactive oxygen derivatives that are capable of reacting with biological tissues and are termed reactive oxygen species (ROS);-

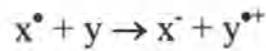
1. Addition.



2. Electron Donation



3. Electron Removal



Reactions are only terminated when two radicals meet.

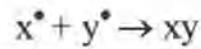
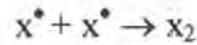


Figure 1.1 Free radical reactions.

The Superoxide Anion

Oxygen can be reduced to form a superoxide radical ion ($O_2\cdot^-$) (Equation 1, Figure 1.2). The superoxide radical is produced by the addition of an electron to ground state oxygen. This results in the intracellular reduction of oxygen and can involve a variety of cellular components, including thiols, hydroquinones, catecholamines, flavins and tetrahydropterins (Freeman & Crapo, 1982). Normally the superoxide anion is short-lived and is converted to hydrogen peroxide by the enzyme superoxide dismutase (SOD).

Hydrogen Peroxide

H_2O_2 (hydrogen peroxide) is the product of superoxide dismutase (SOD) - catalysed disproportionation of $O_2\cdot^-$ (Equation 2, Figure 1.2). Other enzymes that catalyse the formation of hydrogen peroxide are D-amino acid oxidase and amine oxidase. Hydrogen peroxide is not a free radical, but is damaging to the cell for two reasons, (i) it can freely cross biological membranes, and (ii) it is a precursor to the formation of a much more reactive molecule, the hydroxyl radical, $HO\cdot$. Therefore, hydrogen peroxide has the ability to cause cellular damage when appropriate conditions exist.

Hydroxyl Radicals

The hydroxyl radical is generated from hydrogen peroxide by donation of an electron (Equation 3, Figure 1.2). This occurs via the Fenton reaction, (Equations 7, Figure 1.3), which was first described by the Cambridge chemist H.J.H.Fenton, in 1894. The Fenton reaction uses a metal ion catalyst to

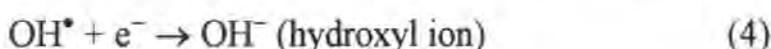
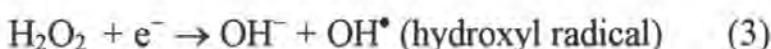
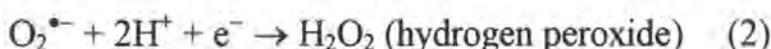
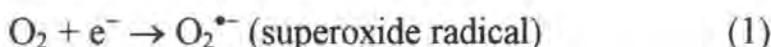
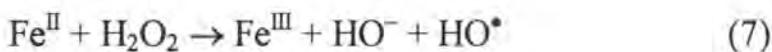


Figure 1.2 The formation of derivatives from the reduction of oxygen.



The Fenton Reaction:



The Haber-Weiss Reaction:

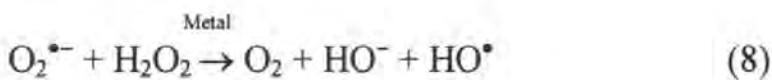


Figure 1.3 The Fenton reaction in which an iron ion is reduced by superoxide radical which in turn reduces hydrogen peroxide to produce hydroxyl radical. The net overall reaction is known as the Haber-Weiss reaction.

reduce hydrogen peroxide to form the hydroxyl ion and the hydroxyl radical (Figure 1.3). Thus; O_2^- reduces iron, which in turn reduces H_2O_2 to form $OH\cdot$. Reductants such as ascorbate can also reduce Fe(III), which means that hydroxyl radicals can be generated in the absence of superoxide radicals (Freeman & Crapo, 1982). The net reaction is known as the Haber-Weiss reaction, (Equation 8, Figure 1.3). The hydroxyl radical is the most reactive of the oxygen radical derivatives. It will react with any biological molecule that it comes into contact with, hence the hydroxyl radical life span is very short as it is so reactive that it does not have the opportunity to travel far from where it is first formed. The hydroxyl radical can also bind to biological molecules, e.g. by reaction with guanine. It can also be converted back into water by pulling off hydrogen atoms from biological molecules, as occurs during lipid peroxidation, (Gutteridge and Halliwell, 1996).

In addition to the Fenton reaction, hydroxyl radicals can be generated directly from hydrogen peroxide exposed to UV-light, which induces homolytic fission of the hydrogen peroxide molecule. Hence UV-irradiation of H_2O_2 containing systems can cause cellular damage from the formation of $OH\cdot$ (Gutteridge and Halliwell, 1996).

1.3 Biological sources of free radicals.

Free radical reactions are essential to many biological processes; for example the electron transport chain and the action of catalytic enzymes involve one-electron transfers that yield free radical intermediates (Freeman & Crapo, 1982). However, some naturally occurring reactions can inadvertently cause cell damage by the generation of ROS. For instance, activated phagocytes can produce oxygen radicals (Weiss, 1982). In addition, haemoglobin releases superoxide radicals; this is very rare and occurs perhaps only once in a thousand cycles of the binding and dissociation of oxygen. However, due to the large amount of haemoglobin present in a human for instance, this

source contributes significantly to the overall production of superoxide radicals in the body (Gutteridge and Halliwell, 1996). Numerous enzymes generate free radicals during their catalytic cycling, for example, xanthine oxidase, aldehyde oxidase, flavoprotein dehydrogenase and tryptophan dioxygenase (Freeman & Crapo, 1982). Peroxisomes are also a potent generator of H₂O₂ and H₂O₂ has been shown experimentally to diffuse out of the peroxisome membranes (Chance *et al.*, 1979). Some free radical species and precursors encountered by cells are listed in Table 1.1.

1.4 Environmental sources of free radicals.

Free radicals can also be generated intracellularly by the influence of environmental factors such as pollution, hyperoxia and geochemical changes. As previously mentioned UV light can give rise to hydroxyl radicals in hydrogen peroxide containing systems. With the thinning of the ozone layer due to pollution effects, UV light could pose a serious problem. UV-B can cause skin cancer and it has been speculated that the incidence of skin cancers will increase as stratospheric ozone is depleted (Newman, 1998). Increased UV-B penetration into the surface waters of the oceans may also decrease phytoplankton photosynthesis (Baird, 1995). As phytoplankton are at the base of many marine food chains, diminished photosynthesis will reduce phytoplankton productivity and could have devastating effects upon higher marine species.

The presence of ozone in the lower troposphere is highly undesirable. Although it is not itself a free radical it is a powerful oxidising agent. Ozone and nitrogen oxides, (NO_•, NO₂•) are a common by-product of industrial combustion processes, and car fumes. In addition, chemical air pollutants: pesticides, tobacco smoke, solvents and aromatic hydrocarbons promote free radical formation. These chemicals either exist as free radicals or free radicals may be formed from them during cellular detoxification processes (Freeman & Crapo, 1982).

Toxins released into the environment also affect oxidative metabolism. A wide range of xenobiotics, of different structures are released into the atmosphere and can potentially cause structural damage to membranes and DNA. Examples of these chemical pollutants are halogenated alkanes and alkenes, pesticides, phorbol esters, herbicides, quinones and transition metals (Stohs, 1995). In addition, many chemical contaminants can undergo heterolytic fission under the influence of UV light to produce reactive free radicals.

1.5 Mechanisms of free-radical mediated tissue damage.

Free radicals have the capacity to react with almost every component of the cell. The effects of these reactions are chemical modifications, which result in tissue damage and / or metabolic changes. Some of these effects are discussed below.

Proteins are susceptible to attack from free radicals primarily because of the reactivity of sulphur-containing groups within them (methionine and cysteine). Tryptophan, tyrosine, phenylalanine, histidine, can also undergo modification induced by free radicals (Freeman, 1982). Some enzymes such as Papain which rely on these amino-acids for their activity can become deactivated in the presence of free radicals (Buchanon & Armstrong, 1978). Proteins which are components of membranes and cytoplasm, can become cross-linked to form dimers. The net effect of protein damage from free radicals is enzyme inactivation, denatured proteins and organelle and cell permeability changes. The extent of free radical mediated protein damage depends upon which amino-acids constitute the protein; their orientation in the protein; the positioning of the protein within the cellular structure; the repair mechanisms available to the protein; and the chemical nature of the free radical which is causing the damage (Freeman & Crapo, 1982).

<i>Species</i>	<i>Sources</i>
O_2^- , H_2O_2 , OH^- ,	Oxygen metabolism potentiated by hyperoxia, inflammation, radiation
NO_2 , O_3 , peroxyacylnitrates, lipid peroxides	Photochemical air pollution By-products of free radical propagation or prostanoid metabolism
Hypochlorite radicals	Inflammation
Semiquinones	Mitochondrial electron transport
Aromatic hydrocarbons	Environmental
Divalent metals	Heme and other metal-containing proteins, free and complexed metals.

Table 1.1 Sources of biological free radicals. A wide range of substrates, metabolic intermediates, and environmental agents either exist as free radicals or can be converted to free radical species by cellular metabolic processes. (Freeman & Crapo, 1982).

Nucleic acids and DNA are also susceptible to free radical damage. Damage to these components can ultimately result in cell death or mutation. Radiation particularly is responsible for this type of damage by inducing chromosomal aberrations (Myers, 1980). Chromosomal damage can cause mutations and initiate the onset of cancer in vertebrates.

Lipids are also susceptible to free radical attack and this is the cause of lipid peroxidation (Figure, 1.4). Lipids readily undergo peroxidation. Lipid peroxidation is indicated by loss of cell membrane unsaturated fatty acids and formation of lipid peroxides. Lipid peroxides can react with integral cell membrane components in much the same way as free radicals. They are also more hydrophilic than unperoxidised fatty acid side chains and try to migrate to the membrane surface to interact with water (Gutteridge and Halliwell, 1996). The net effect is an alteration in intrinsic membrane properties, such as, membrane fluidity and permeability.

Membrane peroxidation is measured by determining the concentrations of malondialdehyde (MDA) present in the tissue. MDA is a small molecular fragment produced from lipid breakdown. Cellular free radical targets are summarised in Table 1.2 and the generation of free radicals and some of the products of free radical damage are shown in Figure 1.5.

1.6 Cellular defences against free radical damage.

Organisms have evolved mechanisms to prevent the damaging effects of free-radical reactions. Some defence mechanisms control the metabolic induction of free radicals, whilst others are inducible and occur when the cell is exposed to elevated free radical concentrations generated by exogenous factors. Free radicals that enter the cell are destined for one of three major fates. They may (i) react with cellular components, (DNA, lipids, proteins); or (ii) be quenched by reactions with low molecular weight free radical scavengers, which are partitioned into either the cellular membranes or

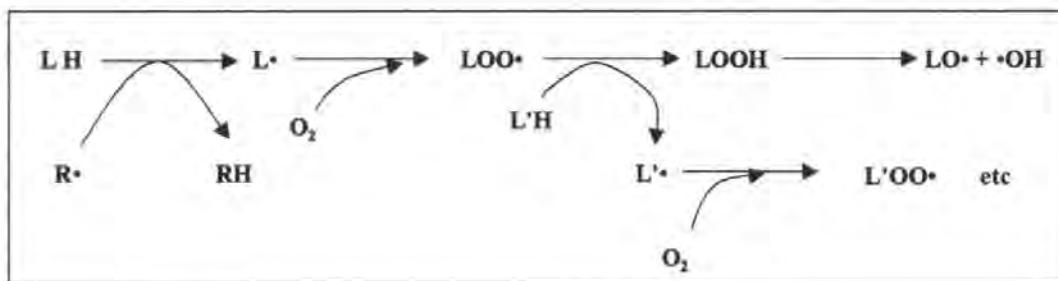


Figure 1.4 A schematic progression of lipid peroxidation. Lipid peroxidation is a chain reaction that leads to the formation of many lipid peroxide radicals. L = Lipid, L' = second lipid, R \cdot = Free Radical species. (Adapted from De Zwart *et al.*, 1999).

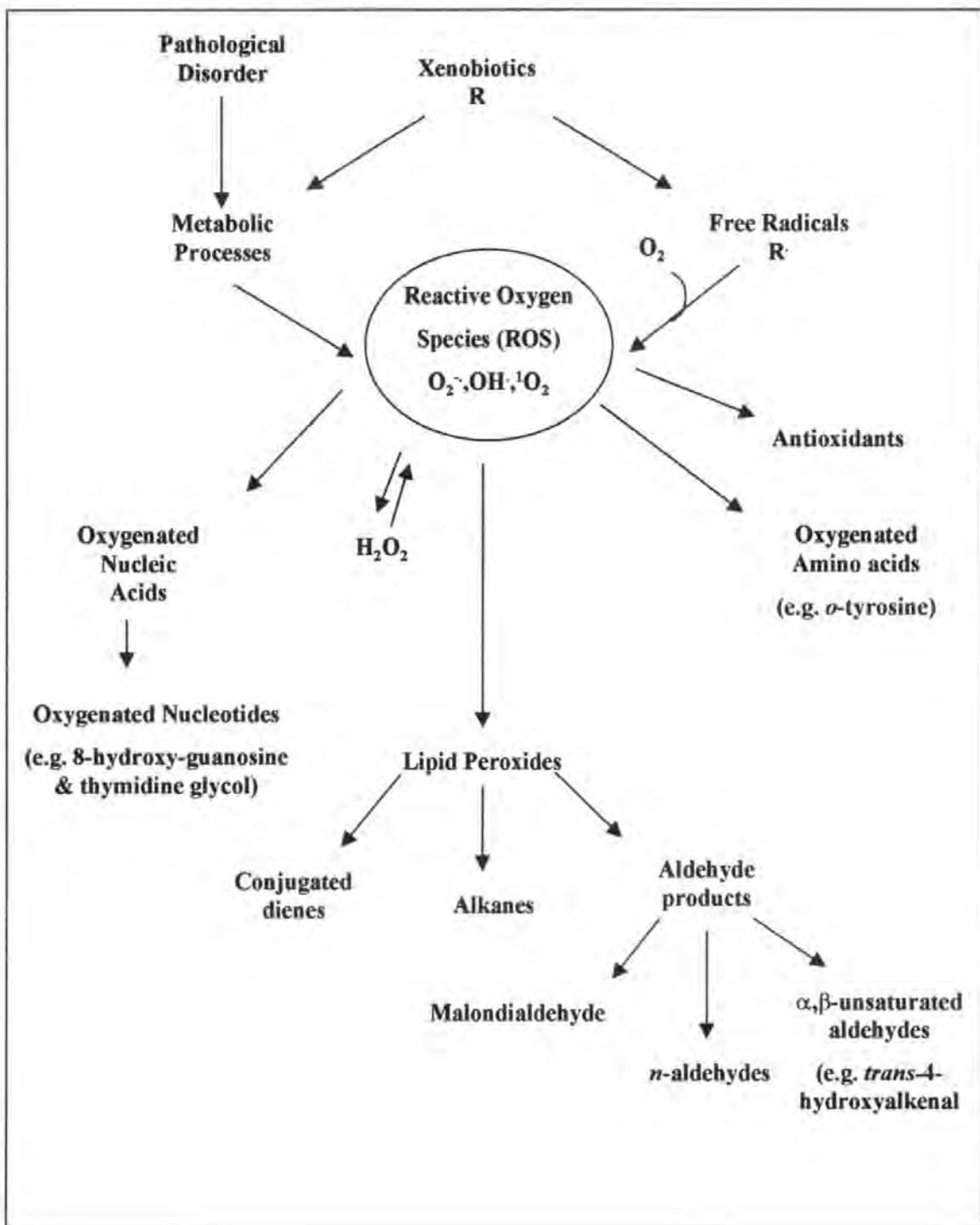


Figure 1.5 The products of free radical damage. Free radicals may react with different cellular macromolecules, such as DNA, cell membranes or proteins, which can lead to the formation of many different products. (Adapted from De Zwart *et al.*, 1999).

<i>Target</i>	<i>Consequence</i>
Small Molecules	
Unsaturated and thiol-containing amino acids	Protein denaturation and cross-linking, enzyme inhibition. Organelle and cell permeability changes.
Nucleic acid bases	Cell cycle changes, mutations.
Carbohydrates	Cell surface receptor changes.
Unsaturated lipids	Cholesterol and fatty acid oxidation, lipid cross-linking.
Cofactors	Decreased nicotinamide and flavin-containing cofactor availability and activity, ascorbate oxidation, porphyrin oxidation.
Neurotransmitters	Decreased neurotransmitter availability and activity.
Antioxidants	Decreased availability.
Macromolecules	
Protein	Peptide chain scission, denaturation.
DNA	Strand scission, base modification.

Table 1.2 The sites and affects of cellular free radical targets. (Freeman & Crapo, 1982).

into the cytosol (glutathione, tocopherol, β -carotene, ascorbate and more recently metallothionein has been established as a defence system against free radicals); (iii) or are metabolised by free radical enzymatic scavengers, (superoxide dismutase). Of particular interest to this research work are the free radical defence systems which center on the low molecular weight molecules, glutathione and to a lesser extent, metallothionein. Glutathione related enzymes are also investigated which are responsible for controlling precursors to free radicals and oxidative stress.

1.7 Glutathione metabolism.

Glutathione production is catalysed intracellularly by γ -glutamylcysteine synthetase and GSH synthetase. It is a tripeptide molecule composed of glutamic acid, cysteine and glycine, (L- γ -glutamyl-L-cysteinylglycine). Glutathione has a significant role in protecting against oxidative stress that is induced both intra-and extra-cellularly. It also is involved in the synthesis of proteins and DNA, enzyme activity and metabolism (Meister and Anderson, 1983). This investigation is focussed upon the role of glutathione in the control of damage associated with oxidative species and xenobiotics.

The protective roles of glutathione are shown in Figure 1.6. Glutathione has a significant function in xenobiotic and anti-oxidant metabolism as it is involved in both Phase I and Phase II detoxification processes. It is also able to react directly with xenobiotics and metabolic intermediates. Phase I chemical reactions modify foreign compounds so that they can conjugate with compounds in Phase II reactions. This then renders the compound more soluble and hence more excretable. If the compound requires further metabolising, it occurs *via* Phase III reactions. However, most compounds do not require an extra step. Phase I metabolism usually involves modifying the compound, for example by an oxidation or reduction process. Glutathione peroxidase functions to

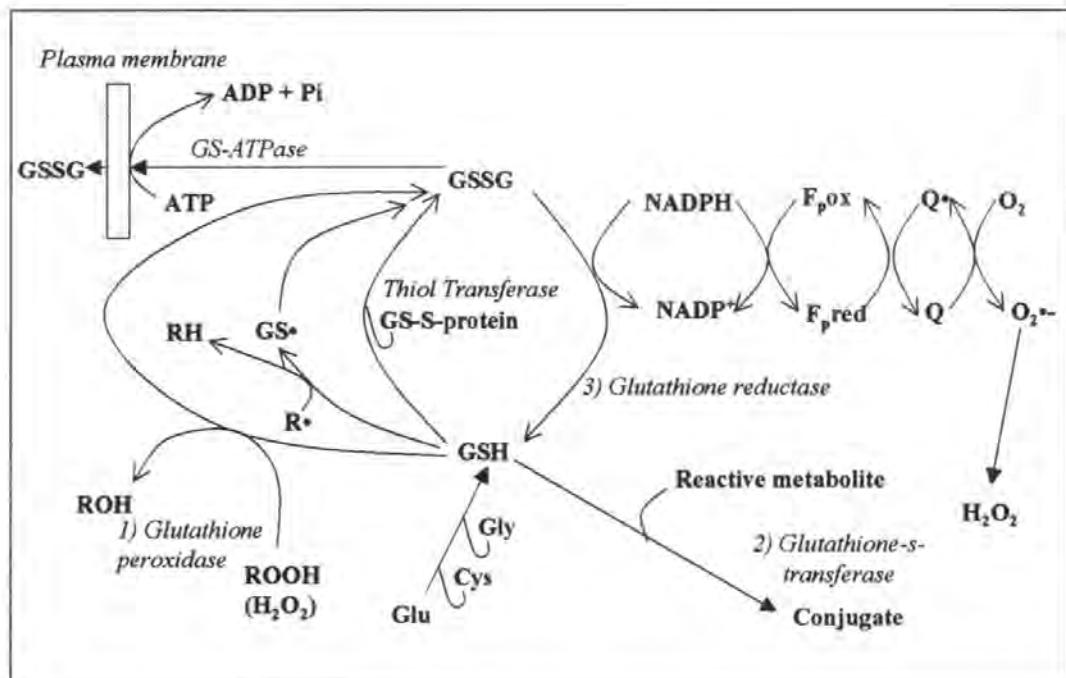


Figure 1.6 The protective roles of glutathione. F_p^{ox} and F_p^{red} are the oxidised and reduced forms of flavoprotein respectively. Q and Q^{\bullet} represent a quinone and a semi-quinone respectively. (Diagram modified from Timbrell, 1994).

reduce peroxides in a Phase I type reaction. Most Phase I type reactions prepare xenobiotics so that they can react more readily in Phase II reactions. Glutathione peroxidase reduces the activity of reactive peroxides. This enzymatic reaction involves glutathione as a substrate, which becomes oxidised by the process (Stage 1, Figure 1.6). Glutathione is also involved in Phase II reactions by the action of glutathione-s-transferase. In this type of reaction glutathione becomes conjugated to the xenobiotic (Stage 2, Figure 1.6), hence making the compound more hydrophilic and more readily excretable.

Thiol Status.

If xenobiotics are present in excess within the cell, the cells' glutathione levels can become depleted. Reactive molecules can oxidise the thiol groups in glutathione as well as other thiols, such as those present in proteins. The net result is a change in the thiol status of the cell. Glutathione reductase reduces the oxidised glutathione produced by these reactions in order to restore the reduced glutathione status (Stage 3, Figure 1.6). If the amount of oxidised glutathione produced from these reactions exceeds the capability of glutathione reductase to restore reduced glutathione levels, the oxidised glutathione is actively removed from the cell. Glutathione conjugates can also be transported out of the cell by this ATP-catalysed reaction. The overall effect is a reduction in thiol status. Unless other detoxification processes compensate for a reduction in glutathione, then the cell can be left vulnerable to toxic compounds. Also the loss of glutathione from the cell leaves other thiol containing compounds more vulnerable to attack from free radicals. Subsequent oxidation of these more vital thiol-containing compounds can result in protein oxidation, formation of mixed disulphides or covalent adducts.

Altered thiol status has been associated with several diseases e.g. Rheumatoid arthritis, (Banford *et al.*, 1982), diabetes (e.g. McLennan, *et al.*, 1991; Mak *et al.*, 1996) and patients suffering from chronic diseases are shown to have lower blood reduced glutathione concentrations (Lang *et al.*, 2000). It is also indicative of oxidative stress induced by a wide variety of chemical and physical stressors (e.g. Lindström-Seppä *et al.*, 1996; and Patel *et al.*, 1990).

Glutathione-s-transferases.

Glutathione-s-transferases (GSTs) are a large family of enzymes, which facilitate the transfer of reduced glutathione to a xenobiotic molecule. Multiple forms of GSTs have been isolated from different species, for example, marine invertebrates (Lee *et al.*, 1988; Fitzpatrick and Sheehan, 1993; and Fitzpatrick *et al.*, 1995a), fish (Pérez-López *et al.*, 1998; Pathiratne & George, 1996) and humans (reviewed by Beckett and Hayes, 1993). They are mainly cytosolic enzymes.

GSTs accept many different types of substrates for glutathione conjugation including aromatic, aliphatic, and halogenated aliphatic compounds, aromatic nitro compounds, unsaturated aliphatic compounds and alkyl halides (Timbrell, 1994). A piscine GST has also been found to efficiently conjugate the end-products of lipid peroxidation (Leaver & George, 1998). Despite their ability to accept a wide variety of substrates they have an absolute specificity for GSH. In each case the glutathione is reacting with an electrophilic carbon atom in an addition or substitution reaction. Even though GST does not directly interact with ROS it plays a secondary role in antioxidant metabolism by conjugating with xenobiotic compounds that induce oxidative stress. Therefore for the purposes of this study it shall be referred to as contributing towards antioxidant mechanisms.

Glutathione Peroxidases.

Glutathione peroxidases (GPs) are also a large family of enzymes that have been found in a wide diversity of species. They catalyse the GSH-dependent reduction of H₂O₂ and other organic peroxidases. Species that have been shown to contain GPs include some marine invertebrates (Gamble *et al.*, 1995; Bell & Smith, 1994; Livingstone *et al.*, 1992; and Pipe *et al.*, 1993), mammals (Faraji *et al.*, 1987), birds and fish (reviewed by Felton, 1995). GPs have not been found in some classes of invertebrates, including arthropods, although some arthropods exhibit GP activity which is associated with the GST enzyme (GST_{PX}) (Felton, 1995). Due to similarity between their structures it is believed that GPs evolved from GST genes or glutaredoxin/thiotransferase genes (Fahey & Sundquist, 1991).

Glutathione peroxidase is a selenium-based enzyme, however GST_{PX} is not and is sometimes referred to as non-selenium dependent GP. Different GP types are able to metabolise different substrates. Common substrates used for GP measurements are H₂O₂, cumene hydroperoxide or *t*-butylhydroperoxide the latter two are good substrates for most glutathione peroxidase isoenzymes.

Glutathione Reductase.

Glutathione reductase (GR) is one of the oldest and most genetically-conserved of enzymes. There is a 55% similarity between aligned DNA sequences between human and *E.Coli* GR genes (Fahey & Sundquist, 1991). Human GR is believed to have become incorporated from a purple bacteria, related to *E.Coli*, via the mitochondria (Fahey & Sundquist, 1991). Virtually all systems that contain GSH have been shown to possess the GR gene. GR has a broad subcellular distribution in insects (Ahmad, 1992) but is limited to the cytosol and mitochondria in mammals (Chance *et al.*, 1979).

GR is a disulfide oxidoreductase and an important class of flavoenzymes, which, also include lipoamide dehydrogenase and thioredoxin reductase (Felton, 1995). Intracellular ratios of GSH: GSSG are maintained at approximately 300:1 through the action of NADPH-dependent glutathione reductase, which catalyses the reduction of oxidised glutathione (Meister & Anderson, 1983; and Ziegler, 1985).

GR functions in the production of free SH groups which are required for several cellular processes; deoxynucleotide synthesis, detoxification of peroxides via GST or GP and maintenance of intracellular thiol status and many other processes (Bucheler *et al.*, 1992).

1.8 Metallothionein and glutathione relations.

Metallothionein (MT) is a low molecular weight molecule, which has a high cysteine content. It forms metal-thiolate clusters and has an important function in metal metabolism and cellular stress response (Christie and Costa, 1984).

The reason that metallothionein has been included in this study is that it has been shown to have an intimate relationship with glutathione in cellular stress responses to oxidative stress and contaminant detoxification (Singhal *et al.*, 1987; and Brouwer *et al.*, 1993a&b). Metallothionein and glutathione both have the sulphhydryl moiety of cysteine, which is susceptible to electrophilic attack. Glutathione has a higher affinity for electrophiles, however when the levels of glutathione become critically low MT acts as an electrophilic 'sink' (Cagen and Klaassen, 1980).

MT's main ability is to be able to sequester metal ions. It is responsible for the transport of metals such as zinc within the body. Metallothionein also has a protective function by binding with metals such as cadmium that can cause tissue damage in acute doses (Timbrell, 1994). Glutathione acts as a first line of defence in preventing metal toxicity before metallothionein is induced. For instance, mice

that were pretreated to diminish their glutathione levels were sensitised to cadmium toxicity. However, mice which had had metallothionein induced prior to reducing their glutathione levels were not sensitised (Singhal *et al.*, 1987; and Chan & Cherian, 1992). In addition, glutathione is involved with preventing copper toxicity. A hepatoma cell line that was copper-tolerant had induced levels of glutathione and metallothionein compared to a normal cell line when both were exposed to copper. When glutathione levels in the copper-tolerant cell line were diminished the incorporation of copper into metallothionein also diminished (Freedman *et al.*, 1989). A similar mechanism was investigated in rabbits where it was also found that GSH mediated the transfer of Cu²⁺ into MT in rabbit livers (Da Costa Ferreira *et al.*, 1993).

The GSH, MT interaction has also been observed in invertebrates. In the American lobster, GSH forms an intermediate complex with isoforms Cu MT I/II that results in the release of copper and glutathione from the metallothionein complex. This mechanism suggests that GSH is used to raise Cu²⁺ levels for biological synthetic processes (Brouwer and Brouwer-Hoexum, 1991, 1992, and Brouwer *et al.*, 1993a). In addition, there is a MT III isoform that forms a stable complex with GSH. The MT III isoform is induced to bind copper when levels of copper are dangerously high. A glutathione-binding site has been identified in CdZn-metallothionein from the Blue crab hepatopancreas, at the MT's N-terminal domain (Brouwer *et al.*, 1993b). This evidence suggests that glutathione has an important function in the metal detoxification processes of metallothionein.

GSH and MT have also been associated with reducing oxidative stress and free radical scavenging. GSH and MT reduce oxidative stress from metal-induced hydrogen peroxide formation in teleost hepatoma cell lines; induction of both thiol-containing compounds afforded better protection than the induction of only MT (Schlenk & Rice, 1998). MT is also induced in rainbow trout gonadal cells during exposure to hydrogen peroxide. Induction of metallothionein prior to free radical exposure provided the animal with greater protection (Kling *et al.*, 1996). Similar results were also established

in cultured mammalian cells, where, MT's act as a scavenger for reactive radical species (Ochi, 1988). Therefore, MT has an involved role in antioxidant processes in addition to its important role of chelating metal ions.

Part II. Oxidative stress as a consequence of disease and environmental pollution.

1.9 Oxidative stress; Why the increased interest?

Aerobic animals maintain a balance between ROS formation (pro-oxidants) and anti-oxidant processes. However, this balance can become disrupted by the influence of exogenous factors such as pollution, or disease, which can result in tissue injury or even mortality. A thorough understanding of oxidative stress and antioxidant processes can help in the treatment of certain diseases or in ameliorating the effect of exposure to contaminants.

Clinicians are interested in oxidative stress, which has been associated with Parkinsons disease, atherosclerosis, neurological disease, inflammatory disease, cancer, lung disease and reperfusion injury (Wolff and Nourooz-Zadeh, 1995). Manufacturers of anti-aging creams research ROS and free-radicals because of the association between free-radicals and the aging process. Ecotoxicologists research this area because increased oxidative stress in organisms is associated with environmental pollution. In addition, nutritionists are interested in ROS and free radicals as foods high in antioxidants are considered to improve health and decrease the risks of diseases, including cardiovascular disease (Gutteridge and Halliwell, 1996).

Free radical production and anti-oxidant defense systems are generally conserved among different species. This means that bioassays are easily transferable and also the comparative causes and effects of oxidative stress can be investigated.

For the purposes of the present research two models of oxidative stress were initially investigated and evaluated in terms of the ease of establishing antioxidant mechanisms with them. The two models of oxidative stress chosen were secondary complications of diabetes and oxidative stress induced in the shore crab, *Carcinus maenas*, by contaminant exposure. Both of these systems possess similar antioxidant mechanisms that may be implicated in preventing tissue damage. The study of oxidative stress in these two systems is well established, but not thoroughly understood. Secondary complications of diabetes are related to oxidative stress (Ceriello & Giugliano, 1997). Thorough understanding of the causes and effects of oxidative stress associated with the disease could lead to successful treatment or prevention of diabetic complications.

The marine environment can potentially become contaminated with anthropogenic waste. The disposal of sewage into the sea has been reassessed and sewage is treated before it enters it. However, the marine environment is still subject to localised pollution from industrial outlets, agricultural run-off, oil spills, litter, marinas, ports and boats. Marine organisms that inhabit these areas can become exposed to this pollution. In extreme cases, such as oil spills the effects on local flora and fauna are immediate and obvious. Whereas, the effects are less obvious in areas that receive gradual and long-term contaminant exposure; for example areas effected by industry. Biomarkers can be used to monitor the initial effects of contamination on marine organisms and these can act as 'early warning systems' of potentially more serious biological disruption. Further information of the benefits of research into these areas are given in the following sections. In addition, further explanations of the sources and effects of free-radical formation are provided.

Model 1.

1.10 The cause of diabetes.

There are at least two forms of diabetes that are caused either by insufficient insulin secretion or the presence of factors that oppose the action of insulin. The net effect is an increase in blood glucose levels (hyperglycaemia).

Insulin dependent diabetes mellitus (IDDM) usually occurs before adulthood and was formally known as juvenile-onset diabetes, it is also referred to as type 1 diabetes. IDDM is an autoimmune disease. At the onset of IDDM the body's lymphocytes and monocytes begin to attack the β cells in the pancreas, killing them and preventing secretion of insulin. The autoimmune attack on the pancreas is very specific and only those cells that are involved with the secretion of insulin, the β cells within the Islets of Langerhans, are attacked. After approximately 80% of the insulin producing β cells have been destroyed the effects of diabetes become apparent (Atkinson & Maclaren, 1990).

Insulin helps the cells in the body to take up biological fuels including glucose. The body becomes dehydrated as it struggles to filter excess glucose through the kidneys. Meanwhile, the body cells starve due to lack of biological fuel and break down their stores of fat and protein. The breakdown of fats produces ketone by-products that added to dehydration, induces fits and finally results in death (Atkinson & Maclaren, 1990).

Non-insulin dependent diabetes mellitus (NIDDM) onset usually occurs in patients over 30 years of age and is known as type 2 diabetes. In contrast to IDDM, the pathophysiology of NIDDM is not clearly established. The underlying mechanisms is due to either diminished insulin secretion (i.e. a defect within the islet of Langerhans), or increased peripheral resistance to the action of insulin (decreased peripheral glucose uptake or increased hepatic glucose output) (Watkins, 1998).

1.11 Sources of diabetes induced free radical formation

Since the discovery of the treatment of diabetes using insulin, the life expectancy of diabetes patients has increased. This development has revealed the second consequence of diabetes, chronic diabetic complications. Insulin injections help diabetic patients to incorporate glucose into their cells so it can be metabolised normally. However, it does not prevent the long-term complications of diabetes, which are believed to occur due to chronically elevated blood glucose levels.

Many theories have been put forward and developed to explain the underlying cause of diabetic complications. They all appear to be justified and many of them overlap. However, a comprehensive explanation has still not been established (Baynes & Thorpe, 1999). The theories that focus upon oxidative stress are of particular interest. Some of the ways in which increased oxidative stress may occur in diabetes mellitus patients are summarised in Table 1.3.

Evidence is available that shows that glucose undergoes autoxidation *in vivo* (Hunt *et al.*, 1988; Greene *et al.* 1999). The reduced oxygen products of this reaction include superoxide and hydrogen peroxide, therefore, autoxidative glycosylation is a reasonable source of oxidative stress. There is also evidence from *in vitro* experiments that glycosylated proteins may form superoxide ions in physiological conditions (Gillery *et al.*, 1988; and Sakuzay & Tsuchiya, 1988).

In addition to the above, antioxidative metabolism in patients with diabetes is believed to be affected (Szaleczky *et al.*, 1999). Patients with diabetes are found to have low concentrations of the protective antioxidants, such as reduced glutathione, and vitamins C and E (Ceriello and Giugliano, 1997; Ashour *et al.* 1999). The decline in these compounds results from competition for NADPH, which is induced by increased influx through the sorbitol pathway (Lee & Chung, 1999). This pathway, which converts glucose to sorbitol, has been implicated in the pathogenesis of many diabetic complications. The sorbitol pathway is shown in Figure 1.7.

<i>Source</i>	<i>Effect</i>
Glucose autoxidation;	Generation of superoxide radicals and hydrogen peroxide
Protein glycosylation and Amadori autoxidation;	Generation of superoxide accompanied by glycoxidation product formation
Decreased antioxidant status;	Linked to sorbitol pathway activation. Reduced levels of antioxidant vitamins and reduced glutathione

Table 1.3 The sources of oxidative stress in diabetes mellitus adapted from, Ceriello and Giugliano, 1997.

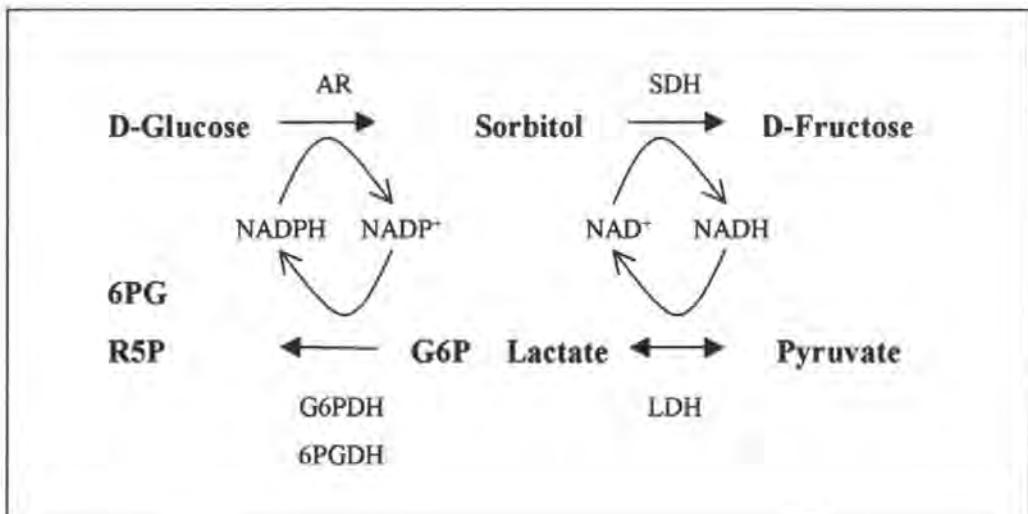


Figure 1.7 The reduction of glucose to sorbitol and oxidation of sorbitol to fructose in the sorbitol pathway. Reduction of glucose to sorbitol by AR (aldose reductase) is coupled to the oxidation of NADPH to NADP⁺. NADP⁺ is reduced to NADPH by the hexose monophosphate pathway. Oxidation of sorbitol to fructose by SDH (sorbitol dehydrogenase) is coupled to reduction of NAD⁺ to NADH. The cytosolic ratio of free NADH/NAD⁺ is in equilibrium with lactate and pyruvate. G6PDH = glucose-6-phosphate dehydrogenase, 6PGDH = 6-phosphogluconate dehydrogenase, LDH = lactate dehydrogenase.

During intracellular hyperglycaemia, glucose is converted to sorbitol. This undergoes further degradation by sorbitol dehydrogenase to produce D-fructose. During the conversion of D-glucose to sorbitol NADPH is oxidised to NADP⁺. It is believed that the increased influx through the sorbitol pathway in hyperglycaemic conditions reduces the cell's reserve of reduced NADPH, which is used as a substrate for many anti-oxidative pathways (Lee & Chung, 1999). Hence, the net effect is a reduction in anti-oxidative defenses within the cell (Szaleczky *et al.* 1999). It is also hypothesised that the altered the NADH : NAD⁺ ratio in the cell (Figure 1.7) induces hypoxic effects which may also play an important role in the pathogenesis of diabetic complications (Obrosova *et al.* 1998; Obrosova *et al.* 1999).

In addition to increased flux through the sorbitol pathway, glutathione concentration can also be reduced by elevated levels of acetoacetate. This ketone body also increases lipid peroxidation in people with type 1 diabetes, (Jain & McVie, 1999).

Recently a new line of oxidative research has received much attention, the AGE hypothesis. This hypothesis further develops the theory of the glycosylation of proteins. The AGE hypothesis (advanced glycation end product) states that chronic accelerated modification of protein by reducing sugars in diabetes alters the structure and function of the tissue proteins which can ultimately lead to diabetic complications (Brownlee *et al.*, 1988; Brownlee, 1996; and Vlassara, 1997). AGEs have been known for a long time and were initially researched by the food industry where they were first discovered. The chain of effects was known as the Maillard reaction. These reactions which produced products, that were brown in colour and had a specific fluorescence, were known to partake in protein cross-linkage reactions. These reactions were prevented in food chemistry by the addition of reducing agents (Saltmarch & Labuza, 1982). Development of this line of research has classified many more compounds as AGE products that are not brown in colour or fluorescent or involved with cross-links of proteins. However they may increase but not necessarily accumulate in

the tissues in ageing or diabetes (Baynes & Thorpe, 1999). It is thought that these AGE compounds are intermediates in the cross-linkage of proteins. The general consensus today proposes a general role for the generation of AGEs due to oxidative stress (Baynes, 1991).

In addition to AGEs there is another group of reactive species that may be distinct from the products of oxidative stress. These are reactive carbonyl precursors of AGEs that increase in diabetes as well as other diseases and influence the chemical modification of proteins. These reactive carbonyl compounds may be formed from oxidation in which case they are described as contributing to 'oxidative stress'. However, they may also be formed, in full, or part, from non-oxidative processes in which case the terminology 'carbonyl stress' is adopted to describe them. The debate between the distinction between the two terms is described by Baynes & Thorpe (1999).

1.12 Diabetic complications associated with altered antioxidant metabolism

Research has shown a relationship between oxidative stress and complications that are associated with diabetes (Reviewed by Ceriello & Giuglano, 1997; Szaleczky, 1999; Vendemiale *et al.*, 1999; Greene *et al.*, 1999, West, 2000). Diabetes mellitus can produce a variety of complications. These complications include microvascular disorders (retinopathy, nephropathy and neuropathy) and also macrovascular disorders (cardiovascular disease, cerebrovascular disease and peripheral vascular disease).

Research has shown that glutathione metabolism is altered in animals and patients with diabetes mellitus (Table 3.1). The complications of diabetes have also been associated with these changes. For example, there is evidence that patients have increased lipid peroxides in their plasma when compared with non-diabetics, especially in the presence of retinopathy. GSSG/GSH ratio, GR, GP and GST were increased in the lens of diabetic rats compared to controls. In this instance the

depletion of essential nonenzymatic antioxidant defences rather than a decrease in enzymatic antioxidant defences were central in increased oxidative stress associated with the diabetic precataractous lens, (Obrosova, *et al.*, 1999). The impairment of lens antioxidative defence could be partially prevented by DL- α -lipoic acid, which is able to penetrate into the lens and is reduced to form a potent antioxidant scavenger (Obrosova, *et al.* 1998). This highlights the importance of oxidative stress in lens metabolic imbalances in diabetes.

Diabetic neuropathy may affect any part of the nervous system. It is a common source of foot problems. Loss of pain sensation due to peripheral neuropathy can lead to prolonged injury to the foot and consequent ulceration (Campbell & Lebovitz, 1996). A reduction of GP activity occurs in peripheral nervous tissue during the early onset of diabetes in mice (Romero *et al.* 1999). In addition, GSH/GSSG redox couples tends towards the more oxidised form (Sytze Van Dam *et al.*, 1999 and West, 2000). Treatment of experimental diabetes with antioxidant scavenging compounds, including oxidised glutathione, helps to ameliorate the effects of diabetic neuropathy (Romero *et al.* 1999; Cameron & Cotter, 1999; Cameron *et al.* 1993).

Accelerated atherosclerotic vascular disease is the leading cause of mortality in patients with diabetes mellitus. Vitamin-E treatment was found to decrease nonenzymatic glycosylation, oxidative stress and red blood cell microviscosity in diabetic patients (Giugliano *et al.*, 1995). Exposure to hyperglycaemia slows the proliferation of cultured human endothelial cells, which may account for vascular lesions that are common in diabetes (Lorenzi *et al.*, 1987). Antioxidants, superoxide dismutase, catalase and reduced glutathione protect human endothelial cells from the delay in replication (Cerriello *et al.*, 1992). Magnesium deficiency is associated with increased free radical-dependent oxidative tissue damage. Research has shown that GSH/GSSG ratios and Mg⁺ concentrations are linked with tissue glucose metabolism and the administration of glutathione and vitamin E can have positive effects on diabetes hypertension (Barbagallo *et al.*, 1999a; 1999b).

Many studies have highlighted that diabetic erythrocytes are in some cases abnormal. Oxidative stress resulting from elevated glucose levels may be responsible for osmotic fragility and related deformity (Jain, 1989).

The likelihood of a diabetic mother bearing a deformed child is twice that of a non-diabetic mother (Borch-Johnsen & Deckert, 1992). Results indicate that GSH depletion and impaired responsiveness of GSH-synthesising enzyme to oxidative stress during organogenesis may have important roles in the development of embryonic malformations in diabetes (Sakamaki *et al.*, 1999). Rat embryos cultured *in vitro*, develop abnormalities in the presence of a hyperglycaemic culture media. This does not occur when free radical scavengers are added to the media (Eriksson & Borg, 1991). These results demonstrate that oxidative stress has a considerable role in the cause of embryonic malformations in diabetes.

Diabetes is also associated with a thrombosis-prone condition. It is hypothesised that this condition is related to hyperglycaemia. However, its relation to the coagulation cascade is still unclear. Evidence from the experimentation using diabetic patients showed that hyperglycaemia may contribute to thrombin activation by possibly inducing oxidative stress, and that glutathione may counter-balance this effect (Ceriello *et al.*, 1995). However this is only one of many pro-thrombin mechanisms.

Although there may be many factors that contribute to diabetic complications, there is undoubtedly evidence that suggests that oxidative stress plays a considerable role. Results indicate that the supplementation of diabetic diets with antioxidants may be useful in preventing the complications that develop with long term diabetes.

Model 2.

The second model investigated was the oxidative stress responses of *Carcinus maenas*, to pollution exposure. Metabolic changes in response to contamination have potential for use as biomarkers. Biomarkers can be applied to monitor areas that may be vulnerable to ecological changes as a result of exposure to anthropogenic waste. A discussion of why environmental monitoring is important is given below. In addition, metabolic changes induced by contaminant exposure are discussed.

1.13 The necessity for ecotoxicological studies.

During the Second World War the solution applied to pollution problems was a ‘dilution solution’. This meant that any waste was dumped into the sea, the hypothesis being that the sea is so vast that the waste would be distributed and diluted to trace levels. The consequence of this practice of pollution control had an adverse affect on environmental health and subsequently human health (Newman, 1998). A series of ecological and human health disasters resulted in governmental and public awareness of pollution problems. From 1960 to 1965 there was a rapid rise in human body burdens of ¹³⁷Caesium which resulted from open air nuclear testing. This problem decreased in impact after the U.S., the former Soviet Union, France, and China ceased open air testing. In addition, there was rising public concern over the effect of pesticides, such as DDT (dichlorodiphenyltrichloroethane), which were accumulating in the food chain of wildlife and causing detrimental effects upon species’ populations, reproduction, and health. This, together with Minamata disease in Japan which was induced by heavy metal poisoning and resulted in nearly one thousand victims of the disease, gave impetus to the science of ecotoxicology (Newman, 1998).

Environmental problems continue to arise despite increased awareness of the resulting problems and increased regulation of waste disposal. The science of ecotoxicology aims to establish the long-term

effects to the environment, also the costs and benefits that will result from the decisions that industry, technology and governments make, which affect our lives.

Ecotoxicology spans many different scientific disciplines, including both the physical sciences and the life sciences. Of particular interest for this research is the application of oxidative stress studies.

1.14 Biomarkers and their implication in ecotoxicological studies.

For the purpose of this study biomarkers are defined as a biological response to a chemical or chemicals that gives a measure of exposure and sometimes, also, of toxic effect (Walker *et al.*, 1997). The definition of a biological response also includes effects that are exerted at a population and community level. Most toxicants exert their effects through interactions with biomolecules, with the exception of some chemicals such as asbestos which cause direct physical damage of tissues and cells. Effects then cascade through the biochemical → subcellular → cellular → tissue → organ → individual → population → community → ecosystem → landscape → biosphere levels of organisation (Newman, 1998). Hence, an understanding of the effects of contaminants at the biochemical level may allow us to predict what will occur at the next few levels. Past experience of other well known chemicals may also allow us to estimate what may occur at higher levels when we are testing a new type of contaminant. In addition, an understanding of biochemical disruption induced by pollutants can act as an early warning system against ecological problems which may occur if the problem is not checked as soon as possible. For example, induced metallothionein concentrations are indicative of the early stages of metal contamination, whereas, a decline in species population may be caused by the later stages of metal contamination. The advantage of locating toxicological disruption at a cellular level means that very often the contamination can be treated and the effects reversed relatively quickly. When toxicological damage is exhibited at a community level,

ecologically irreversible damage has usually already occurred. Biomarkers also allow us to monitor the improvement in the environment after a pollution incident.

The analysis of toxicological effects at a biochemical level has other benefits. The cost of chemical water analysis can be very high and time consuming, whereas bio-assays are relatively a lot cheaper and quicker. If a sample population of animals from a monitored area is exhibiting stress then it is worth while carrying out expensive chemical analysis of the water to establish which contaminants are causing the effect. The other advantage of bio-assays is that they show that the contaminant is causing a biological effect. A contaminant describes a compound that has been located in an environment where it is not usually found, for instance agricultural run off can introduce nitrates into aquatic systems. However, unless the contaminant is influencing the environment it has entered, e.g. by inducing algal blooms or by poisoning organisms, is it behaving as a pollutant or causing any harm? Biomarker assays confirm whether the chemical is causing a biological effect.

Some biomarkers are also advantageous in allowing us to assume which type of contaminant is causing the effect, for instance, an increase in metallothionein levels in an organism is indicative of heavy metal exposure (Pedersen, 1996). Other biomarkers are useful in establishing toxic effects such as DNA damage that is caused by aromatic hydrocarbons. Another direct toxic effect is demonstrated by disturbances to the transthyretin-retinol binding protein complex by a metabolite of 3,4,3',4', tetrachlorobiphenyl. In addition the measurement of clotting proteins in blood is indicative of the inhibition of the Vitamin K cycle by anticoagulant rodenticides (Walker, 1995).

1.15 Mechanisms of xenobiotic induced oxidative stress.

Many xenobiotic compounds have the potential to increase oxidative stress in the cells of organisms. These xenobiotic compounds include nitroaromatics, quinones and biphenyls, ethanol and carbon

tetrachloride (Newman, 1998). The latter chemical CCl_4 has been used extensively as a model compound for free radical damage in the liver. CCl_4 undergoes bioactivation by P_{450} and is reduced to a trichloromethyl radical ($\text{CCl}_3\cdot$), which in the presence of oxygen is converted into a peroxy radical ($\text{Cl}_3\text{COO}\cdot$) (Recknagel *et al.*, 1989). Other xenobiotics, which are also known to induce massive radical damage, are redox cycling compounds; these compounds are reduced to form free radical species by single electron reduction. They then donate their electron to molecular oxygen, hence producing superoxide free radicals and regenerating the parent molecule. Some examples of compounds that undergo redox cycling via P_{450} reductase and/or P_{450} include paraquat, diquat, menadione and doxorubicin (De Zwart *et al.*, 1999) (e.g. paraquat Figure 1.8). Evidence has shown that microsomes from various aquatic organisms also catalyse the reduction of these compounds (Matkovics *et al.*, 1987; Washburn & DiGiulio, 1989; and Livingstone *et al.*, 1989).

Xenobiotics also cause oxidative stress indirectly by interfering with processes, which have evolved to cope with oxidative stress, and which mop up free radicals. Free radicals can form covalent bonds with a variety of biomolecules. They covalently bind to membrane components such as enzymes and receptors, which results in changing their orientation and functions (Slater, 1984). Free radicals also cause damage by reacting with sulphhydryl groups of proteins and other biomolecules, (e.g. glutathione), hence altering their function (Slater, 1984).

Other contaminants such as metals can have an effect on enzyme function and substrate pools. Metals can influence protein-mediated catalysis, protein transport and gas exchange by modifying protein structure (Ulmer, 1970). Metals also bind to a wide range of electron donor groups, hence inhibiting substrate function. They may induce changes in the secondary and tertiary protein structure of enzymes, which can result in lowered or increased enzyme activity; this makes many enzymes useful as biomarkers of contaminant exposure. Some metals that are present in enzymes are responsible for their quaternary structure. Substitution of the normal stabilising metal for another can

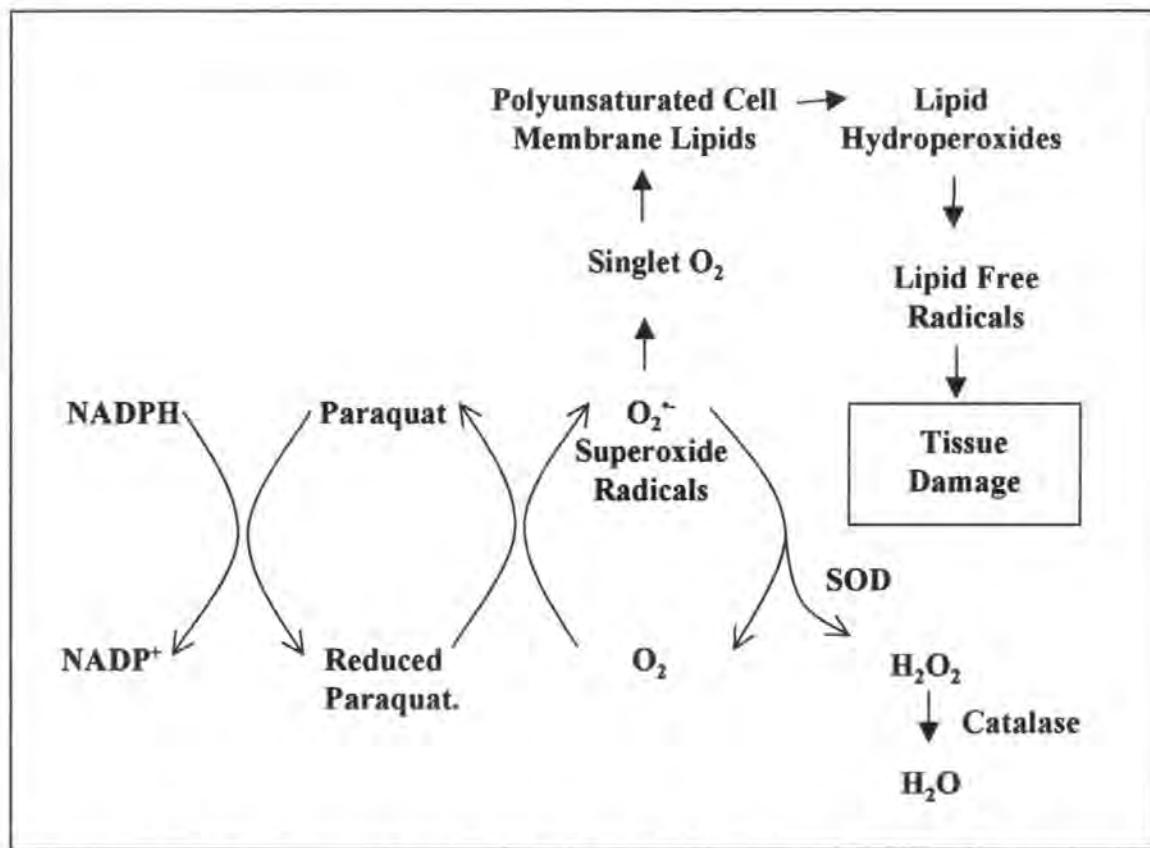


Figure 1.8 The proposed mechanism of toxicity of paraquat, involving the mechanism of redox cycling which is induced by different toxins. Redox cycling produces active oxygen species, which can deplete NADPH and glutathione and potentially cause peroxidation of membrane lipids. (Figure adapted from Timbrell, 1994).

result in interference of the coming together of peptide chains to form stable and functional multimeric enzymes (Newman, 1998). Alternatively substitution of these metals can assist in denaturation of the enzyme (Ulmer, 1970). Metals are also present at the active site of biomolecules, such as cytochrome c and haemoglobin; displacement of the metal by another can also change the functioning of these proteins.

1.16 Biomarkers of oxidative stress.

There has been a great deal of interest in the use of marine invertebrates as biomonitoring of pollution-mediated oxidative stress. Many invertebrates are filter feeders and contaminants that are present in their habitat become concentrated in their tissues. Because of this concentration effect the presence of these contaminants is often easier to establish from animal tissue than it is from the surrounding medium. As many invertebrates are at the base of the food chain the bioaccumulation of the metals at this level can have devastating effects on organisms at higher trophic levels as they digest food that have a high contaminant content.

Research to date has identified which types of contaminants induce metabolic changes within different organisms. Oxy-radical production is found to be a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis*, as reviewed by Livingstone *et al.* (1990). Oxy-radical production has been shown to decrease the stability of digestive gland lysosomes in the common mussel (Winston *et al.*, 1991). This can result in the leakage of potent hydrolytic enzymes into the cytosol, which can result in enzyme degradation and protein damage to other organelles. Oxidative stress is also thought to be a contributing factor towards coral bleaching; it is believed to be induced by increased temperatures or UV light (Lesser, 1997). Oxidative stress induced by cadmium has been shown to increase lipid peroxidation in Atlantic Croaker tissues (Thomas & Wofford, 1993)

and copper toxicity was found to have the same effect in the gill and digestive tissues of the mussel, *Mytilus galloprovincialis* (Viarengo *et al.*, 1990).

There are many metabolic systems of invertebrates that are sensitive to xenobiotic exposure and can be utilised as biomarkers of toxicity; some of which include superoxide dismutase, glutathione-s-transferase, glutathione peroxidase and glutathione reductase activities, catalase, thiol status, P₄₅₀ system, multi-drug resistance systems and concentrations of antioxidants such as glutathione, vitamin E and beta-carotene. These biomarkers have a direct or indirect role in the metabolic control of oxidative stress. For the purposes of this study attention is focussed on glutathione and its related enzymes.

Most laboratory-based research has been performed on the effects that individual contaminants have on organisms. A list of the different effects that metals or organic pollutants have upon the glutathione and related enzymes of different species is given in Tables 1.4 and 1.5. The tables do not show a universal response of organisms to any one type of contaminant. The enzyme activities of different organisms respond differently to the same contaminant. These tables demonstrate that glutathione and its related enzymes do not provide us with a universal biomarker that is applicable to all species. It cannot be assumed that a change in enzyme activity is related to a certain type of contaminant. Despite this, glutathione and its related enzymes have been successfully applied as biomarkers in field experimentation and have been shown to be efficient biomarkers of pro-oxidant contamination.

Field biomarker studies have highlighted some biochemical mechanisms that result from pollution exposure. A study by Regoli and Principato (1995) showed that different tissues within the same animal have variable enzyme activities in response to exposure to the same contaminant. This

Author	Pollutant	Species	Tissue	Effect
Canesi <i>et al.</i> 1999	Copper	Mussels, (<i>Mytilus galloprovincialis</i>)	Gill	Decrease in total glutathione Increased GST activity
			Digestive Gland	Decrease in total glutathione Increased GST activity
	Organic Mercury		Gill	Decrease in total glutathione Increased GST activity
			Digestive Gland	Inhibition of glutathione synthesis rate Decrease in total glutathione Increased GST activity Inhibition of glutathione synthesis rate
Thomas and Wofford, 1993	Cadmium	Atlantic Croaker (<i>Micropogonias undulatus</i>)	Hepatic Ovarian	Decrease in GP activity Total glutathione concentration constant.
Regoli <i>et al.</i> 1997a	Copper 20 $\mu\text{g l}^{-1}$	Antarctic Scallop (<i>Adamussium colbecki</i>)	Digestive gland	Decrease in total glutathione concentration Decrease in GST activity
	Mercury 5 $\mu\text{g l}^{-1}$			No change in GP activity No change in GR activity Decrease in total glutathione concentration Decrease in GST activity No change in GP activity No change in GR activity
Regoli <i>et al.</i> 1998a	Copper 20 $\mu\text{g l}^{-1}$ Mercury 5 $\mu\text{g l}^{-1}$	Antarctic scallop (<i>Adamussium colbecki</i>)	Digestive gland	Decrease in total glutathione concentration Decrease in GST activity Decrease in total glutathione concentration Decrease in GST activity
Rafter, 1982	Copper	Human (<i>Homo sapiens</i>)	Leukocytes	Decrease in GR activity Decrease in reduced glutathione concentration.
Thomas and Juedes, 1992	Lead	Atlantic croaker (<i>Micropogonias undulatus</i>)	Liver Intestine	Increased total glutathione concentration.
Patel <i>et al.</i> 1990,	Mercury	Blood Clam (<i>Anadara granosa</i>)	Erythrocytes	Decrease in GR activity
Doyotte <i>et al.</i> 1997	Copper	Freshwater bivalve (<i>Unio tumidus</i>)	Gill	Decreased GP activity
			Digestive gland	Decreased GR activity Decreased total glutathione concentrations Decreased GP activity Decreased GR activity Decreased total glutathione concentrations Decrease in GR activity
Fukino <i>et al.</i> , 1986	Selenium Zinc	Male Wistar Rats	Kidney	Increased total glutathione concentrations.

Table 1.4 Responses of glutathione concentrations and related enzyme activities in response to heavy metal contamination in different tissue types and species of animal.

Author	Pollutant	Species	Tissue	Effect
Wofford and Thomas, 1984	Cadmium	Striped Mullet (<i>Mugil cephalus L.</i>)	Liver	Increased total glutathione concentrations
Reddy <i>et al.</i> 1996	Copper	Crab (<i>Oziotelphusa senex senex</i>)	Hepatopancreas	Increased GST activity.
	Cadmium			Increased GST activity.
	Phosalone			Decreased GST activity.
Roche and Bogé, 1993.	Copper	Marine Fish (<i>Dicentrarchus labrax</i>)	Red blood cells	Decrease in GP activity
	Zinc			Decrease in GP activity
	Chromium (1-100µM) (0.5&1mM)			Increased GP activity in low concs of chromium Decreased GP activity in high concs of chromium.
Gwozdzinski <i>et al.</i> 1992	Copper	Human	Erythrocytes	No effect on GP activity.
	Mercury			No effect on GP activity.
	Copper	Fish (<i>Dicentrarchus labrax</i>)	Erythrocytes	Decreased GP activity.
	Mercury			No effect on GP activity.
Ringwood <i>et al.</i> 1998	Copper	Oyster (<i>Crassostrea virginica</i>)	All tissues	No effect on glutathione concentrations.
Allen, 1995	Cadmium	Cichlid (<i>Oreochromis aureus</i>)	Kidney	Increased reduced glutathione levels
Yan <i>et al.</i> 1997	Lead	Green Mussel, (<i>Perna Verdis</i>)	Gill	Increased glutathione concentrations
	Mercury		Digestive Gland	Increased glutathione concentrations
			Kidney	No effect on glutathione concentrations
Reddy, 1997	Copper	Edible crab (<i>Scylla serrata</i>)	Gill	Increase glutathione concentrations
	Cadmium		Digestive Gland	Increased glutathione concentrations
	Copper		Kidney	Increased glutathione concentrations
	Cadmium		Gill	Increased GP activity
				Increased GST activity
				No effect on GP activity
				No effect on GST activity
				Increased GP activity
				Increased GST activity
				No effect on GP activity
				No effect on GST activity

Table 1.4 (contd) Responses of glutathione concentrations and related enzyme activities in response to heavy metal contamination in different tissue types and species of animal.

Author	Pollutant	Species	Tissue	Effect
Thomas and Wofford, 1993	Aroclor 1254	Atlantic Croaker (<i>Micropogonias undulatus</i>)	Hepatic Ovarian	Increase in GP activity Total glutathione concentration constant.
Dierickx, 1984	Quinones o-chlorinal chlorophenoxyalkyl acids	<i>Tubifex tubifex</i>	Whole animal	Decreased GST activity
		Rat	liver	Decreased GST activity.
Fitzpatrick et al. 1995	Hydrocarbons PCBs Organochlorines	Mussel (<i>Mytilus galloprovincialis</i>)	Digestive gland	Decreased GST activity.
Porte et al. 1991	PCBs PAHs	Mussel (<i>Mytilus galloprovincialis</i>)	Digestive gland	No effect on GP activity.
Boryslawskyj, et al. 1988	Organochlorine (Dieldrin)	Freshwater mussel. (<i>Sphaerium corneum</i>)	Whole soft Tissue	Increased GST activity.
Doyotte et al. 1997	Dithiocarbamates thiram (bis-dimethyl thiocarbonyl disulphide)	Freshwater bivalve. (<i>Unio tumidus</i>)	Gill	Decreased GP activity
				Decreased GR activity
				No change in glutathione concentrations
			Digestive gland	Decreased GP activity
				Decreased GR activity
				Decreased glutathione concentrations
Solé et al. 1994	Organochlorine	Mussel (<i>Mytilus galloprovincialis</i>) Oyster (<i>Ostrea edulis</i>) Western oyster (<i>Crassostrea gigas</i>) Clam (<i>Tapes semidecussata</i>)	Digestive gland	Increased GP activity.
Förlin et al. 1996	PCBs	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Liver	Increased GST activity Increased GR activity.

Table 1.5 The responses of glutathione concentrations and related enzyme activities to organic pollution in different tissue types and species of animals.

observation of differential enzyme responses to pollution within different tissues of the same animal is also shared by other researchers including Doyotte *et al.* (1997) and Cossu *et al.* (1997). Field studies have also highlighted possible adaptation of the animals to a contaminated environment. Adaptation was observed in *Mytilus galloprovincialis* that were native to contaminated sites and had similar GR and GP activities to control animals; however, animals which were transplanted to the polluted sites from a clean site, or exposed to contamination in the laboratory, exhibited varying GR and GP activities (Regoli & Principato, 1995). Adaptation of mussels was also observed in a transplantation field experiment by Cossu *et al.* (1997). The adaptation of animals to polluted environments can cause problems when interpreting biomarker data. It may be more effective to implement continual biomonitoring of a site using indigenous organisms.

Metal toxicity has caused a reduction of thiol status of many different organisms in several field and laboratory experiments (Regoli & Principato, 1995; Doyotte *et al.*, 1997; Regoli *et al.*, 1997a, 1998a; Cossu *et al.*, 1997; Canesi *et al.*, 1999). However, this is not a universal response to metal toxicity as Yan *et al.* (1997) and Allen (1995) have demonstrated contrary responses. In many cases glutathione status has been proven to be a useful and effective biomarker of xenobiotic exposure, although many negative results are not published. Thiol status is greatly affected due to direct interaction with contaminants and via xenobiotic metabolising enzymes. Patel *et al.* (1990) showed that the blood clam, *Anadara granosa*, benefited by glutathione supplementation during mercury exposure as it reduced toxic effects. In addition, Doyotte *et al.* (1997) showed that reduced glutathione concentration was coupled with reduced GR activity and this was accompanied by a concomitant increase of malonaldehyde production. Other investigations have also coupled decreased enzyme activities with increased lipid peroxidation (Regoli & Principato, 1995; Cossu *et al.*, 1997; Thomas and Wofford, 1993; Roche & Bogé, 1993). These results prove that contaminant induced oxidative stress can ultimately lead to physiological damage.

The measurement of antioxidant enzymes is not as useful if we want to associate observed effects with a possible cause. However, Porte *et al.* (1991) were successful in relating enzyme effect with the amount of contaminant loading in mussels. Results showed correlations between cytochrome P₄₅₀, catalase and SOD levels with whole body PAH body burdens in mussels. In addition, Solé *et al.* (1994), found good correlations between the body burdens of PAHs in different tissues, of four marine invertebrates, and several cytochrome P₄₅₀ components. In addition, they observed a relationship between GP activity and accumulation of organochlorinated compounds. Other attempts at correlating results have been less successful. Lee (1988) observed a significant rise in GST activities in marine invertebrates from two polluted sites in Norway. However, this activity was not associated with tissue concentrations of PAHs and PCBs, the real reason for this response could not be determined from the available data. Fitzpatrick *et al.* (1997), also observed a change in GST specific activity in *Mytilus edulis* that were exposed to sites receiving tannery discharges. The cause for this effect also could not be evaluated from the parameters measured. Oxidative enzymes have proved to be useful as biomarkers in highlighting the presence of xenobiotic-induced stress; which if left unchecked could have significant effects upon the health of the population.

1.17 Summary

Antioxidant stress systems are essential for the survival of an organism inhabiting an aerobic environment. An understanding of them can provide us with the knowledge of how to prevent or treat cellular damage resulting from oxidative stress, when our own antioxidant systems become depleted or overcome by pro-oxidants. Oxidative stress is incurred from exposure to pollution, uv-light, food additives, disease etc. For this reason an interest into antioxidant metabolism has been

provoked from many different research areas, drug companies, cosmetic industries, food companies, medical research.

For the purposes of this study attention has focussed upon oxidative stress that is associated with the long-term complications of diabetes and the effects of xenobiotic exposure to *Carcinus maenas*. Long-term complications of diabetes are linked to oxidative stress and are thought to result from the toxic effects of hyperglycaemia. An understanding of the mechanisms that link oxidative stress to long-term diabetic complications could provide insight into effective treatment for the condition. The second model of oxidative stress is useful in assessing the impact of anthropogenic waste upon marine organisms. When antioxidant processes are triggered in a species in response to contamination we can assume that the contaminant is having an effect on that organism. This effect may be the early warning signs of a larger ecological damaging event that could occur if the waste continues to be deposited in that environment. Biomarkers provide a means of monitoring an environment so that action can be taken before an ecologically significant pollution incident occurs.

1.18 Aims and Objectives.

The initial aim of this project was to apply reliable enzyme assays and related anti-oxidant measurements to the tissues of two organisms used as models of oxidative stress. The assays chosen had to be simple to apply, sensitive, specific and have a low experimental error.

After the analytical techniques were selected they were applied to the two models of oxidative stress. The two models were assessed with regard to the ease with which they could be applied to future investigations. Criteria considered were the number of subjects that were required to give significant results, ease of obtaining tissue samples, control over the experimental model, and the ease of manipulating experimental conditions.

Further investigations used the *Carcinus maenas* model of oxidative stress. The aim was to establish how crabs anti-oxidant and xenobiotic metabolising system was affected by a complex mixed effluent; then to investigate how additional stressors, seasonality, nutritional changes and the presence of glutathione-depleting agents affected glutathione metabolism. There were many objectives to this part of the investigation as listed below:

- (i) To establish how these stressors influence glutathione metabolism.
- (ii) To assess how the parameters measured would be affected by environmental changes, hence determining their reliability as biomarkers.
- (iii) To assess the importance of the role that glutathione has in antioxidant metabolism, whether glutathione antioxidant metabolism is crucial in the controlling of oxidative stress.
- (iv) To uncover any mechanisms of antioxidant pathways that have not been previously reported in *Carcinus maenas*.

To achieve these objectives additional parameters had to be incorporated such as, metallothionein concentrations, total antioxidant scavenging ability and a physiological measurement of tissue health, i.e. lysosomal neutral red retention time.

Finally, the object was to incorporate the biochemical and physiological parameters developed into a field study of pollution using *Carcinus maenas* as a bio-indicator species of pollution. The objective of this study was to assess how effectively glutathione enzymes, metallothionein levels and lysosomal neutral red retention time, could be applied as biomarkers *in situ*. The field trial had to fulfil the following aims;

- (i) to effectively apply biomarkers that were established in the laboratory to a field situation,
- (ii) to establish which biomarkers were most effective in highlighting contaminated areas,

- (iii) to assess the effectiveness of using *Carcinus maenas* as a bio-indicator species,
- (iv) to compare biomarker techniques with conventional toxicity tests,
- (v) and to investigate the potential of using a novel application of statistical analysis to the data.

Analytical method selection and evaluation

Several procedures were investigated for their potential to be applied to oxidative stress studies. Analytical procedures selected for use fulfilled certain criteria; they had a high degree of reproducibility and they were specific to the measurements required.

The following parameters were selected GST, GP, GR assays, total GSH/GSSG concentrations, metallothionein concentrations (MT), total antioxidant scavenging ability, neutral red retention time and metal ion quantification. Several methods were investigated to quantify total glutathione concentrations. The method that was the most simple to use and which gave the most reproducible results was eventually employed.

Section 2.1

MATERIALS AND METHODS

2.1.1 Chemicals and suppliers

Chemicals were obtained from SIGMA unless stated otherwise.

2.1.2 Instrumentation

Spectrophotometric assay analysis

All enzyme assays and the quantification assays for metallothionein and protein, were conducted using a Perkin-Elmer lambda 7 UV/VIS spectrophotometer fitted with a thermostated cell holder. Enzyme assays using human tissue were carried out at 37°C and those using crab tissue at 25°C. Other assays were performed using a plate reader (Dynatech Laboratories MRX).

Metal ion analysis

Cu, Cd and Zn, were quantified using a Varian Liberty 200 inductively coupled plasma - atomic emission spectrometer, (ICP-AES).

2.1.3 Sample collection and tissue preparation

Human blood tissue collection

Human blood samples were collected from patients attending the Diabetic Clinic at Derriford Hospital, Plymouth, South Devon. Local ethical committee aproval had been obtained by Dr Millward, Consultant Physician, Derriford Hospital for collecting the blood samples from the subjects. Control blood samples (age and sex matched) were taken from local volunteers. The blood was taken from the patient's median vein in their forearm with a 10ml *Vacutainer*® containing EDTA, as an anti-coagulant.

Human peripheral blood mononuclear cells (PBMC) preparation for enzyme analysis

5ml of human blood containing EDTA as anti-coagulant was diluted with an equal volume of RPMI media in a sterile tube. 10ml of Histopaque™ was carefully pipetted into the bottom of the tube, which was then centrifuged for 30 minutes at 1700 rpm. The mononuclear layer of cells suspended between the histopaque layer and the plasma were removed and washed twice with RPMI by centrifuging at 1000rpm for 10 minutes. The cells were re-suspended in 5ml of physiological saline (0.9% NaCl) and counted using a coulter counter. The lymphocytes were lysed by a sonication probe (3 x 15s, at 40% output power). The resulting suspension was centrifuged at 1000rpm for 3 min and the supernatant was decanted ready for immediate enzyme analysis.

Crab collection

Crabs were caught inter tidally, using baited drop nets, from Bantham Estuary in South Devon and transported to the aquarium in buckets, (8 crabs / 5 litres of aerated water). The crabs were covered with seaweed in order to reduce stress arising from transportation.

Maintenance and exposure of the crabs to pollutants.

The crabs were kept at 15°C in a temperature-controlled aquarium in aerated tanks having a 12/12h light: dark regime. The crabs were kept for three to four days within the aquarium prior to experimentation so that they became acclimatised to laboratory conditions. Pollutant exposures were carried out in glass tanks that had been thoroughly acid washed in 10% nitric acid. Two replicate tanks were used for each experimental group, which contained aerated, biologically filtered seawater with a salinity of 32%. The tanks were arranged on shelves in a randomised block design. Animals were fed irradiated cockles three times weekly and the water changed every three days twenty-four hours after feeding. Pollutant exposures were renewed with each water change. The used water was removed and the tanks were thoroughly cleaned and fresh water was added before the fresh contaminant mixture was added.

Crab tissue preparation for enzyme, glutathione and total antioxidant scavenging ability analysis

Approximately 1ml haemolymph was removed from each crab from the unsclerotized coxal membrane between the third walking leg and the body using a hypodermic syringe. The sample was immediately injected into vials, snap frozen in liquid nitrogen and stored at -80°C ready for enzyme activity analyses. Another 50µl of the fresh haemolymph was pipetted into a vial containing an equal volume of 5% 5-sulfo-salicylic acid (SSA) and also stored at -80°C until required for glutathione analysis.

Crabs were sacrificed by destruction of the thoracic ganglion. The gill tissues, muscle tissues, endocuticle and midgut gland were removed and snap frozen in liquid nitrogen for storage prior to tissue homogenisation. The gill, endocuticle and midgut gland were thawed and homogenised on ice in 1 part wet weight of tissue: 3 parts homogenisation buffer, respectively (Homogenisation buffer; 0.01 M Tris- HCl pH 7.6 and 0.03% of NONIDET P-40 detergent). The muscle tissue was prepared using a 1:6 ratio, as it is a much denser tissue. The homogenate was further disrupted with a sonication probe for 15s at 40% output power and centrifuged for 30 minutes at 15,000g, 4°C. The resulting supernatant was aliquoted into vials and frozen at -80°C until required for enzyme analysis. 50µl of the supernatent was reserved and placed in a vial containing an equal volume of 5% SSA and then snap frozen ready for glutathione analysis.

2.1.4 Measurement of low molecular weight compounds

i) Measurement of total glutathione levels

The measurement of glutathione has many associated problems. Glutathione is one of many thiol-containing compounds present in biological tissues. Therefore methods of glutathione measurement which incorporate a marker of thiol groups, run the risk of reaction with other thiol containing compounds. Some methods of glutathione quantification are designed to overcome this problem by separating the components of the mixtures by size and then combining the “glutathione extract” with an appropriate thiol marker. The alternative method incorporates the enzymatic selection of glutathione and quantifying the amount of glutathione present by the activity of a cyclic reaction.

Glutathione exists in both oxidised and reduced forms within the tissues of animals. The oxidised and reduced forms of glutathione will be referred to as GSSG and GSH respectively through the remainder of this thesis. Care must be taken when measuring these forms independently so that the reduced glutathione does not become oxidised when it is extracted from the tissue. There is also

risk that the glutathione may undergo spontaneous thiol exchange with other thiol containing compounds. As glutathione is present in both oxidised and reduced forms in addition to conjugation with proteins and other compounds the total glutathione status was determined for the present experimentation purposes.

Initially a HPLC method of determining glutathione concentrations by Paroni *et al.* (1995) was investigated. The tissue sample was derivatised with a fluorescent marker, o-phthaldialdehyde, followed by HPLC separation and quantification of the glutathione by the intensity of fluorescence. Six weeks of intensive experimentation failed to provide an adequate working system for glutathione measurements using this method. The method was abandoned in favour of a procedure for the quantification of total glutathione levels using a cyclic enzymatic method.

This assay was first proposed by Owens and Belcher (1965), it was later developed by Tietze, (1969) and improved by others such as Griffith (1980) and Andeson (1985). The method depends upon the reaction of reduced glutathione with dithionitrobenzoic acid (DTNB) to produce a coloured compound (which can be measured spectrophotometrically at 412nm). The precise nature of the reaction is not known, however evidence has been put forward to support a process of cyclo-reduction. GSSG is reduced enzymatically to GSH (Figure 2.1,(1)), which reacts with DTNB to produce a coloured ion and a mixed disulphide (Figure 2.1, (2)). This disulphide reacts with further quantities of GSH to liberate another ion and GSSG (Figure 2.1, (3)), which then re-enters the cycle (Owens & Belcher, 1965).

Preparation of tissue samples for glutathione analysis

The haemolymph, gill and muscle - 5% SSA solutions were thawed and centrifuged at 10,000rpm for 10 min to separate the precipitated protein from the supernatant. The supernatant was decanted off and used for glutathione analysis.

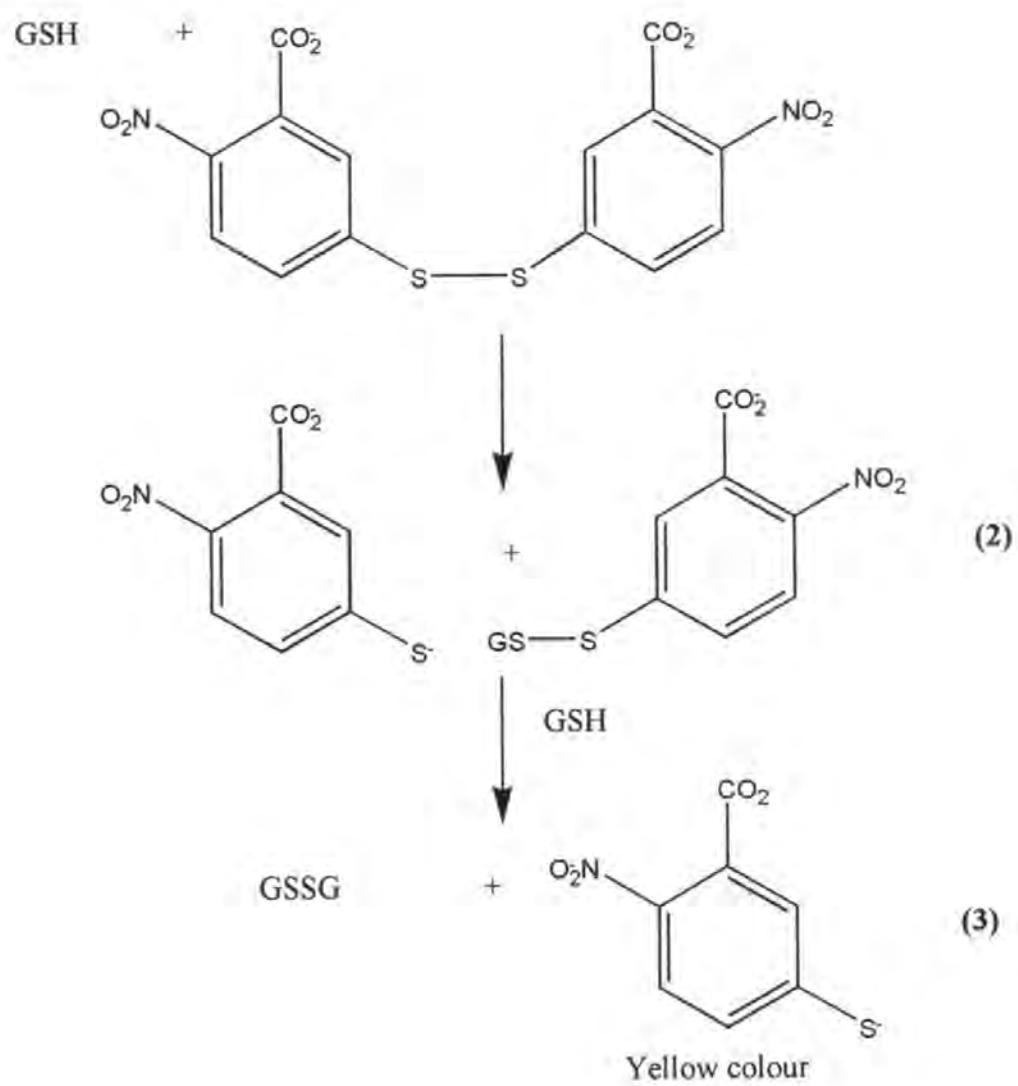


Figure 2.1 The chemical reactions involved in the cyclic quantification of glutathione.

Enzymatic determination of total glutathione levels

Glutathione measurements were made on a 64 well spectrophotometric plate and read in a plate reader. Each well contained either 10 μ l of 5–40 μ M standard GSH solution or the prepared tissue sample. The reference wells contained distilled water instead of sample or standard. 150 μ l of 0.1M KHPO₄ - 5mM Na₂EDTA buffer pH7.5 and 20 μ l of 6mM DTNB were added to each well. The reaction was initiated by adding 20 μ l of glutathione reductase (5.32 U/ml) using a multi-channel pipetter. The plate reader was programmed to shake the plate for 15 seconds prior to reading and the absorbencies of the wells were read at 410nm every 15 seconds for six minutes. All tissue samples, standards and references were run in duplicate on the same plate. The concentration of glutathione in the sample was determined from the reduced glutathione calibration curve.

Evaluation and validation of the assay

To ensure that the concentration of glutathione is directly related to the activity of the reaction, the concentration of glutathione was plotted in a graph against change in absorbance, (ΔA) (Figure 2.2). The graph showed a linear relationship between GSH concentrations and absorbance which passed through zero. An enzyme reaction occurred within the reference cell, which contained water in place of a sample. This spontaneous reaction first recorded by Owens & Belcher (1965) is believed to occur due to the presence of glutathione which is simultaneously extracted during the glutathione reductase purification process. Therefore it is essential that a blank containing the GR solution is run alongside the sample assay in order to cancel out this spontaneous reaction. Six replicates using this assay were completed with haemolymph. The coefficient of variation for this assay was found to be 3%

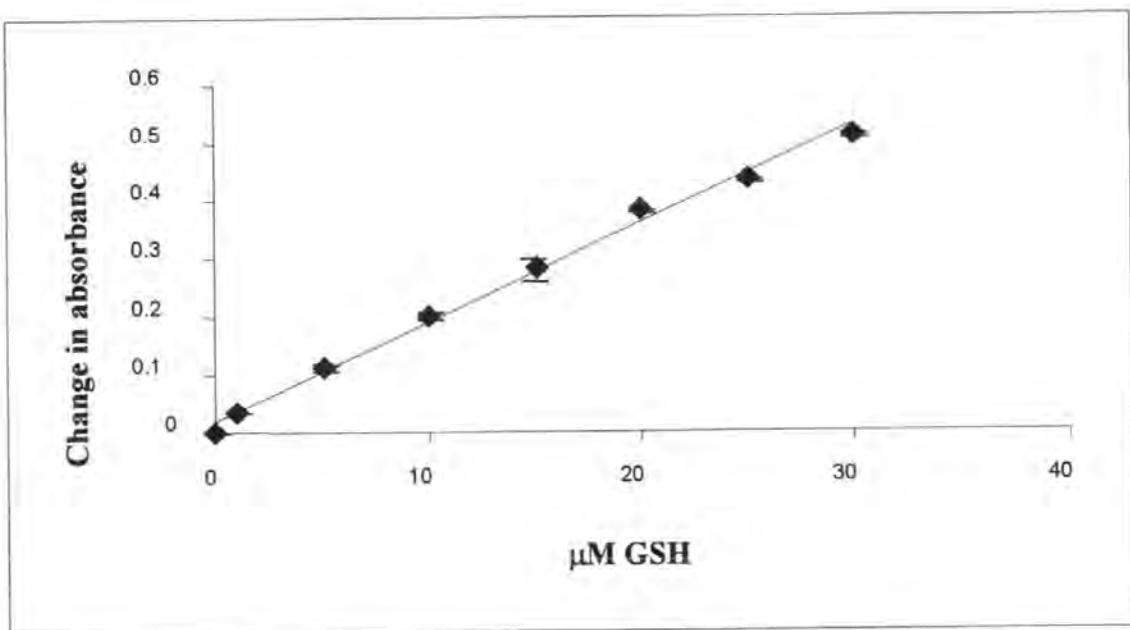


Figure 2.2 Calibration curve of the enzymatic cyclic quantification of reduced glutathione observed spectrophotometrically at 412nm. ($R^2 > 0.9$)

n = 6

Mean = 1.5 μ M

SD = 0.05

C.V. = 3%

The enzymatic cyclic quantification of glutathione was found to be simple and rapid. Up to 40 samples could be determined in duplicate in approximately 3 hours including buffer and standard preparations. Disadvantages lie with this method because of an inability to differentiate between oxidised, reduced and protein bound glutathione concentrations. The HPLC method was investigated to evaluate the possibility of measuring oxidised, reduced and bound glutathione, however this experimentation failed to produce a reliable method of analysis. The enzymatic method measures total glutathione which includes all three glutathione derivatives.

Glutathione quantification was included in the experimental sections because of the simplicity of the method and also because glutathione contributes towards several antioxidant pathways.

ii) Measurement of metallothionein levels

The assay for metallothionein quantification was adapted from a method by Viarengo *et al.* (1997) by S. Pedersen (1996). Metallothionein levels were quantified by using a partially purified metalloprotein obtained by acidic ethanol/chloroform fractionation of the tissue homogenate. Care was taken to prevent the oxidation of sulphhydryl groups, the contamination of the sample by low molecular weight thiols and degradation caused by proteolytic enzymes. This was achieved by rapid extraction steps and the addition of reducing agents, dithiothreitol (DTT) and chemicals that prevent the action of proteolytic enzymes, phenylmethylsulfonylfluoride (PMSF) to the sample mixture achieved this.

The final metalloprotein extract was reacted with dithionitrobenzene (DTNB) and the concentration read spectrophotometrically against a calibration curve, which used glutathione as the thiol-containing standard.

Preparation of tissue for metallothionein analysis

Approximately 1g of wet weight gill tissue was ground in liquid nitrogen to produce a fine powder after the nitrogen had evaporated. The powder was weighed accurately in a 10ml beaker. 3ml of ice cold 1mM DTT and 30µl of 0.5mM PMSF in ethanol were added. The mixture was sonicated (3 x 15s) at 40 % output power using a sonication probe and then a further 30µl ice cold PMSF was added. The resulting mixture was placed in an ultracentrifuge (Beckman TL-100) and centrifuged for 55,000rpm at 4°C for 70 minutes to obtain the cytosolic fraction. The resulting supernatants were decanted into vials and snap frozen ready for ethanol / chloroform extraction of metallothionein.

Ethanol / chloroform extraction of metallothionein

The rest of the analysis followed the procedure of Viarengo *et al.* (1997). 500 µl of absolute ethanol and 40µl chloroform kept at -20°C were added to 500µl of thawed cytosolic fraction and mixed vigorously. The mixture was then centrifuged at 7000rpm for 10 minutes at 4 °C. The supernatant was poured into a new tube and three times its volume of -20°C ethanol was added and the mixture mixed thoroughly again for 15s and left for 1 hour at -20°C. The samples were then centrifuged at 7000rpm for 10 minutes. The supernatant was decanted, the pellet washed with 2ml of washing buffer (87% ethanol, 1% chloroform and 12 %, 20mM Tris-HCl, 0.5M sucrose, pH 8.0) and centrifuged again at 7000rpm for 10min at 4°C. The pellet was placed on ice and dried in a fume cupboard under a continuous flow of nitrogen or argon gas to prevent oxidation. The dried pellets

were dissolved in 300 μ l of 5mM Tris-1mM EDTA buffer (pH 7) and mixed. A calibration curve was prepared from 0.5 to 16 mg/ml GSH dissolved in the Tris-EDTA buffer. 4.2ml of 0.43mM DTNB solution were added to 0.3 ml of the standards and the samples. The samples were incubated for 15 minutes to allow the colour to develop and then read with a API UNICAM UV/VIS UV-4 spectrophotometer at 412nm. The concentrations of the metallothionein samples were calculated from the GSH calibration curve.

Calculation of metallothionein levels

wet weight of tissue in sample, (g / ml)	=	initial weight of the sample, (g) x volume of sample purified (0.5ml) initial volume of buffer added to ground sample (3ml)
Metallothionein (μ g/g wet weight tissue)	=	Concentration of metallothionein read from GSH standard curve (μ g / ml) Amount of wet weight tissue in sample (g/ml)

Evaluation and Validation of the Assay

The method was validated at Plymouth University by Pedersen (1996). Comparisons of the method with an enzyme-linked immune assay for the quantification of metallothioneins in Crustacea was investigated by Pedersen (1996) and the results were found to be similar. Accurate and reproducible metallothionein concentrations were obtained by prompt extraction from the fresh tissue. Care had to be taken to ensure minimal oxidation of thiol groups. Reproducibility of metallothionein concentrations improved with practice of the method and rapid extraction steps. Six repeats of metallothionein quantification were carried out upon the cytosolic fraction of gill tissue from three crabs that had been pooled together. The coefficient of variation of the method was found to be 6%

n = 6

Mean = 18 μ g MT / g wet weight tissue

SD = 1.1

C.V. = 6%

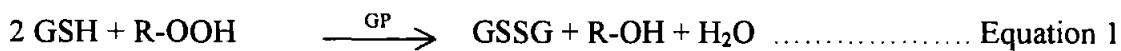
The advantage of the assay is its simplicity especially when compared with other methods that could be used for determining metallothionein concentrations, e.g. immuno-assays and HPLC separation. Experiments have shown that although it is relatively time consuming it offers a simple, cheap way of quantifying metallothionein concentrations for anti-oxidant studies.

2.1.5 Enzyme assays

i) Glutathione peroxidase assay, (adapted from Braven et al., 1989)

Several forms of glutathione peroxidase enzymes have been isolated. Most of them are selenium based and their activities are generally measured by using hydrogen peroxide, cumene hydroperoxide or t-butylhydroperoxide as substrates. Glutathione peroxidase has a restricted phylogenetic distribution and has not been detected in arthropods, (Felton, 1995). The GP activities observed using crab haemolymph tissue and the tissues of other arthropods, including insects, are likely to be due to the peroxidase activity of glutathione transferase GST_{PX}. GP activity that is associated with GST_{PX} is also known as non-selenium dependent glutathione peroxidase activity. The crab haemolymph did not exhibit GP activity when H₂O₂ was used as a substrate. This may be because ROOH is the preferred substrate of GST_{PX}, (Felton, 1995).

The glutathione peroxidase assay involved the GP catalysed oxidation of reduced glutathione with t-butyl hydroperoxide to generate oxidised glutathione, (see Equation 1). None of the reactants or the products of this reaction can be distinguished spectrophotometrically. Therefore, the reaction is coupled to the reduction of the oxidised glutathione by GR, which simultaneously oxidises NADPH (see Equation 2). Equation 1 shows the rate-determining step. GP activity is provided from the tissue sample. The other substrates and enzyme are available in excess within the assay mixture.



Spectrophotometric measurement of GP activity

The assay solution was made up to 3ml in quartz cuvettes and contained 20 μ l of prepared tissue (see Section 2.1.3) and 50mM Tris-HCl Buffer pH 7.6, 4mM GSH and 0.2mM NADPH. The mixture was incubated for 10 minutes and the reaction was initiated with 75 μ l of 15mM t-butyl hydroperoxide. The reaction was followed spectrophotometrically for 1 minute and read against a reference containing the assay mixture and 100 μ l of distilled H₂O instead of the enzyme sample.

Evaluation and validation of the assay

The haemolymph from three crabs were pooled and six repeats of the assay were performed. The coefficient of variation for the assay was calculated to be 1.7%.

n = 6

Mean change in absorbance = 1.19

SD = 0.02

C.V. = 1.7%

A typical enzyme activity curve of GP using 20 μ l of crab haemolymph is shown in Figure 2.3. The activity curve of GP was linear over the duration that the absorbance measurements were taken. Figure 2.4 shows that there is a linear relationship between enzyme activity and the amount of tissue preparation added to the assay mixture. The assay proved to be simple and rapid, up to 40 samples could be run in duplicate within three to four hours. The assay proved to be reliable and could be effectively applied to investigate glutathione metabolism in response to oxidative stressors.

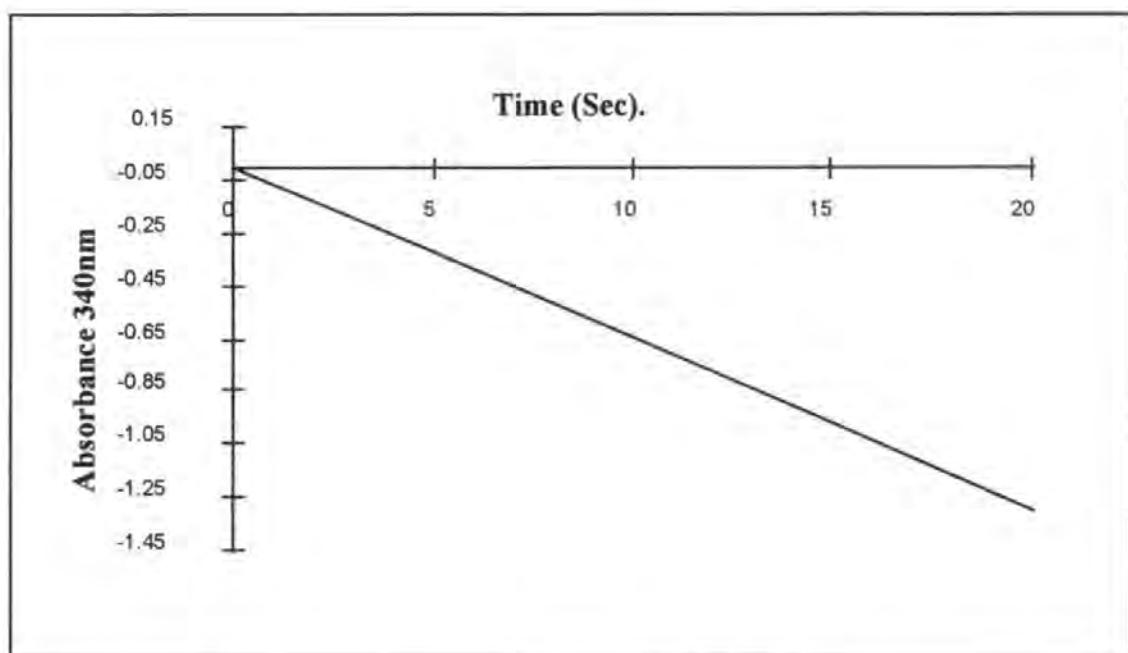


Figure 2.3 The enzyme activity curve of GP using 20 μ l of crab haemolymph enzyme preparation.

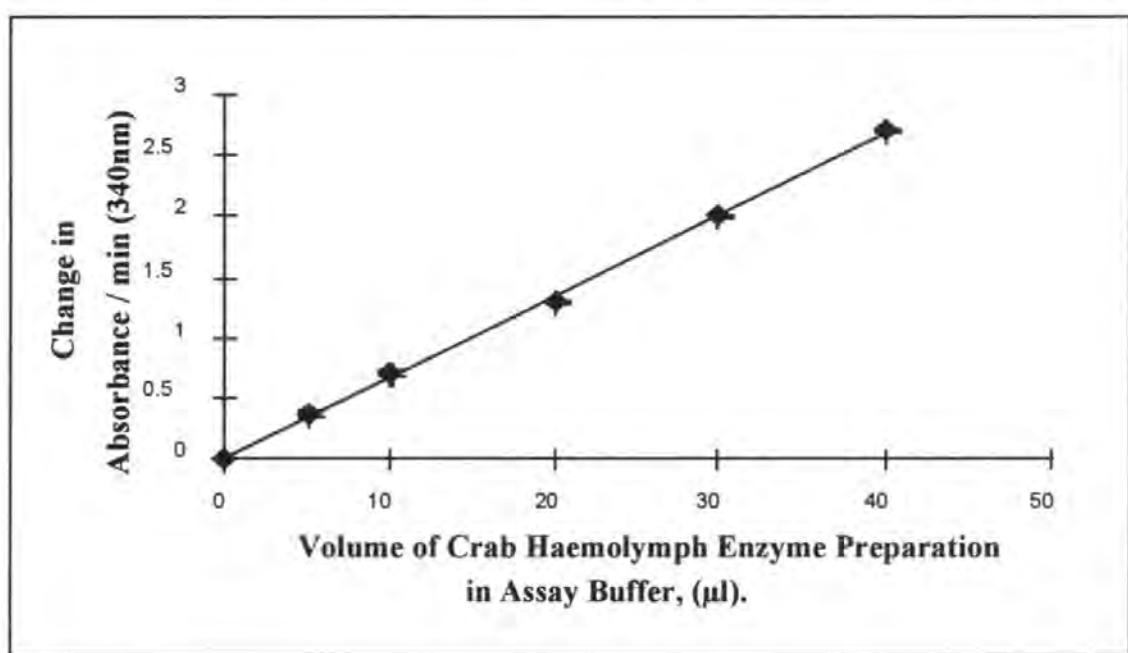


Figure 2.4 The change of absorbance/min of GP activity with increasing volumes of crab haemolymph added to the enzyme assay buffer.

ii) Glutathione reductase assay (adapted from Braven et al. 1989)

The glutathione reductase assay involves the reduction of oxidised glutathione by glutathione reductase with the concomitant oxidation of NADPH, (Equation 3). The assay follows the oxidation of NADPH spectrophotometrically at 340nm.



Spectrophotometric measurement of GR activity

The assay solution made up in quartz cuvettes contained; 2.8ml of 0.22M sodium phosphate buffer - 2mM EDTA pH7.0, 150µl of 20mM GSSG and 100µl of prepared enzyme solution (Section 2.1.3). The reaction was initiated using 150µl of NADPH. The reaction was followed spectrophotometrically for three minutes and read against a reference containing the assay mixture and 100µl of distilled H₂O instead of the enzyme sample.

Evaluation and validation of the assay

The enzyme activity trace of glutathione reductase using crab tissue as the enzyme source is shown in Figure 2.5. The trace is linear over the period that the measurements were taken. There was also a linear relationship between the volume of enzyme used in the assay mixture and the GR activity produced from the trace (Figure 2.6). GR activity of tissue preparations from different animals produced different enzyme activities, demonstrating that the GR assay is sensitive to inter-individual variability. The haemolymph from three crabs were pooled and the GR activity was established six times. The coefficient of variation for the assay was calculated to be 4.5 %.

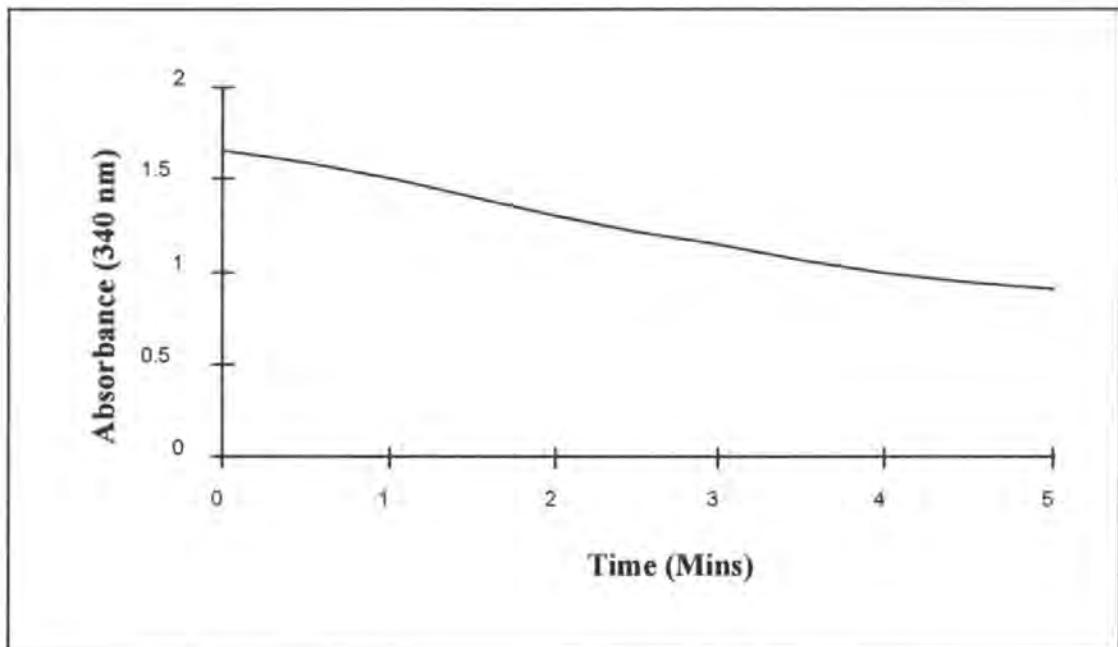


Figure 2.5 GR enzyme activity curve from using 200 μ l of crab gill homogenate enzyme preparation.

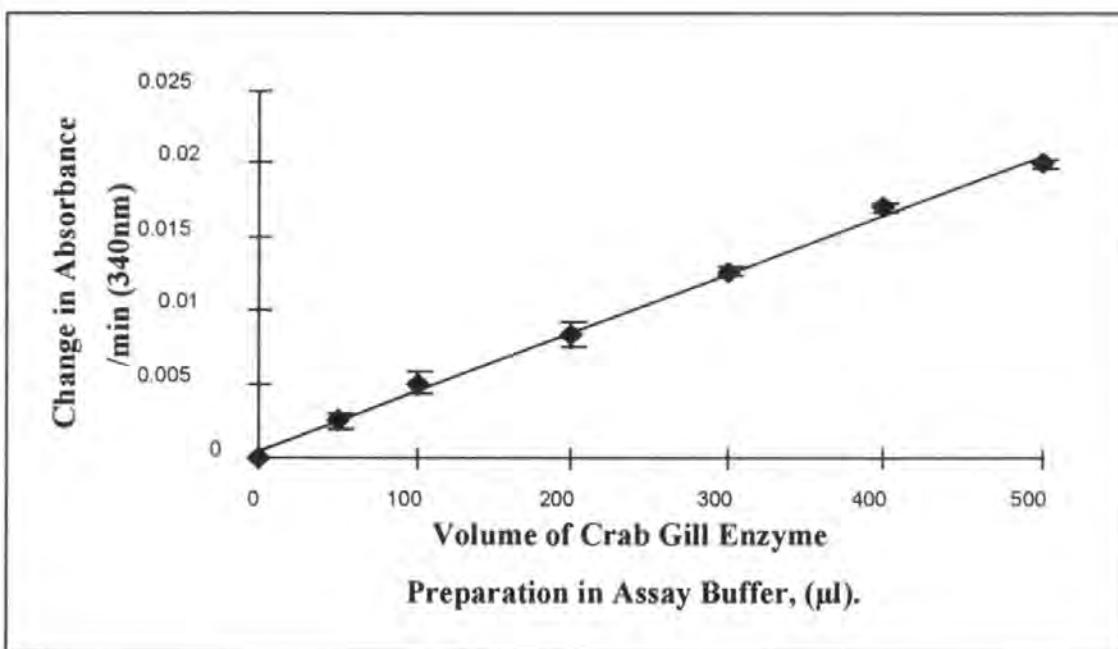


Figure 2.6 Change of absorbance/min of GR activity with increasing volumes of crab gill tissue homogenate added to the enzyme assay buffer.

n = 6

mean change of absorbance = 0.075

standard deviation = 0.0032

C.V. = 4.2%

The assay was rapid and efficient. GR has a significant role in maintaining levels of reduced glutathione; therefore GR activity is of interest when investigating glutathione metabolism. The GR activity of crab gill tissue was generally measured in preference to haemolymph, as it has more GR activity. However, measurements of both of these tissues have been used in some of the experimental sections.

iii) Glutathione-s-transferase assay, (Adapted from Habig et al. 1974)

The enzyme assay is measured by the GST catalysed reaction between reduced glutathione and 1, chloro, 2,4,-dinitrobenzene (CDNB). The GST activity is proportional to the rate of formation of a product that can be observed spectrophotometrically at 340nm.

When Habig *et al.* (1974) first developed this assay they commented that the concentration of the substrates investigated were limited by either high absorbance or low solubility; in most cases the substrate concentration was equal to or less than its K_m . When the initial velocity of an enzyme catalysed reaction is plotted against the concentration of the substrate of the reaction a rectangular hyperbolae is produced, (Figure 2.7). When all substrate sites within the enzyme are saturated the enzyme activity cannot increase any further, hence producing the flat area of the hyperbolic curve. Curves of this type are given by reactions of the form;

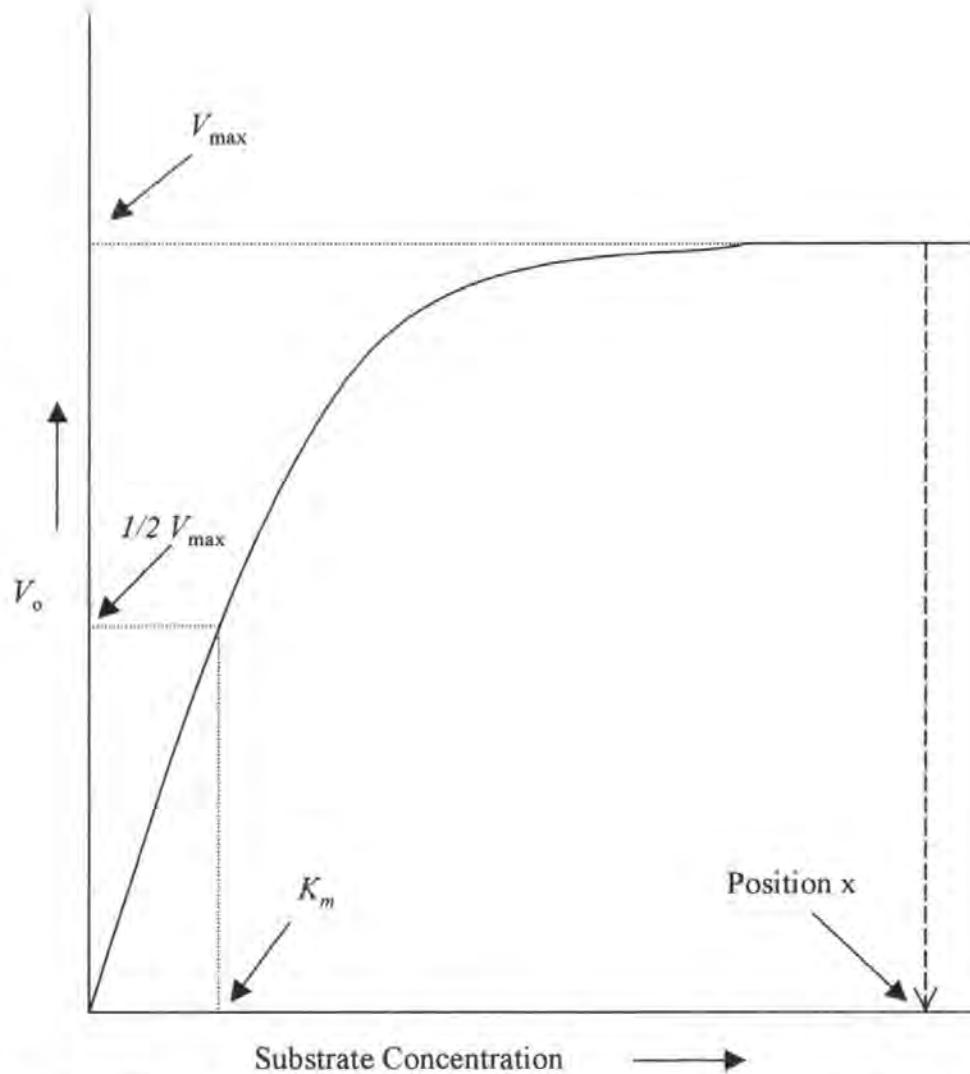
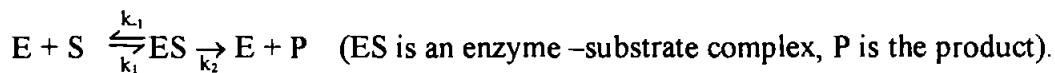


Figure 2.7 Plot of initial velocity of enzyme activity versus concentration of substrate [S], for a typical enzyme-catalysed reaction. Position x indicates where an ideal substrate concentration would lie, i.e. where the substrate concentration is a lot higher than the concentration required for V_{\max} to occur. This concentration allows for human error in measuring the substrate and assures that it is in excess in the assay mixture.

$A + B \rightleftharpoons C \rightarrow D$ when $[C]$ reaches a steady state we can express this in enzyme terms as;



From this equation we can derive several kinetic equations;

- 1) $[E]^o = [E] + [ES]$, where $[E]^o$ = initial concentration of the enzyme
- 2) Steady state assumption: rate of ES formation = Rate of disappearance of E and S ;

$$k_1 [E] [S] = (k_2 + k_{-1}) [ES].$$

- 3) Rate of the reaction is described as $v = d[P] / dt = k_2 [ES]$, (where v is the initial velocity).

To use these equations to measure the rate of the reaction $[E]$ must be eliminated. If we rearrange equation 1 with respect to $[E]$ and substitute it into equation 2 we have;

$$k_1 [S] ([E]^o - [ES]) = (k_2 + k_{-1}) [ES] \quad \therefore k_1 [S] [E]^o = [ES] (k_2 + k_{-1} + k_1 [S])$$

$$\therefore \text{from equation 3} \quad v = \frac{k_2 k_1 [S] [E]^o}{k_2 + k_{-1} + k_1 [S]} \quad \dots \dots \dots \quad (4)$$

If we divide the top and bottom of equation 4 by k_1 and $(k_2 + k_{-1}) / k_1 = K_m$ (K_m is known as the Michaelis constant and is constant for a given E, S , system) this gives us;

$$v = \frac{k_2 [E]^o [S]}{K_m + [S]} \quad \dots \dots \dots \quad (5)$$

From this equation we can establish certain criteria for measuring rates of enzyme activity. If $[S] \gg K_m$ such that the substrate constantly saturates E , and we fix $[S]$ for a series of experiments then $v \propto [E]^o$. This therefore gives the basis of enzyme assays that are demonstrated in Figures 2.4 and 2.6. The concentration of the enzyme is proportional to the rate of the reaction; (the rate of the reaction is measured in these figures as changes in absorbency). To fulfil this criterion it is necessary to work with substrate concentrations that are in the region of the flat area of the

hyperbola (Figure 2.7). Under ideal experimental conditions [S] is used in concentrations that are approximately 100 times greater than K_m . This ensures that any slight change of substrate concentration does not effect the rate of the reaction. On account of substrate solubility limitations the assay by Habig *et al.* (1974) used a CDNB concentration of 1mM, despite the measured K_m for the GST enzyme being from between 0.06 and 0.1mM depending upon the isoenzyme of GST. This falls below the requirements of enzyme assays. Therefore assays were carried out under identical conditions and measurements were therefore relative to one another.

An alternative substrate (4-chloro-3, 5-dinitrobenzoic acid) was investigated as a potential substitute for CDNB for the GST assay. This substrate is more soluble than CDNB on account of it being a more ionic compound. However results from experimentation showed it to be a poor substrate. Despite the observation made by Habig, (1974), GST activity has been extensively measured using CDNB as the substrate, by many workers including Boryslawskyj *et al.*, 1988; Dierickx, 1984; Al-Ghais & Ali 1995; Cookson & Pentreath, 1996; Viganò *et al.*, 1993; Jung & Henke, 1997; Ogasawara *et al.*, 1989; and Reddy, 1997.

Spectrophotometric measurement of GST activity

A quartz cuvette contained 1.8 ml of 0.1M Potassium Phosphate buffer pH 6.5 and 1mM GSH and 1.0mM CDNB (1chloro-2,4-dinitrobenzene). The CDNB solution was initially prepared in ethanol such that the final assay solution was no more than 4% ethanol. The assay mixture was then left to incubate for 10 min at 25°C. The reaction was initiated with 100 μ l of enzyme preparation and followed spectrophotometrically at 340°C for 3 minutes. The reference cell contained the assay mixture and 100 μ l of distilled water instead of sample.

Evaluation and validation of the assay

An example of a typical GST activity curve is shown in Figure 2.8. The activity curve is linear for the initial three minutes in which the change in absorbance is calculated. Figure 2.9 shows that the change in absorbance over 1 minute is proportional to the amount of enzyme in the assay mixture. The enzyme preparation from the gill tissue of one crab was used in six repeats of the assay in order to calculate the experimental error of the method. The coefficient of variation for the assay was found to be 6.6%.

Mean = 0.144

S.D. = 0.0095

C.V. = 6.6%

Reliability of the assay increased with familiarity. To help prevent experimental error that may occur from using a substrate that is not available in excess, all GST assays for each of the experimental sections were performed using the same substrate mixture, on the same day. The substrate solution was kept in a water bath during the experimental period at 25°C, (the same temperature at which the assay was followed). All assays were also performed in duplicate, all samples were run singularly and then repeated to allow for fluctuations in conditions that may have occurred during the progression of the day.

GST assay was found to be sensitive and responsive to contamination exposures. The experimental error of the assay was low despite the flaw in maintaining substrate concentrations.

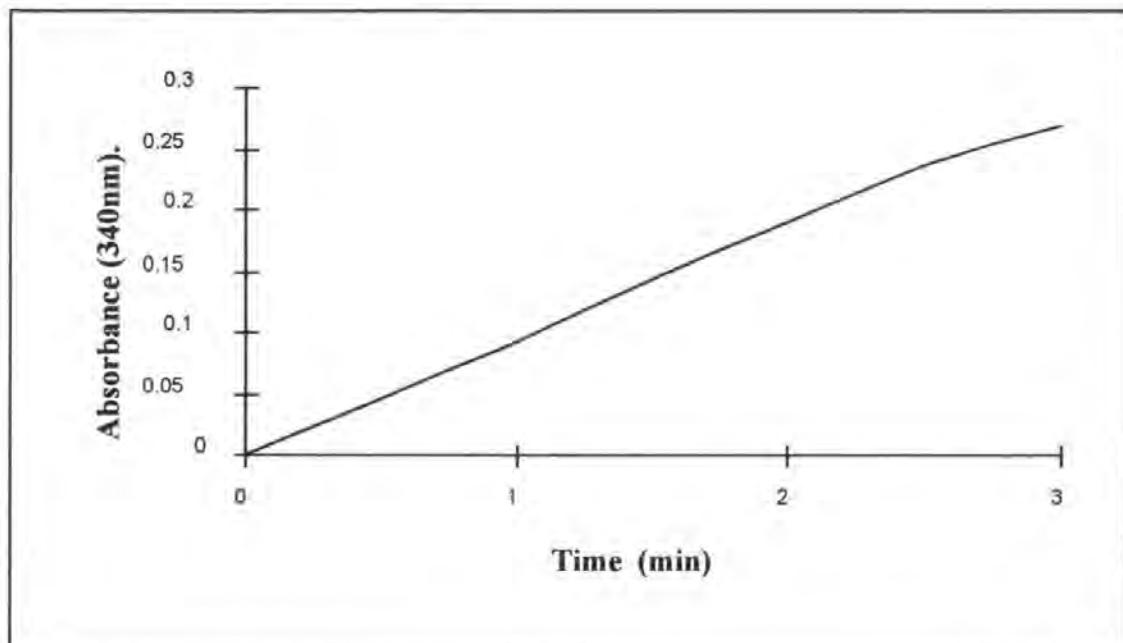


Figure 2.8 The enzyme activity curve of glutathione-s-transferase using 100 μ l of crab gill homogenate.

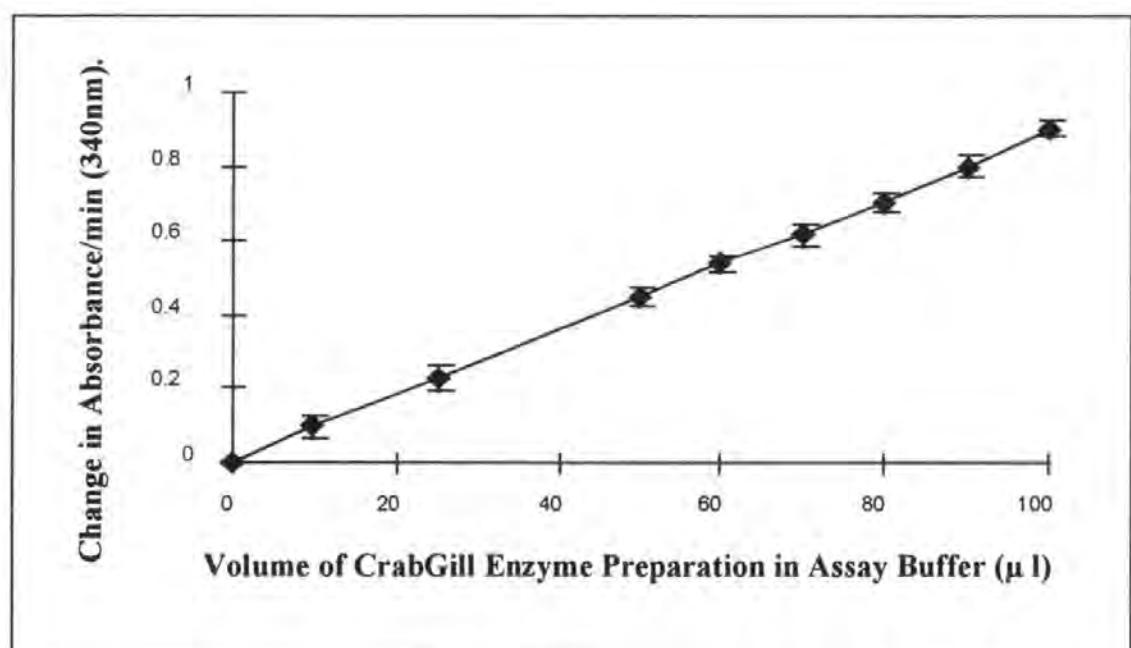


Figure 2.9 The change in absorbance/min of GST activity with increasing volumes of gill tissue homogenate added to the enzyme assay buffer. ($R^2 > 0.9$).

Calculation of enzyme activities

$\Delta A = \epsilon c l$ where ΔA = change in absorbance (for 1 minute)
 ϵ = extinction coefficient of substrate (6220M for NADPH and 9600M for CDBN).
 l = length of light path, (1cm)
 c = concentration of substrate metabolised / min

$$\therefore c (\text{moles/l}) = \frac{\Delta A}{\epsilon (\text{moles/l})}$$

However, to measure the rate of the reaction we need to know how many moles of substrate were metabolised / min

$$\therefore \text{No. of moles metabolised / min} = \frac{c (\text{moles/l}) * \text{total volume of assay mixture in the cuvette (ml)}}{1000}$$

Rate = moles of substrate metabolised / min / g protein* or moles of substrate metabolised / min / 10^6 lymphocytes

*g protein measured using BCA protein assay kit.

2.1.6 Physiological measurements

Lysosomal neutral red retention time (derived from Lowe et al. 1995a)

The lysosomal neutral red retention time is based upon the ability of lysosomes within tissue cells to retain a toxic dye, neutral red (NR). The healthier the cell, the longer the dye is retained within their lysosomal compartments. The test was terminated when dye loss from the lysosomes to the cytosol was evident in 50% (numerically assessed within each field of view) of the crab haemocytes.

Preparation of the neutral red solution.

A stock solution of neutral red (NR) dye was prepared by dissolving 20mg of NR in 1 ml of dimethyl sulfoxide (DMSO). The solution was filtered through a $0.5\mu\text{m}$ Millipore filter to remove traces of undissolved material. NR is photosensitive so it was stored in a dark container until required.

Slide preparation

0.5 ml of crab haemolymph was taken *via* the unsclerotized coxal membrane between the crab body and third walking leg using a sterilised syringe containing an equal volume of anticoagulant (0.45M NaCl, 0.1 M Glucose , 30 mM Trisodium Citrate, 23 mM Citric acid, and 10mM EDTA pH 4.6 as described by Smith & Ratcliffe, 1978). The mixture was placed in an eppendorfTM vial and kept on ice. A 40µl aliquot of the haemocyte suspension was placed onto microslides coated with polylysine solution, which improved cell adherence. Excess solution was tipped off the slides after 15 minutes. The crab haemocytes were stained using 50 µl of NR working solution, which was prepared by placing 2 µl of stock solution into 1000 µl crab physiological saline (0.5M NaCl, 11mM KCl, 12mM CaCl₂.6H₂O, 26mM MgCl₂.6H₂O and 45mM Tris/HCl, pH 7.4).

Measurement of the neutral red retention time

The slides were left to stain for 15 minutes in a humidity chamber. A cover slip was placed over the cells and the cells were checked every 15 minutes for the first hour and then every 30 minutes using a light microscope. After examination the microslides were returned to the humidity chamber. The NR retention time was taken to be the length of time it took for the lysosomes to lyse and release the dye into the cytoplasm in 50% of the cells observed in each field of vision using a light microscope.

Validation of the neutral red retention time technique.

The technique was initially established by Lowe *et al.* (1995a), using mussels as the experimental animal. Astley (1998) further developed the technique, at the University of Plymouth for use with crabs *Carcinus maenas*. The method is found to be easier to apply to mussels than to crabs as the end point of the neutral red retention time is more obviously distinguishable, however, its

application to crabs still provides a means of detecting cellular stress induced by contaminant exposure. The technique was found to have a relatively low degree of experimental error. Blind experiments were run with crabs exposed to copper contamination for two weeks. The control animals had a longer neutral red retention time than the exposed animals. The two groups were successfully distinguishable from each other on this basis.

Neutral red retention time has also proved to be a sensitive biomarker of contamination in many field studies, (Regoli, 1992, Lowe *et al.* 1992 and Lowe *et al.* 1995a) and laboratory based experiments, (Lowe *et al.* 1995b). Moore, (1980 and 1985), has also reviewed the application of cytochemical determination of cellular responses to environmental stressors.

2.1.7 Determination of metal concentrations in crab tissues

Metal analysis was completed using ICP-AES (Inductively Coupled Plasma – Atomic Emission Spectrometer), which allowed determination of metal concentrations using small amounts of tissue samples. The use of the ICP/AES to analyse tissue samples is still rather novel as previously it has been used to analyse water and chemical samples, (Ebdon & Evans, 1988). The method has previously been successfully applied to the analysis of metal concentrations within fish tissue samples, (Campbell *et al.* 1999).

Acid digestion of tissue samples

Tissue samples, crab haemolymph, muscle and gill were collected and dried in an oven at 85°C for 48 hrs. The weighed tissue samples were digested in 5 times their weight / volume of acid of 75% conc. nitric acid and 25% conc. hydrochloric acid. When the tissues were fully digested the solutions were made up to 10 times their volume with pure de-ionised water.

Measurement of zinc, copper and cadmium in acid digested tissue samples.

All reagents used for trace metal samples were spectrophotometric grade or equivalent. All glassware was acid washed with 2M nitric acid followed by deionised water before use. Analytical runs contained procedural blanks.

The ICP/AES automatically samples a small volume of the tissue-acid mixture and was programmed to read the levels of the required ions against calibration curves that had been performed on the same day before tissue analysis.

Validation and evaluation of the technique

The correlation coefficient of the calibration curves of all the metals analysed approximated 0.99, which demonstrates a high degree of accuracy.

2.1.8 Total antioxidant status assay

The assay was carried out using a commercial kit available from Calbiochem® (Cat. No. 615700). It relies upon the ability of the antioxidant levels in the samples to inhibit the oxidation of ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline]), the oxidation of which, forms a coloured compound that has an absorbance at 400nm. The reference wells contained distilled H₂O instead of sample and hence induced the greatest colour change. The antioxidant scavenging ability of tissue samples is calculated from the colour change, which occurs after 3 minutes from a standard of known antioxidant scavenging ability (Miller *et al.*, 1993 and Miller and Rice-Evans, 1996).

Method

The number of samples analysed with the kit can be increased from the number recommended by Calbiochem® by using a plate reader. The kit comprises of a buffer, (phosphate buffered saline), chromogen, (Metmyoglobin and ABTS®, concentrations not given), substrate (stabilised H₂O₂) and a standard (1.5mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). 250µl of chromogen were placed in each well of the plate reader, 5µl of de-ionised water, 5µl of standard and 5µl of sample were added to the blank, standard and sample wells respectively. The absorbency was read at 405nm at time 0 minutes (A₀). 50µl of substrate were pipetted into each well simultaneously using a multi-channel pipetter. The plate was incubated at 25 °C and was re-read after 3 minutes (A). The antioxidant concentrations in the sample wells were calculated as follows:-

Calculation of total antioxidant status.

$$\Delta A = A - A_0$$

$$\text{Antioxidant Concentration (mM)} = 1.5 \text{ mM} * \frac{(\Delta A_{\text{Blank}} - \Delta A_{\text{Sample}})}{(\Delta A_{\text{Blank}} - \Delta A_{\text{Standard}})}$$

Validation and evaluation of the total antioxidant status kit.

The validation of the antioxidant assay kit is available with the kit from Calbiochem®. The intra-assay and inter-assay coefficients of variation are 1.2% and 2.4% respectively. The assay is linear for antioxidant levels up to 2.5mM.

The assay procedure requires the absorbance to be read at 400nm. The plate reader used had filters of fixed wavelength and 405nm was the closest to the required wavelength. An absorbance spectra was run to find whether the coloured product formed had a significant absorbance at 405nm, (Figure 2.10). The spectra showed a strong absorbance at 405nm so measurements could be made with that filter. Validation of the kit was also carried out using the amended multi-well plate technique. The gill tissue from a crab was prepared as described in Section 2.1.3 and serial dilutions were prepared. The total antioxidant scavenging ability of the gill tissue preparations were evaluated and a correlation curve was plotted between these results and the concentration of gill tissue used. The curve that was produced was linear and passed through zero. The correlation coefficient was equal to 1.0. As the assay of all the samples was run on a plate reader with the standard and the blank simultaneously present the multi-well plate reader method was found to be very reliable. The method was rapid and sensitive. The method was simpler to use than potential alternatives that require oxygen probe measurements.

2.1.9 BCA protein assay

Protein levels were determined using the BCA protein assay kit from PIERCE No. 23225. It uses BSA (bovine serum albumin) standards. The kit combines the biuret reaction, (the reduction of Cu⁺² to Cu⁺¹ by protein in an alkaline medium) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu⁺¹) using bicinchoninic acid (BCA) (Smith *et al.*, 1985 and Brenner & Harris, 1995 and Wielchelman *et al.*, 1988). The product is a purple-coloured compound formed by the chelation of two molecules of BCA with one cuprous ion. This complex exhibits a strong absorbance at 562nm that is linear with increasing protein concentrations over a broad working range of 20µg/ml to 2000µg/ml.

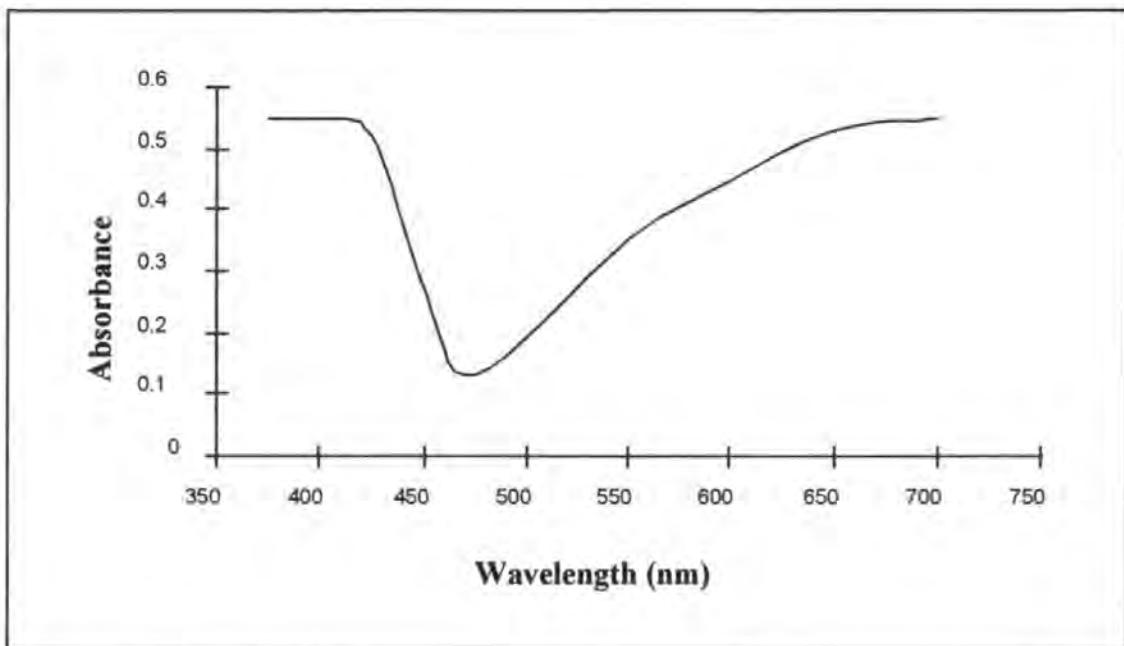


Figure 2.10 Absorbance spectra of the Total Antioxidant Status Kit standard, in assay buffer solutions. The spectra shows a high absorbance at 630nm after the addition of buffers.

Method

50 parts of BCA Reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.2N sodium hydroxide, exact proportions are not provided), were combined with 1 part BCA Reagent B (contains 4% cupric sulfate), to provide the working reagent. Protein standards were prepared using the BSA standard ampule, (2.0 mg/ml bovine serum albumin in a solution of 0.9% saline and 0.05% sodium azide). Standard protein solutions were diluted to ranges between 0 to 40 µg/ml. 5µl of the unknown sample or standard were pipetted into a plastic cuvette and 2 mls of the working reagent added to it. The colour was allowed to develop for 2 hours at room temperature and the absorbance of the tubes measured at 562nm using a spectrophotometer. All measurements were made in duplicate and the mean absorbance of the blanks was deducted from the absorbances of the standards and samples. The concentrations of the samples were determined against the standard calibration curve.

Validation and evaluation

The validation and details of the kit were provided. The assay was extremely efficient and all calibration curve coefficients were found to approximate to 1.0. The assay was ultimately included to evaluate protein concentrations of crab tissues as the final colour continues to develop very slowly after the initial incubation period. This means that the samples can be analysed long after the protocol has been run, up to 12 hours later. Also, it allows for large numbers of samples to be run consecutively without the product precipitating out of solution, which can occur when analysing large numbers of samples using other methods.

Section 2.2

STATISTICAL ANALYSIS.

The statistical analysis performed for the experimental sections is provided in each method section. The statistical package Statgraphics plus version 3.1 (Statistical Graphics Corp) was used for all data analyses. Where possible data was analysed using one-way analyses of variance (ANOVA), which tests for statistical differences between means of the data from different groups of samples (Sokal & Rohlf, 1995). The test used to differentiate between the groups of data was the Tukey HSD (Honestly Significant Differences) test (Sokal & Rohlf, 1995). This test is based upon the studentised range distribution and has a relatively small Type I error. As ANOVA assumes normality of the data and equal variance among treatment groups, the Shapiro Wilks W statistic and the z scores for skewness and kurtosis were used to check for normality, and Bartlett's test and Cochran's C test were used to test for equal variance (Sokal & Rohlf, 1995). Correlations between data sets for each experiment were tested using simple regression analysis (Sokal & Rohlf, 1995).

Non-parametrically distributed data.

If the variances among treatment groups were found to be unequal then the data were transformed. The log transformation technique was generally applied but other transformations were also utilised such as arc-sine transformation. The aim of transformations to normality is to achieve a normal distribution, to equalise variances or to get a linear or additive relationship. If none of the transformations tried managed to make the data meet the assumptions of analysis of variance, then an analogous non-parametric method was incorporated into the statistical analysis of the data.

Non-parametric statistical analysis.

The Kruskal-Wallis test was used with data that were not normally distributed (Sokal & Rohlf, 1995). Non-parametric tests are also known as *distribution-free methods*, since they are not dependent upon a given distribution (such as the normal distribution in the case of ANOVA) (Sokal and Rohlf, 1995). The null hypothesis is concerned with the distribution of the variates. The Kruskal-Wallis test tests the null hypothesis that the medians of each group are the same. The data from all groups are combined and ranked from the smallest to the largest, the average rank is then computed for each group. The p-value from this analysis indicates whether there is a statistically significant difference amongst the medians at the 95% confidence level. Toxicological data are often non-parametric because animals exposed to contamination have variable abilities of tolerating the toxicological stress. Therefore, the Kruskal-Wallis test is very useful for analysing this type of data.

Graphical representation of the data.

Much of the data is displayed as Box and Whisker plots (Sokal and Rolfe, 1995). Both parametric and non-parametrically data are displayed in this way as the plots provide lots of information. The Box and Whisker plot for the normally distributed data is shown on the left of Figure 2.11 and the box and whisker plot for non-normally distributed data is displayed on the right. The line across the middle of each diagram represents the median. A cross in the middle of the box represents the mean. The horizontal lines above and below the median line represent the upper and lower quartiles and cover 50% of the data. The whiskers, which lie either side of the box, cover all data points within 1.5 quartile ranges from upper and lower quartiles. Any points outside the 1.5 quartile range but which lie within 3 interquartile ranges from the upper and lower quartiles are represented as a square and are known as suspected outliers. Any point outside this range that is, outside 3 interquartile ranges are represented by a box with a cross through it and are known as outliers. The

notches on either side of the median, (the left diagram), are used in non-parametric statistical analyses. If these areas do not overlap between groups of data they are considered to be statistically significantly different to one another.

This type of diagrammatic representation allows consideration of the distribution of the data as well as an indication of statistical differences that lie between exposure groups.

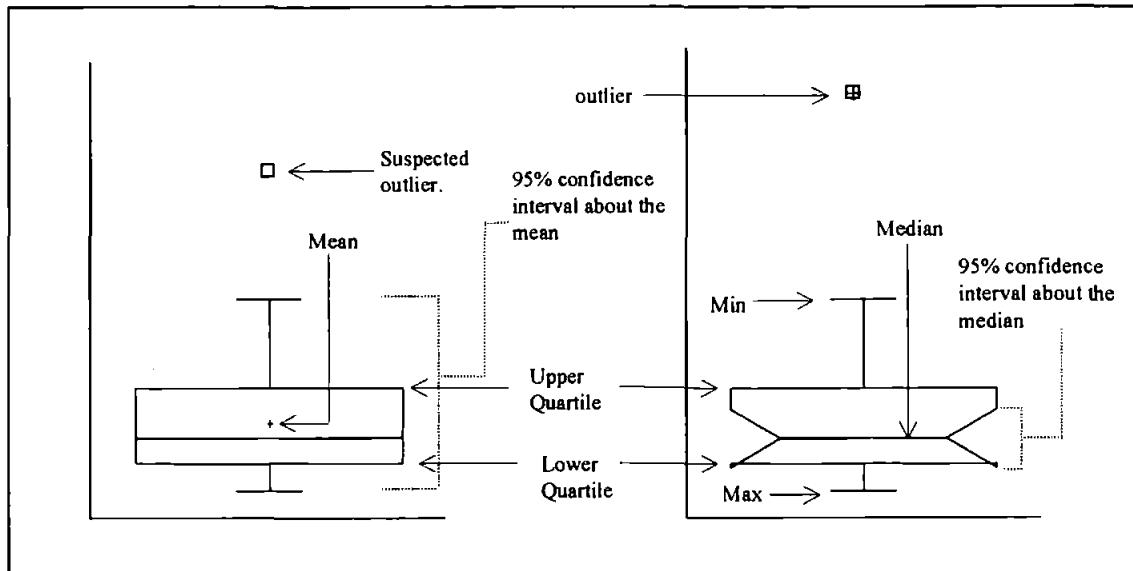


Figure 2.11 Labeled diagrams of box and whisker plots used to represent normally (on the left) and non-normally (on the right) distributed data.

A preliminary experiment to test

two models of oxidative stress.

Section 3.1

INTRODUCTION.

Two models were chosen to study mechanisms of oxidative stress. These were (i) the complications of long term diabetes, i.e. retinopathy, nephropathy and neuropathy and (ii) the effect of a contaminant, cadmium, upon the antioxidant mechanisms of the common shore crab, *Carcinus maenas*. The ease of which each model could be used as a monitor of induced oxidative stress was compared. A reliable model was essential so that future investigations of glutathione antioxidant mechanisms could be undertaken.

Oxidative stress is induced in the tissues of diabetic patients due to their exposure to hyperglycaemia (West, 2000). It is also associated with the complications which can occur during long term diabetes (Ceriello & Giugliano, 1997; Baynes & Thorpe, 1999; and McDermott *et al.*, 1994). Research has shown that diabetes and its complications are associated with alterations in glutathione metabolism, see Table 3.1. Alterations in the glutathione metabolism of erythrocytes have been thoroughly investigated, whereas research into lymphocyte glutathione metabolism has not. However, abnormalities of differential lymphocyte surface antigen expression may provide some insight into diabetes pathogenesis and represent a non-invasive screening tool for the detection of the prediabetic state (Pinies *et al.*, 1997; Smerdon *et al.*, 1993; Faustman *et al.*, 1989; Topliss *et al.*, 1983 and Roll *et al.*, 1994).

During this investigation GR and GP activities were measured in the lymphocytes of diabetic patients. The lymphocytes and erythrocytes of diabetics are rheologically abnormal (Ernst & Matrai, 1986). Research has shown that in red blood cells this may be due to oxidative stress, which induces osmotic fragility and altered deformability of red blood cells (Jain, 1989).

Author	Animal	Tissue	Glutathione Metabolism in diabetics compared to controls
Ceriello, et al. 1996	Human (Type I)	HUVEC cultures exposed to elevated glucose levels	Increased GP
Dominguez, et al. 1998	Human (children and adolescents) (Type I)	Erythrocytes	Decreased GP
Bravi, et al. 1997	Human (Type II)	Erythrocytes	Decreased GSH, Decrease GSH/GSSG ratios
Yoshida, et al. 1995	Human (Types II)	Erythrocytes	Decreased GSH
Thornalley, et al. 1996	Human, (Type I & II) with diabetic complications	Erythrocytes	Decreased GSH
Matkovics, et al. 1997/98b	Human (Type II)	Blood plasma Haemolysate	Increased GSH Decreased GSH Decreased GP
Matkovics, et al. 1997/98a	Human (Type I)	Blood plasma Haemolysate	Increased GSH Increased GSSG Increased GSH Increased GSSG Increased GP Decreased GR
Ndahimana, et al. 1996	Human (Type I) with neuropathy	Erythrocytes	Decreased GP
Murakami, et al. 1989	Human (Type II)	Erythrocytes	Decreased GSH Increased GSSG Decreased GR Unaltered GP
Sundaram, et al. 1996	Human (Type II)	Whole blood Haemolysate	Decreased GSH Increased GP
Ruiz, et al. 1999	Human (Type I)	Erythrocytes	Decreased GP in patients with poor & medium metabolic control
Kharb, 2000	Human pregnancies	Whole blood	Decreased GSH/GSSG
Aaseth & StoaBirketvedt 2000	Human (Type II over weight patients)	Erythrocytes	Reduced GSH Reduced GP
Hartnett, et al., 2000	Human (Type I & Type II patients with retinopathy)	Blood serum	Decreased GP
Stahlberg & Hietanen, 1990	Human (Type I)	Erythrocytes	Decreased GR Unaltered GP
Iwakiri, et al. 1995	Streptozotocin – induced diabetic rats	Intestine	Decreased GP Decreased GR (After insulin administration)
Makar, et al. 1995	Diabetic mice	Brains	Unaltered GP Increased total GSH and GSSG
Kakkar, et al. 1998	Streptozotocin – induced diabetic rats	Liver and Pancreas	Increased GP
Saito-Yamanaka, et al. 1993	Streptozotocin – induced diabetic rats	Liver	Decreased total GSH and GSSG Decreased GST
Mak, et al. 1996	Streptozotocin – induced diabetic rats	Kidney	Increased GP Unaltered GR
Pieper, et al. 1995	Streptozotocin-induced diabetic rats	Aorta Iliac artery Femoral artery	GP no change

Table 3.1 The effect of diabetes and related complications upon glutathione metabolism. Increased or decreased GP, GR or GST refers to increased or decreased activity of those enzymes.

Glutathione metabolising enzymes were used as a biomarker of oxidative stress in order to indicate whether the same situation exists for lymphocytes of diabetic patients. Increased glutathione status and GST and GR activities in lymphocytes of uremic patients have been associated with a compensatory mechanism against the accumulating toxic wastes in renal plasma (El-Rashidy *et al.*, 1984). Diabetic patients do not have this advantage in controlling the toxic effects of glucose, as a reduction in antioxidant mechanisms such as GSH is a feature of the disease (Yoshida *et al.*, 1995; Giugliano *et al.* 1995; West, 2000; Kharb, 2000). Glutathione prevents diabetic neuropathy in streptozotocin-induced diabetic rats (Bravenboer *et al.*, 1992). Also, antioxidant treatment prevents the development of peripheral nerve dysfunction in streptozotocin rats (Cameron *et al.*, 1993). The administration of glutathione supplements to diabetic patients reduced hypertension (Ceriello *et al.*, 1997) and the possibility of thrombin formation (Ceriello *et al.*, 1995). The aim of this part of the experiment was to assess whether diabetes induces an alteration in activity of the antioxidant enzymes, GR and GP of lymphocytes.

The second part of the investigation analysed the toxic effects of cadmium upon glutathione metabolism in the crab, *Carcinus maenas*. Cadmium is a non-essential heavy metal that has been shown to have toxicological effects on crabs above a threshold concentration (Rainbow, 1985). Glutathione ameliorates these effects (Cookson & Pentreath, 1996; Peters *et al.*, 1995; Kang & Enger 1988; Shaikh *et al.*, 1999; and Eisenmann and Miller, 1995). Cadmium exposure has also been shown to induce changes in glutathione status. For example, long term cadmium exposure in the cichlid, *Oreochromis aureus*, increased glutathione concentrations in the kidney (Allen, 1995). An increase of glutathione concentration also occurs in the livers of striped mullet after four weeks exposure to cadmium (Wofford & Thomas, 1984) whereas, Rhizobacteria that were exposed to cadmium had reduced glutathione levels (Hultberg, 1998). Cadmium exposure is also reflected in alterations of glutathione metabolising enzymes. Atlantic croaker exposed to cadmium suffered a decrease in GP activity in both their hepatic and ovarian tissues, whereas glutathione concentrations

in these tissues remained unaltered (Thomas and Wofford, 1993). The responses of glutathione and antioxidant enzymes towards cadmium toxicity cannot be considered to be common to all species and tissues (See Table 1.4). However an interaction between glutathione concentrations and metallothionein induction has been observed in many species as a result of cadmium toxicity (Chan & Cherian, 1992; Schlenk & Rice, 1998; Brouwer *et al.*, 1993a; Kawata & Suzuki, 1983; and Singhal *et al.*, 1987).

The aim of exposing crabs to different concentrations of cadmium was to elucidate changes in GP, GR and GST activities in the crab haemolymph. In addition, metallothionein concentrations of gill tissues were measured to observe whether an increase in cadmium concentrations was correlated with an increase in metallothionein formation. Alterations of the parameters measured in response to cadmium toxicity have potential as biomarkers of cadmium toxicity in the crab, *Carcinus maenas*. In addition, they may provide a model for studying antioxidant mechanisms.

Section 3.2

MATERIALS AND METHODS.

3.2.1 Measurement of GP and GR activities in PBMC

Blood was collected as described in Methods 2.1.3, from three groups of volunteers; (i) diabetic patients with Type 1 diabetes, that do not yet exhibit complications; (ii) diabetic patients with Type 1 diabetes and diabetic complications; and (iii) a control group without diabetes from October-December, 1995. Clinical characteristics of the subjects are shown in Table 3.2. Peripheral blood mononuclear cells (PBMC) were extracted from the blood samples and counted (as described in Methods 2.1.3). GP and GR enzyme activities were measured in the PBMC extracts.

Uncomplicated patients (n=25)

These patients had had IDDM for at least 9 years but remained free of retinopathy (fewer than five dots or blots per fundus) and proteinuria (urine Albustix negative on three consecutive occasions over 12 months).

Diabetic neuropathy patients (n=10)

Overt neuropathy was defined if there was any clinical evidence of peripheral or autonomic neuropathy. Peripheral neuropathy was defined clinically; most of the patients having all of the following: (a) symptoms of pain, numbness, or paraesthesiae in the feet, or hands, or both; (b) sensory signs such as loss of vibration sense as assessed with a tuning fork, light touch , or pin prick, and temperature using warm and cold stimuli; (c) loss of ankle reflexes; (d) evidence of past or present foot ulceration.

Autonomic neuropathy was diagnosed if there was orthostatic hypotension, gastroparesis, or a flaccidneuropathic bladder. None of these patients had another obvious cause of peripheral neuropathy as they had negative syphilis serology and autoimmune profiles for vasculitis and; vitamin B12 and folate concentrations within normal limits (Heesom *et al.*, 1998).

Patients of the diabetic neuropathy group displayed symptoms for either peripheral or autonomic neuropathy or both.

Diabetic nephropathy patients (n=17)

Nephropathic patients have had diabetes for at least 10 years with persistent proteinuria (urine Albutix positive on at least three occasions over 12 months or three successive total urinary protein excretion occasions rates >0.5g/ 24 hours) in the absence of hematuria or infection on midstream urine samples; diabetic nephropathy was always associated with diabetic retinopathy (Heesom *et al.*, 1997).

Diabetic retinopathy patients (n=16)

Retinopathic patients had retinopathy defined as more than five dots or blots per eye, hard or soft exudates, new vessels or fluorescein angiographic evidence of maculopathy or vitreous hemorrhage. None of these patients had proteinuria (Heesom *et al.*, 1997).

3.2.2 Exposure of crabs (*Carcinus maenas*) to cadmium.

Crabs were exposed to 0, 0.5 or 2ppm cadmium for 14 days and their haemolymph sampled. They were then sacrificed and their gill tissues were removed. A control set of crabs was kept in clean seawater along side the exposed crabs. Eight crabs were held at each exposure concentration. GP,

GST and GR activities were assayed in the haemolymph and metallothionein concentrations were determined in the gill tissue.

3.2.3 Data analysis.

Data analysis of GP and GR activities for the lymphocytes of diabetic patients showed that only the GR activities were normally distributed. GP activities from the diabetic patients and the GP and GST activities from crab haemolymph were not normally distributed. The median and minimum and maximum values are displayed for these data. The data were log transformed in order to normalise the distribution so that ANOVA analysis could be performed on it. GR data from the crabs were normally distributed so that parametric analyses could be performed directly upon them.

Section 3.3

RESULTS.

3.3.1 GP and GR Activities of PBMC.

The ages of diabetic and normal volunteers ranged from 17 to 68 years. The distribution of the volunteer's ages was normal and the average age was 39. The statistics of the clinical characteristics of the patients are shown in Table 3.2. There were no statistically significant differences observed among the groups, (Figures 3.1 and 3.2). The GR activity of diabetic patients with complications was slightly higher than in the other two populations, $p = 0.054$ (Table 3.3).

Statistical correlations were performed between enzyme activities and age; and enzyme activities and age of onset of complications. No positive correlations were found. There were also no statistical correlations between GR and GP activities; or enzyme activities and type of diabetic complication suffered by the patient; or enzyme activities and age of subjects or duration of diabetes.

3.3.2 Metabolic changes of crabs exposed to cadmium

The GR activities of haemolymph from crabs exposed to 0.5 and 2 $\mu\text{g l}^{-1}$ of cadmium were not statistically different to those from control animals (Table 3.4). The GP and GR activities of haemolymph in the same crabs significantly decreased at the highest concentration of cadmium exposure compared to 0 and 0.5 $\mu\text{g l}^{-1}$ Cd exposures, $p = 0$ and 0.01, respectively, (Figures 3.3 and 3.4, respectively).

Linear regression analysis performed between the different data sets showed that there were no correlations between the activities of the different enzyme activities or between enzyme activities and metallothionein concentrations.

<i>Group</i>	<i>Diabetes and no associated complications</i>	<i>Diabetes with associated complications</i>
<i>x</i>	25	22
Age	34±13	42±13
Male	9	12
Female	16	10
Duration of Diabetes	21±12	25±11
Age of diabetes onset	21±13	20±10
Patients with DN	-	17
Patients with DNu	-	10
Patients with DR	-	16

Table 3.2 Clinical characteristics of patients with Type 1 diabetes. DN = diabetic nephropathy, DNu = Diabetic neuropathy, DR = Diabetic retinopathy.

Assay	Statistics	Control	<i>Diabetic Patients</i>	<i>Diabetic Patients</i>
			<i>Without Complications</i>	<i>With Complications</i>
GR	n	25	25	22
	Mean	1.17	1.28	1.48
	SD	0.65	0.52	0.63
GP	Median	1.49	1.06	1.49
	Min	0.43	0.0	0.0
	Max	6.39	2.38	2.49

Table 3.3 The GP and GR activities of PBMC from patients with or without diabetes and / or diabetic complications. The mean and standard deviations are displayed for the GR activities and the median and minimum and maximum values are displayed for the GP activities. GR and GP activities are measured as µmoles of NADPH oxidised / hour / 10⁶ lymphocytes.

Box-and-Whisker Plot

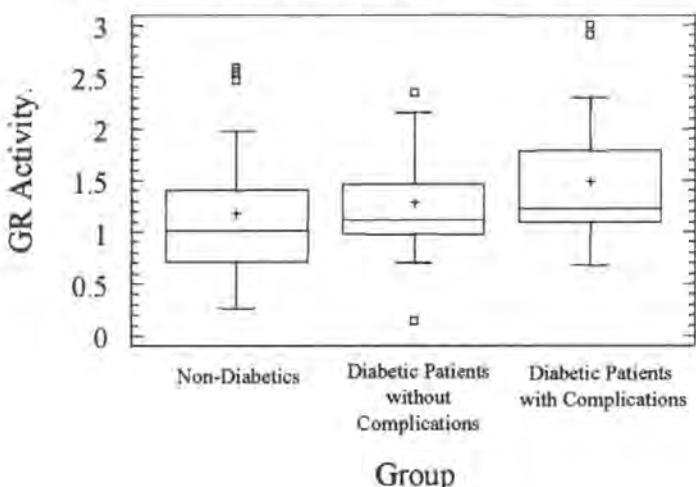


Figure 3.1 GR activity of lymphocytes from donors with or without diabetes and / or diabetic complications. GR activity is measured as μ moles of NADPH oxidised / hour / 10^6 white blood cells. There are no statistical differences between the groups.

Box-and-Whisker Plot

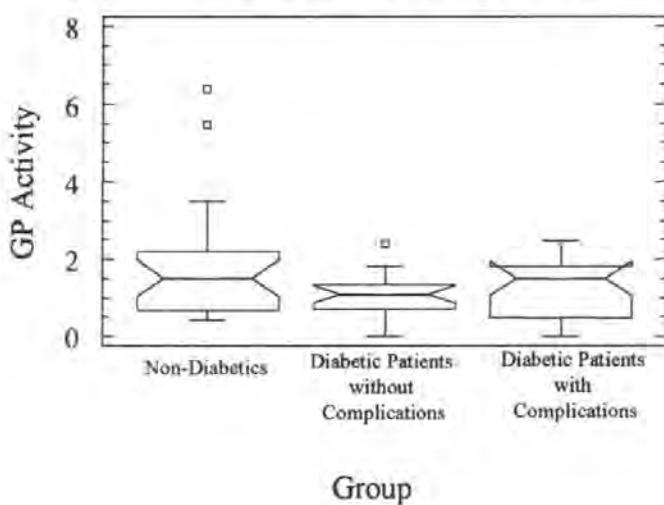


Figure 3.2 The GP activity of lymphocytes from donors with or without diabetes and / or diabetic complications. GP activity is measured as μ moles of NADPH metabolised / hour / 10^6 white blood cells. There is not a statistical difference between the groups.

Exposure	0	0.5	2	
n	6	8	8	
Statistics for normally distributed data	mean	SD	mean	SD
MT	13.9	5.4	19	7.2
GR	2.47	1.28	2.56	1.2
Statistics for non-normally distributed data	Median	Min / Max	Median	Min / Max
GST	1.9	0.47	2.06	0.93
GP	0.16	3.38 0.05	0.17	2.26 0.08
		0.48	0.25	*0.76 *0.05
			0.07	

Table 3.4 Results of parameters measured in the crab, (*Carcinus maenas*), exposed to increasing concentrations of cadmium. The mean and SD are given for the normally distributed data and the median and minimum and maximum values are given for the non-normally distributed data. * Signifies values that are statistically significantly different to the rest of the population.

MT is measured as $\mu\text{g} / \text{g}$ wet weight tissue. GR activity is measured as nmoles of NADPH oxidised / min / g protein. GP and GST activities are measured as μmoles of substrate metabolised / min / g protein.

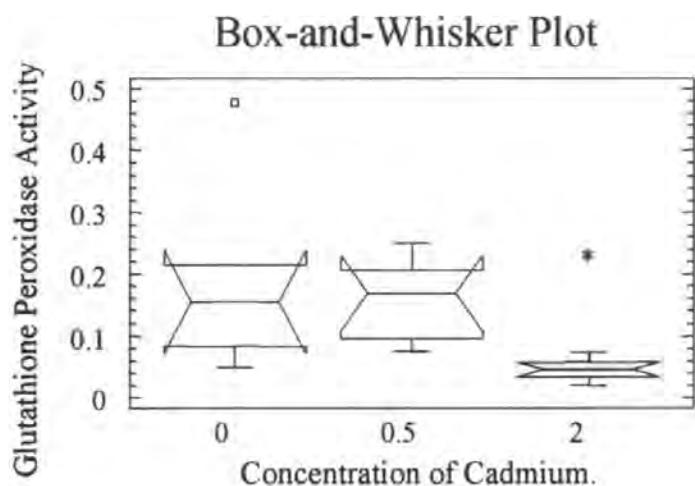


Figure 3.3 Glutathione Peroxidase activity of crabs, *Carcinus maenas*, exposed to increasing concentrations of cadmium toxicity. The units of cadmium concentration are $\mu\text{g l}^{-1}$. GP activity is measured as $\mu\text{moles of substrate metabolised / min / g protein}$. * Represents statistically significant response, $p = 0.00$.

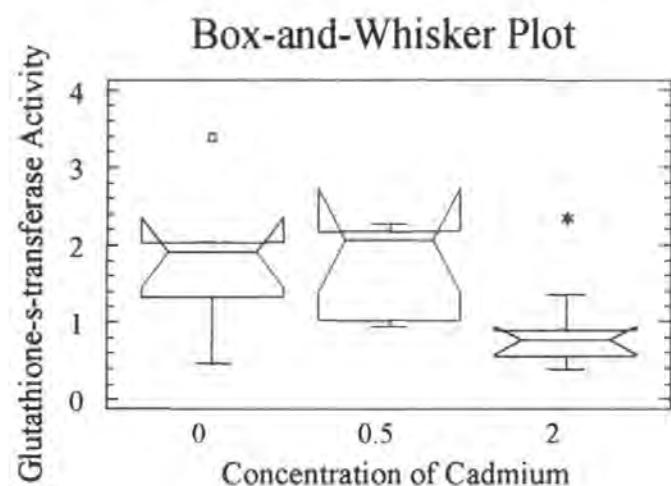


Figure 3.4 Glutathione-s-transferase activity of *Carcinus maenas* haemolymph exposed to increasing concentration of cadmium toxicity. GST activity is measured as $\mu\text{moles of substrate metabolised / min / g of protein}$. Units of cadmium concentration are $\mu\text{g l}^{-1}$. * Represents exposure at which a statistically significant response is observed, $p = 0.01$.

Box-and-Whisker Plot

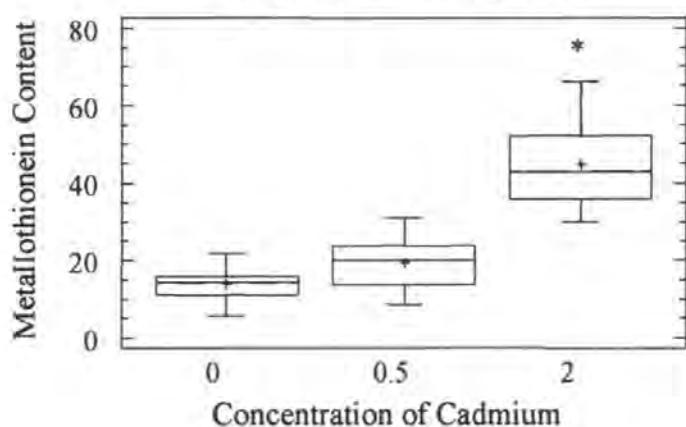


Figure 3.5 Metallothionein content of gill tissue exposed to increasing concentration of cadmium toxicity. * Represents statistically significant response, $p = 0.00$. Metallothionein concentration is measured as $\mu\text{g} / \text{g}$ wet weight tissue. Units of cadmium concentration are $\mu\text{g l}^{-1}$.

Metallothionein content of crab gill significantly increased in crabs exposed to 2 $\mu\text{g l}^{-1}$ of cadmium, (Figure 3.5). There was a slight but insignificant difference in metallothionein concentrations between the 0 and 0.5 $\mu\text{g l}^{-1}$ cadmium exposures.

Section 3.4

DISCUSSION.

GP and GR activities of PBMC.

GR and GP activities of subjects with or without diabetes were similar. A non-significant increasing trend in GR activity was observed in diabetic patients with complications compared to controls. However, in past investigations, using erythrocytes, the GR activities from diabetic patients indicate a decline in activity (Matkovics *et al.* 1987/98a,b, Murakami *et al.* 1989 and Iwakiri *et al.* 1995). GR activity is believed to be susceptible to oxidative damage, which may account for a decline of GR activity in erythrocytes (Baker, 1996). However, in order for GR activities to be affected by oxidative stress the conditions have to be severe. For example, the kidney tissue of diabetic rats experiencing oxidative stress had induced GP activities, whereas GR activities remained unaltered (Mak *et al.* 1996). Therefore this level of oxidative stress was sufficient to induce GP activity but insufficient to affect GR activity. The lymphocytes of diabetic patients do not suffer from the fragility that erythrocytes do. This may be due to lymphocytes not being as susceptible to oxidative damage as erythrocytes; possibly because they have a greater antioxidant scavenging ability. Further comparative measurements of antioxidant enzymes and compounds in the lymphocytes and erythrocytes of patients would have to be made to verify this hypothesis.

GP activity remained unaltered between the groups investigated, which is consistent with the findings of Akkus *et al.* (1996). However, there was a slight non-significant decline between the GP activities of non-diabetics and those diabetics without complications. The GP activities of erythrocytes differed depending upon the type of diabetes (Type 1 or 2), the age of the patient, and the extent of diabetic complications. Children and adolescents suffering from Type 1 diabetes were reported to have decreased GP activity in their erythrocytes (Dominguez *et al.*, 1998), as did Type 1 diabetics of all ages with neuropathy (Ndahimana, *et al.*, 1996). However, other patients of all ages

with Type 1 diabetes had increased GP activity (Matkovics *et al.*, 1997/98a). Another study showed no change in GP activities of Type 1 diabetics (Stahlberg & Hietanen, 1990). The GP activities in erythrocytes of Type 2 diabetic patients were shown to be depressed (Matkovics, 1997/98b) and also to increase in a similar population (Sundaram *et al.* 1996). This fluctuation of GP activities in different sample populations was explained by Ruiz *et al.* (1999), who discovered that GP activities decreased in the patients of Type 1 diabetes who had medium to poor metabolic control. Fluctuations of GP activities determined in the erythrocytes of different sample populations may be due to the amount of metabolic control the majority of the sample population exhibits. In this instance metabolic control was determined by the amount of glycosylated haemoglobin (HbA_{1c}). This measurement is frequently made in studies of this type. However, this assay was not included in the present study as HbA_{1c} measurements have proved to be variable in past experiments. Metabolic control is also referred to as glycemic control and is sometimes measured as blood glucose levels. Glycemic control of diabetic mice has shown to prevent the early decrease of glutathione peroxidase activity in peripheral nerve of diabetic mice (Romero *et al.*, 1999).

The data obtained from this investigation did not show correlations between enzyme activity and the age of the patient; or duration of the disease. However, a correlation between age and GR activity has been observed in erythrocytes and lymphocytes of humans by Al-Turk *et al.* (1987). The study could have been made more sensitive if larger sample numbers had been used. This would be necessary if future studies were to be made into this subject.

The findings of the present study did not support the hypothesis that diabetes induces an alteration in the enzyme activities of GR and GP in lymphocytes from diabetic patients. However, increased oxidative status of PBMC from diabetic patients has been associated with low intracellular reduced glutathione and the unbalanced profile of key enzymes involved in GSH metabolism such as gamma-glutamyltransferase (γ -GT) and GST, (Graber *et al.*, 1999). The number of diabetes patients that were used in the study just described were similar to the numbers used in the current

study. Therefore we can conclude that if altered GP and GR activities were strongly associated with diabetes in PBMC then this would have been evident from the sample sizes used. However, if changes of GP and GR activities are weakly associated with diabetes then it may be necessary to use larger populations of patients. The population of the groups were limited by the ability to obtain blood samples during the study period.

Metabolic changes of crabs in response to cadmium exposure.

GP and GST activities fell at high levels of cadmium exposure. At low levels of exposure there was not a significant difference in activity compared to that in control animals. This suggests that GP and GST would be useful as biomarkers of high levels of cadmium exposure. A fall of enzyme activity is associated with the enzyme becoming deactivated by the metal. Enzyme deactivation can occur by inducing changes to the secondary and tertiary protein structure of the enzyme; the metal binding to an electron donating group; or by substituting a metal that stabilises the enzyme structure by another (Newman, 1998). The results of this investigation do not elucidate which of the mechanisms are causing the significant decrease in enzyme activity. No change was observed in GR activity in response to cadmium exposure.

Metallothionein concentrations of crab gill tissue increased in a typical exposure-response manner with cadmium exposure. Metallothionein has a protective role against cadmium toxicity in rat livers (Chan & Cherian, 1992), in the abdominal muscle of the coral prawn *Metapenaeopsis crassissima* (Francesconi *et al.*, 1998), and in the intestine of green sea urchins, *Strongylocentrotus droebachiensis*, (Aspholm & Hylland, 1998). The potential for using metallothionein as a biomarker of toxicity has been extensively researched (Pedersen, 1996) and has proven to be effective. This study also shows that metallothionein measurements of crab gill tissue have potential as a biomarker of cadmium toxicity. There were no correlations observed between the enzyme

activities measured in the crab haemolymph and the metallothionein concentrations measured in the crab gill.

Summary

The crab (*Carcinus maenas*), has proved a simple and effective model of cadmium toxicity. The animals are kept in a controlled environment during the experimental period, which reduces variability of enzyme activities among individuals. Therefore, fewer animals are required to achieve results that show significant differences among sample groups. Laboratory based exposures also allow more control over the amount of stress that the animals are exposed to. For these reasons, crabs exposed to contamination were the favored model for observing metabolic changes in response to oxidative stress.

**The effect of mixed effluent on the
anti-oxidant systems of *Carcinus maenas*.**

Section 4.1

INTRODUCTION.

There has been a significant amount of research on the responses of biomarkers to various contaminants. The majority of these investigations have used exposures consisting of just one or two contaminants, e.g. Förlin *et al.* (1996); Boryslawskyj (1988); Dierickx (1984); Spicer & Weber (1992); Thomas & Juedes, (1992); Reddy *et al.* (1996); and Ringwood *et al.* (1998). The effects of combinations of chemicals on these biomarkers have yet to be thoroughly investigated despite the fact that animals in the wild are more likely to encounter anthropogenic waste which comprises a mixture of chemicals rather than a single contaminant.

Results obtained from previous investigations of using glutathione metabolism as a biomarker of contaminant exposure indicate considerable variation in response (Tables 1.4-1.5). In some species, glutathione and its related enzymes are induced by exposure to a particular contaminant, whilst in others they are inhibited. The reason for this cannot be ascertained from these investigations; there may not be a generic response of glutathione metabolism to contamination or these results may reflect a seasonal variation of biomarker responses.

Marine species exhibit seasonality, including seasonal changes of metabolism and behaviour, as reported in *Mytilus edulis* by Livingstone (1981); Livingstone & Clarke (1983); Ahmad & Chaplin (1979); Sheehan & Power (1999). Seasonality of behaviour and animal distribution have been reported in *Carcinus maenas* (Attrill & Thomas, 1996; Aagaard *et al.* 1995; and Crothers, 1967). Tissue composition and contaminant body burdens in marine invertebrates are also responsive to seasonal changes (Smaal & Vonck, 1997; Hühnerfuss *et al.* 1995; Hutchinson & Manning, 1996; Kreeger, 1993; and Campbell, 1969). The activities of antioxidant enzymes fluctuate over the year

(Viarengo *et al.*, 1990; Looise, *et al.*, 1996; Nies *et al.*, 1991; Kirchin *et al.*, 1992; Butow *et al.*, 1997; Weinstein, 1995; Power and Sheehan, 1996; Solé *et al.*, 1995; and Livingstone & Clarke, 1983).

Despite a broad acknowledgement that changes in enzyme activities are associated with seasonal change, little research has focused upon seasonal variability of responses to contamination or environmental stress. Chapple *et al.* (1998) reported that the mussel, *Mytilus edulis* was more tolerant of heat stress in the summer than during the winter. These observations were correlated with an increase in stress-70 protein levels during the summer months. Olsson *et al.*, (1996) reported that seasonal changes of water temperature affected the inducibility of renal metallothionein in the rainbow trout. In addition, Lochmiller *et al.* (1999) found that P450 levels in cotton rats inhabiting petrochemical waste sites had induced P450 enzymes during the summer compared to rats from a control site. However, during the winter there was not a significant difference between their P450 levels. If antioxidant enzymes are to be applied as biomonitoring tools it is necessary to fully understand seasonal changes of their activities as well as seasonal changes in their inducibility to toxicity, as their reliability may be compromised during different months of the year.

In the investigations described here, a suite of biomarkers were measured in *Carcinus maenas* exposed to a mixed effluent for fourteen days during the summer and in a second group of crabs exposed during the winter. The mixed effluent was dosed at multiples of the environmental quality standard recommended by the Environmental Data Services (1992). The biomarkers measured included neutral red retention time, metallothionein protein levels and total free radical scavenging ability. Particular attention was focussed on responses of glutathione metabolism (glutathione peroxidase, glutathione-s-transferase and glutathione reductase activities, total glutathione levels). The responses of some of these biomarkers were compared to show seasonal variability in activities as well as seasonality of response to contaminant exposure. The total antioxidant scavenging

abilities of different tissues in *Carcinus maenas* were measured to estimate the extent to which each tissue contributes to the animals' antioxidant defence system during a winter exposure. In addition, the total scavenging ability of different tissues taken from crabs exposed to increasing concentrations of mixed effluent were also measured.

Section 4.2

MATERIALS AND METHODS

4.2.1 Exposure of crabs to a mixed effluent

Carcinus maenas were captured and exposed to a mixed effluent in the middle of July (1997) and a second group of crabs was captured and exposed in November, to represent a summer and winter exposure.

A stock mixed effluent was prepared in seawater, which when diluted 1ml : 1000ml (stock : seawater) gave a 1 times dilution comprising of 2.5µg/l Cd, 5µg/l Cu, 40µg/l Zn, 21µg/l NH₃ and 5µl/l engine oil (distributed by Vauxhall). This was added to the crab tank water in multiples to give 10x, 32x and 100x environmental standards at each exposure level. 8 crabs were used for each exposure.

On the fourteenth day of the experiment, the crabs were sacrificed and haemolymph and gill tissues were dissected out. In addition, muscle tissue, heart tissue, midgut gland and epidermis were removed from the winter-exposed crabs (see Section 2.1). Protein content was established in all the tissues using the BSA protein assay kit (PIERCETM). GST and GR activities were measured in gill tissue. GST activity and total glutathione concentrations were determined from the haemolymph. In addition, GP activity, total glutathione and metallothionein concentrations were measured in gill tissue and GP activity and neutral red retention time were assayed using the haemolymph obtained from the crabs that were used during the summer experiment.

Following the winter exposure of crabs to a mixed effluent; GR activity in haemolymph and GST activities of haemolymph, heart and muscle tissue were measured. Protein content was determined for the epidermis, midgut gland and muscle tissues; total anti oxidant scavenging ability were determined for the haemolymph, gill, muscle, epidermis and midgut gland tissues.

Selected tissues were dissected from the crabs to determine the amount that each of the tissues contributes to the total antioxidant scavenging ability in undisturbed and stressed animals. Tissues selected were the haemolymph, the muscle, the epidermis, the midgut gland and the gill. Eight crabs were dissected and the weight of midgut gland, epidermis, muscle and gill tissues established. The haemolymph contributes approximately 33% to the wet weight of the animal (Depledge, 1989). The weight of the haemolymph was therefore calculated for each animal, i.e. 33% of the whole animal weight. The weights of all five tissues were pooled and average relative proportions of each tissue calculated.

The metal concentrations in the tissues of the crabs were evaluated using the method described in Section 2.1. Gill tissues exposed to 100x mixed effluent during the winter were pooled so that two measurements of metal concentrations were made for each exposure level. Insufficient muscle and haemolymph tissue were available from this experiment to measure the levels of metals in them. Therefore, these tissue samples were pooled from the mixed effluent exposed crabs used for experiments described in Chapter 6.

Dr.K.Astley made determinations of metallothionein concentrations and the lysosomal neutral red retention time of crabs exposed to mixed effluent during the summer.

4.2.2 Statistical analysis of seasonal data.

The normally and non-normally distributed data are shown in Table 4.1. ANOVA analysis was performed on the normally distributed data. GR activities were square root transformed so that the data complied with assumptions underlying ANOVA analysis. The distribution of haemolymph protein content data was not normalised by data transformation. Therefore, non-parametric data analysis was employed, and the Kruskal-Wallis Test was used to establish statistically significant differences in protein content among exposure levels.

4.2.3 MDS analysis of seasonal data.

The seasonal data were also analysed using the PRIMER programme (See Chapter 7). PRIMER is a multi-dimensional scaling program. It analyses all biomarker data for each given exposure level collectively, data sets which are most similar are positioned closer together on the MDS plot. The ANOSIM program computes similarities between the data groups, so that exposure levels can be classified as statistically similar or dissimilar to one another. All data points were transformed into percentages of the highest value for each data set (e.g. if the highest value for GST activity was 6, then a GST activity of 3 is equal to 50%). This prevented numerically high data sets from dominating the MDS analysis. The data was finally analysed using the ANOSIM program and pairwise group tests.

4.2.4 Analysis of data from crabs exposed during the summer.

Metallothionein and neutral red retention time data were log transformed to normalise their data distributions. ANOVA analysis was performed upon the log transformed data.

4.2.5 Analysis of data from crabs exposed during the winter.

Total antioxidant scavenging ability of crab epidermis (measured /g wet weight tissue), and muscle tissue (measured /g protein), the GST activities of heart tissue and the total protein content of muscle tissue were analysed using ANOVA statistics. The GST activities of crab muscle tissue were log transformed to normalise the data distribution. ANOVA analysis was performed on the transformed data.

4.2.6 Linear regression analysis

Linear regression analysis was performed between different parameters at different exposure levels to establish whether relationships existed between the parameters measured. A simple linear regression model was used in preference to any other regression model as this produced correlations that were most reproducible for different experiments. Therefore, the correlations made in the different experimental sections (Chapters 4-6), can be compared. The p- value for each of the linear correlation's describes the significance of the relationship, the correlation coefficient describes how powerful the relationship is and the R-squared value describes the percentage of the population that the model corresponds to.

Section 4.3

RESULTS

4.3.1 Metal analysis

Estimates of metal concentrations in muscle, gill and haemolymph tissues from the crabs, which were either held in clean water or in water contaminated with 100x mixed effluent, are shown in Figures 4.1a-c. The gill tissue accumulated all of the metals that were measured, i.e. zinc, copper and cadmium. Most significant was the accumulation of cadmium, which was eight times more concentrated in the gills of crabs at the 100x exposure, compared to controls.

The concentration of cadmium in the haemolymph tissues increased slightly. There were no other apparent accumulations of the metals in either the haemolymph or the muscle tissues.

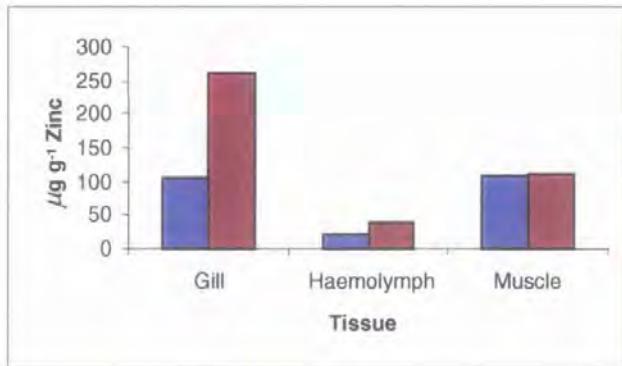
4.3.2 Seasonal Variability of Biomarker Parameters.

The results of the seasonal data are shown in Table 4.1

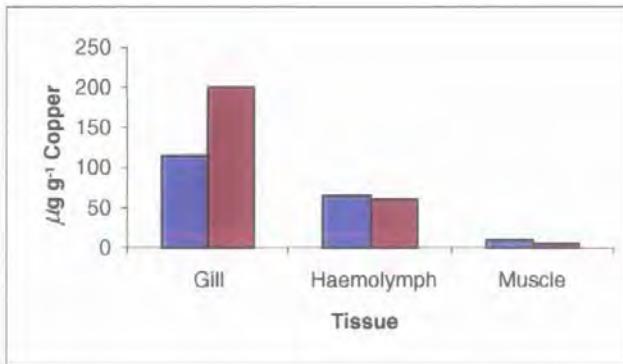
i) Protein content of haemolymph and gill tissues

The extractable protein content of haemolymph during the winter decreased to approximately <50% of that measured during the summer, $p = 0.00$. However, the protein content was not altered in response to increasing contamination exposures in either the winter or the summer exposures (Figure 4.2a).

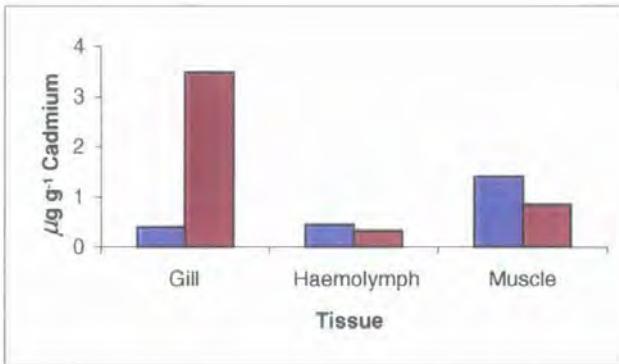
The protein content of gill tissue remained unaltered between seasons (Figure 4.2b). The exception was when crabs were exposed to high levels of contamination during the winter; protein levels were reduced compared to crabs exposed during the summer ($p < 0.001$). The fall in gill protein concentration at the 100x exposure was also significantly lower than the values measured for the rest of the winter exposures ($p = 0.04$).



4.1a



4.1b

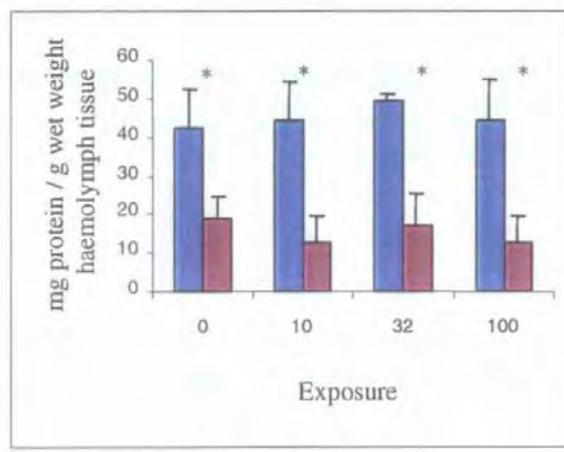


4.1c

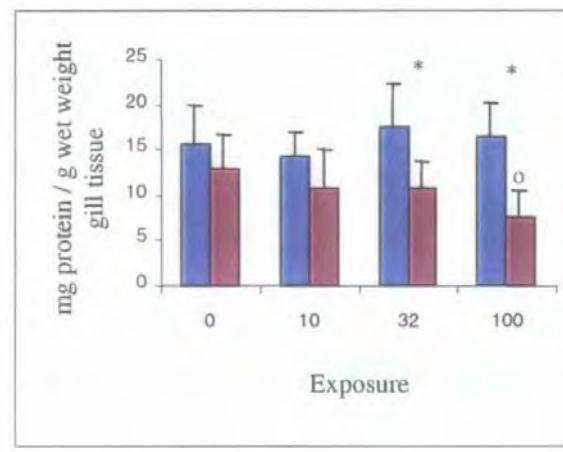
Figures 4.1a-c The concentration of metals which have accumulated in different tissue types within the crab, *Carcinus maenas*, exposed to a 0x exposure and a 100x mixed effluent exposure, measured as $\mu\text{g g}^{-1}$ of dry weight tissue. The tables show the mean values of the metal concentrations that were measured, 2 measurements were taken for each exposure level and the pooled tissues of four crabs were analysed for each measurement.

Season	Summer										Winter										
	Exposure Statistics for normally distributed data		0		10		32		100		Exposure Statistics for non-normally distributed data		0		10		32		100		
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD		median	Min/Max								
Gill protein content.	15.7	4.2	14.3	2.7	*17.3	4.9	*16.5	3.7	13.0	3.7	10.8	4.4	10.6	2.9	7.5	•2.9					
GST activity in gill tissue	*0.30	0.08	*0.21	0.05	*•0.2	0.04	*0.2	0.06	0.11	0.07	0.11	0.04	0.07	0.03	0.12	0.05					
GST activity in haemolymph tissue.	4.76	2.92	4.25	1.88	4.3	2.89	3.42	2.10	3.66	1.59	5.58	4.44	2.69	1.23	4.37	3.50					
Statistics for non-normally distributed data.	median	Min/Max	median	Min/Max	median	Min/Max	median	Min/Max	median	Min/Max	median	Min/Max	median	Min/Max	median	Min/Max	median	Min/Max	median	Min/Max	
GR activity in gill tissue	0.97	0.10	*1.28	0.82	*0.78	0.47	*•0.4	0.02	2.33	0.99	3.69	2.17	2.85	2.06	3.36	2.20					
[GSH] in haemolymph tissue	0.34	0.238	0.398	0.25	0.32	0.11	0.28	0.14	0.45	0.18	0.78	0.14	0.54	0.24	0.42	0.14					
Haemolymph protein content	*42.4	18.9	*44.0	19.5	*49.5	46.1	*44.6	26.8	18.7	11.8	10.5	6.03	21.3	5.1	10.8	5.0					
		50.6		50.7		51.9		58.35		30.5		23.7		23.5		22.4					

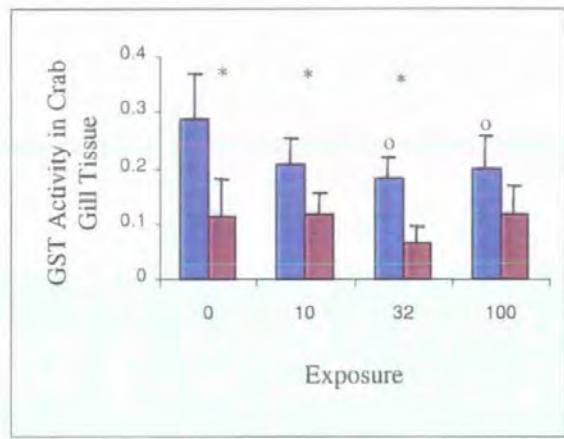
Table 4.1 Results of biomarker and physiological parameters from a summer and winter exposure to a known mixed effluent. The mean and standard deviations are shown for normally distributed data and the median and range of values are shown for the non-normally distributed data. Protein levels in gill and haemolymph are measured as mg protein / g wet weight tissue. GST activity is measured as μ moles of substrate metabolised / min / g protein for gill tissue and in nmoles of substrate metabolised / min / g protein for haemolymph. GR activity is measured as nmoles of substrate metabolised /min / g protein. [GSH] is as μ moles of GSH / g protein. * highlights statistical differences between summer and winter measurements. • signifies significant differences between measurements to one or more other exposures within that season



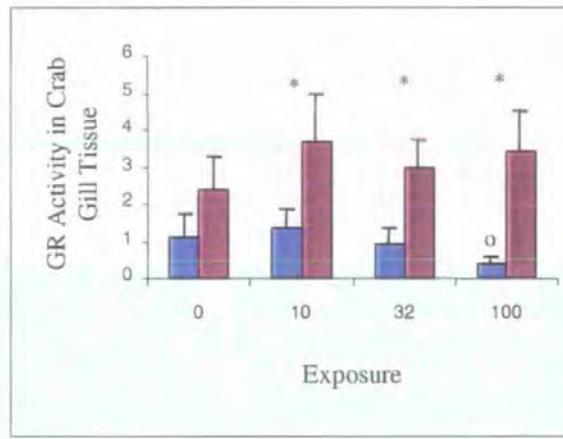
4.2a



4.2b



4.2c



4.2d

Figures 4.2a-d The seasonal variability of protein levels and enzyme activities observed in the crab, (*Carcinus maenas*), during a summer █ and winter █ exposure to a mixed effluent. GST activities are measured as mmoles / min / g protein. GR activity is measured as μ moles / min / g protein. o above the bars for either a winter or summer exposure signifies exposures which are significantly different from the control exposure during that particular season. * signifies a significant difference between the winter and summer exposure.

ii) Enzyme analysis and GSH concentrations.

Neither GST activity or GSH concentration in haemolymph altered with the changing seasons. However, there were differences between seasons for the GST and the GR activities of gill tissue. Gill GST activity was generally higher during the summer exposure than during the winter ($p < 0.05$). However, activities from the 100x exposure level measured during the summer approached those measured in the winter ($p = 0.000$). Analysis of the summer GST enzyme activities showed that they decreased at the 32 and 100x exposure levels, compared to controls ($p < 0.01$, Figure 4.2c). GR activities were significantly higher during the winter than the summer except in the control population, ($p = 0.000$). The GR activities measured during the summer also decreased at the highest exposure compared to 0 and 10x exposures, ($p = 0.003$, Figure 4.2d).

iii) Results of multi-dimensional scaling (MDS) of seasonal data.

Multiple dimensional scaling of the data using PRIMER identified two clusters, which represented the winter and summer exposures (Figure 4.3). The ANOSIM program, which analyses the similarities between the groups, showed that the summer and winter exposures were statistically different $p < 0.001$. Pairwise tests for the different groups gave significance levels of 78.5%, 39% and 61% for the summer exposure groups; 0 & 10x; 0& 32x; and 10x & 32x respectively. All other pairwise groupings were significantly different to one another, i.e. only the 0, 10 and 32x exposures during the summer exposure were similar.

The difference in biomarker responses to a mixed effluent during the summer and the winter is further presented in the MDS plot (Figure 4.4). The summer exposures are grouped closer together than the winter exposures, except the summer 100x mixed effluent exposure, which is positioned closer to the winter group.

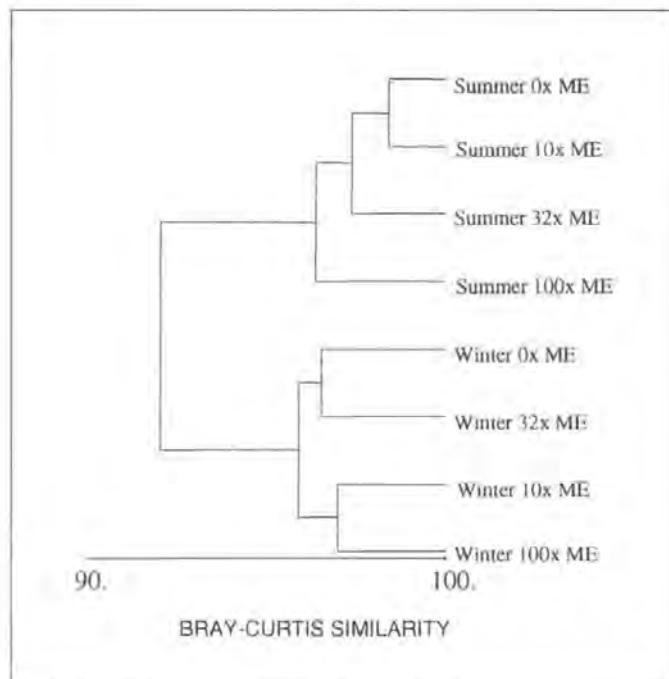


Figure 4.3 A dendrogram of the group averaged data from a similarity matrix of the measured parameters during a winter and summer exposure of crabs, to a mixed effluent, as derived from the PRIMER program. (ME = Mixed effluent).

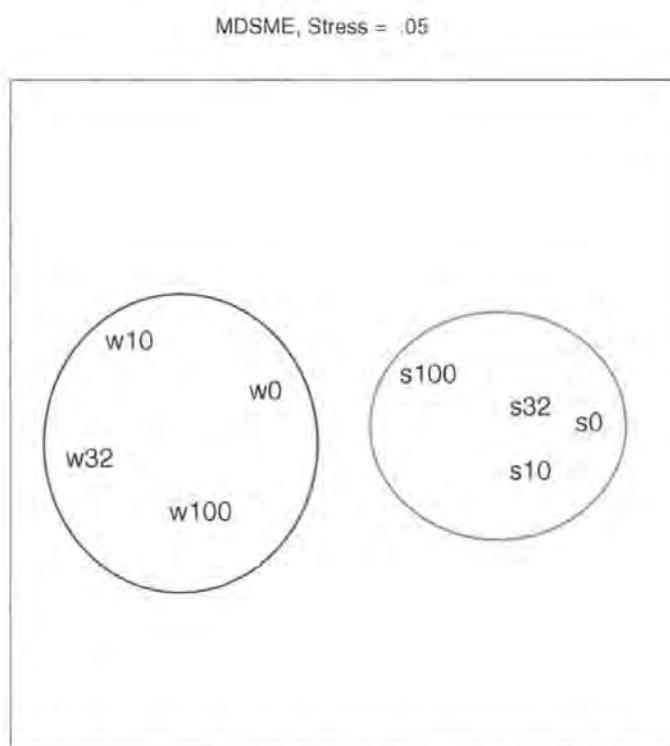


Figure 4.4 The group averaged data MDS plot as derived from the PRIMER program. The results fell out into the winter (blue circle) and summer (red circle) exposure groups. (w = winter exposure, s = summer exposure).

4.3.3 Biomarker results from the summer exposure

Biomarker values measured during the summer are displayed in Table 4.2.

i) Enzyme analysis and GSH concentrations.

No statistically significant differences were observed among exposure levels for GP activities of gill tissue and haemolymph. In addition, total glutathione concentrations of haemolymph remained unaltered upon exposure to increasing concentrations of mixed effluent.

ii) Metallothionein concentrations.

Metallothionein concentrations increased in a dose responsive manner with increasing concentrations of mixed effluent exposure. The metallothionein levels at the highest exposure were statistically different from all other exposure levels ($p < 0.001$, Figure 4.5).

iii) Lysosomal neutral red retention time

The lysosomal neutral red retention time decreased with increasing contamination exposure. The neutral red retention time was lowest at the 100x exposure, ($p = 0.001$, Figure 4.6).

4.3.4 Biomarker results from the winter exposure

The biomarker and physiological responses obtained for the winter exposure of crabs to the mixed effluent are presented in Table 4.3.

i) Enzyme activities

The GR activity of crab haemolymph remained unaltered upon contaminant exposure. However, GST activities of heart and muscle tissues responded differently in crabs exposed to the mixed

<i>Exposure</i>	<i>0</i>	<i>10</i>	<i>32</i>	<i>100</i>				
Statistics for normally distributed data.	mean	SD	mean	SD	mean	SD	mean	SD
GP activity in Gill tissue	0.27	<i>±</i> 0.09	0.25	<i>±</i> 0.10	0.22	<i>±</i> 0.08	0.28	<i>±</i> 0.08
GP activity in Haemolymph tissue	0.16	<i>±</i> 0.05	0.16	<i>±</i> 0.05	0.17	<i>±</i> 0.06	0.16	<i>±</i> 0.04
[GSH] in gill tissue	4.59	<i>±</i> 1.87	3.10	<i>±</i> 0.72	4.55	<i>±</i> 1.22	5.40	<i>±</i> 2.21
Statistics for non-normally distributed data.	median	Min/ Max	median	Min/ Max	median	Min/ Max	median	Min/ Max
Metallothionein	26	9 47	33	22 74	52	12 57	*75	51 138
Neutral red retention time (mins)	60	30 120	30	15 45	30	15 60	*15	0 30

Table 4.2 Results of biomarker parameters for the summer exposure of crabs (*Carcinus maenas*) to a known mixed effluent. The mean and the standard deviations are given for the normally distributed data and the median and min and max values are given for the non-normally distributed data.

Glutathione peroxidase activity is measured as mmoles of substrate metabolised / min / g of protein. [GSH] is measured as µmoles of GSH / g protein. Metallothionein (MT) is measured as µg / g wet weight tissue. And neutral red retention time is measured in minutes. * signifies exposure which is significantly different to one or more of the other exposure levels.

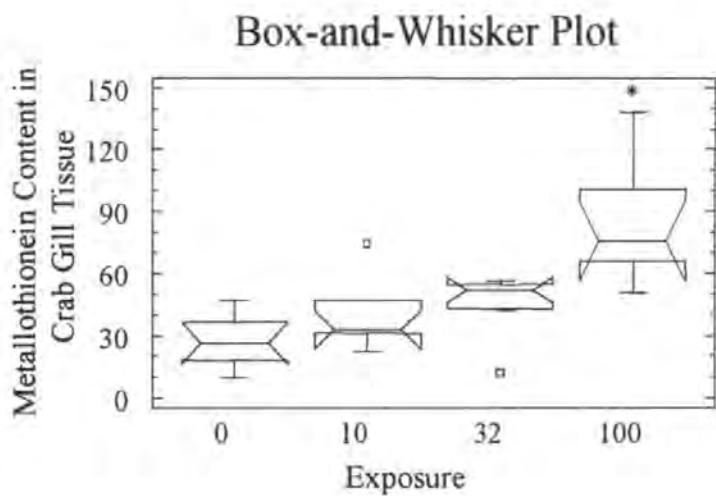


Figure 4.5 The metallothionein concentrations in crab gill at increasing concentrations of mixed effluent exposure. Metallothionein concentrations are measured as $\mu\text{g} / \text{g}$ wet weight tissue. * signifies exposure which gives a metallothionein concentration that is significantly different to concentrations all the other exposures, $p < 0.001$

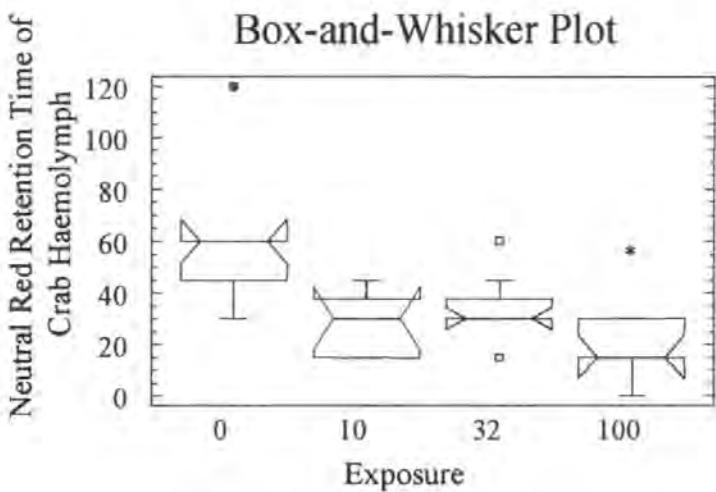


Figure 4.6 The neutral red retention time of crab haemolymph at increasing exposures of mixed effluent. Units are measured as minutes. * signifies exposure which gives a neutral red retention time which is significantly different to the control group, $p=0.001$

Exposure	0		10		32		100	
Statistics for normally distributed data.	mean	SD	mean	SD	mean	SD	mean	SD
Muscle protein Content	0.025	0.01	0.028	0.01	0.03	0.01	*0.01	0.004
Heart protein Content	19	6	13	7	17	8	13	7
GST Activity in Heart Tissue	0.25	0.64	0.30	0.14	0.26	0.11	*0.43	0.13
Endocuticle protein content	0.04	0.01	0.02	0.008	0.038	0.016	0.023	0.016
Statistics for non-normally distributed data.	Median	Min/ Max	Median	Min/ Max	Median	Min/ Max	Median	Min/ Max
MGG protein content	0.02	0.017 0.03	0.02	0.018 0.02	0.017	0.004 0.028	0.014	0.013 0.017
GST Activity in Muscle Tissue	0.16	0.11 0.43	0.19	0.07 0.25	0.11	0.05 0.20	*0.23	0.13 0.72
GR Activity in Haemolymph Tissue	0.297	0.21 0.4	0.493	0.22 0.78	0.475	0.18 0.93	0.393	0.17 1.01

Table 4.3 Results of biomarker and physiological parameters for a winter exposure of crabs (*Carcinus maenas*) to a known mixed effluent. The mean and standard deviations (SD) are shown for the normally distributed data and the median and the min and max values are shown for the non-normally distributed data.

GR activity is measured in nmoles of substrate metabolised / min / g protein. GST activity is measured in μ moles of substrate metabolised / min / g protein. Protein concentrations are measured in mg protein / g wet weight of tissue. *signifies exposures that are significantly different to one or more of the other exposure levels.

effluent. In both instances the GST activities increased at the 100x exposure level. The heart tissue GST activities at the 100x exposure were raised compared to the 0x exposure level ($p = 0.02$, Figure 4.7). Whereas, the muscle tissue GST activities at the same exposure were raised compared to all of the other exposures ($p = 0.04$, Figure 4.8).

ii) Protein concentrations.

Mixed effluent exposure did not affect the protein concentrations of mid gut gland, epidermis and heart tissues. However, the protein levels of muscle and heart tissues from crabs exposed to the highest mixed effluent concentrations decreased (Figures 4.9 & 4.10, respectively). This decrease was particularly evident for the muscle tissue ($p < 0.001$).

iii) Total antioxidant scavenging ability.

Total antioxidant scavenging ability was standardised against both protein content and grams of wet weight tissue (Table 4.4 and 4.5). Only the total antioxidant scavenging abilities of the crabs' muscle and epidermis tissues were affected by mixed effluent contamination. The antioxidant scavenging ability of the crab muscle, when calculated / g of protein, increased at the 100x exposure (Figure 4.11, $p = 0.00$). Whereas, the total antioxidant scavenging ability of crab epidermis tissue decreased with increasing contaminant exposure when calculated / g wet weight tissue, ($p = 0.02$, Figure 4.12). The antioxidant scavenging ability of the epidermis at the 100x exposure was significantly reduced.

4.3.5 The contribution of different tissues to the crabs overall antioxidant scavenging ability

The proportions that selected crab tissues occupy within the crab are presented in a pie chart (Figure 4.13). The percentage proportions that the tissues contribute towards the total antioxidant

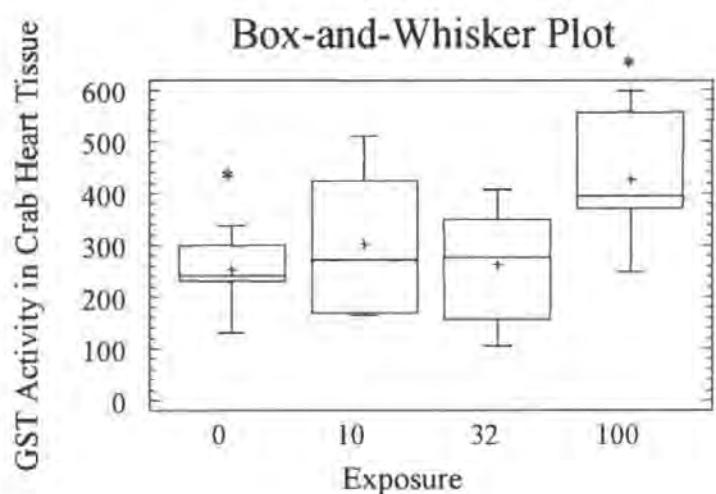


Figure 4.7 The GST activities of crab heart tissue after exposure to increasing concentrations of a mixed effluent. * signifies exposure at which enzyme activities are significantly different to the GST activities of the control group, $p = 0.02$

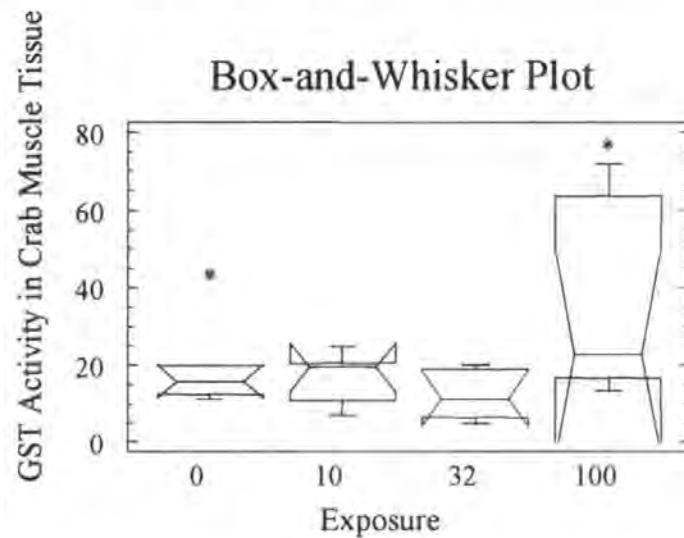


Figure 4.8 The GST activities of crab muscle tissue after exposure to increasing concentrations of mixed effluent. * signifies exposure at which the GST activities are significantly higher than the activities of the control group, $p = 0.04$

GST activities are measured as $\mu\text{moles} / \text{min} / \text{g protein}$.

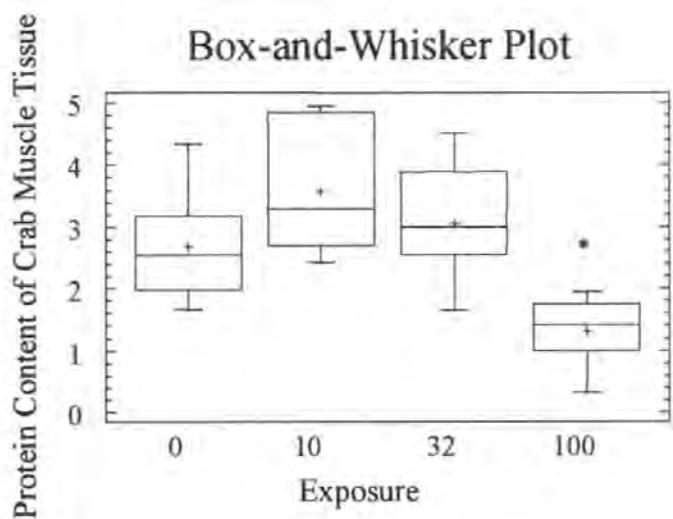


Figure 4.9 The protein content of crab muscle tissue in crabs exposed to increasing concentrations of mixed effluent. * signifies the exposure at which the protein content of the crab muscle tissue is significantly lower than at all other exposure levels, $p < 0.001$

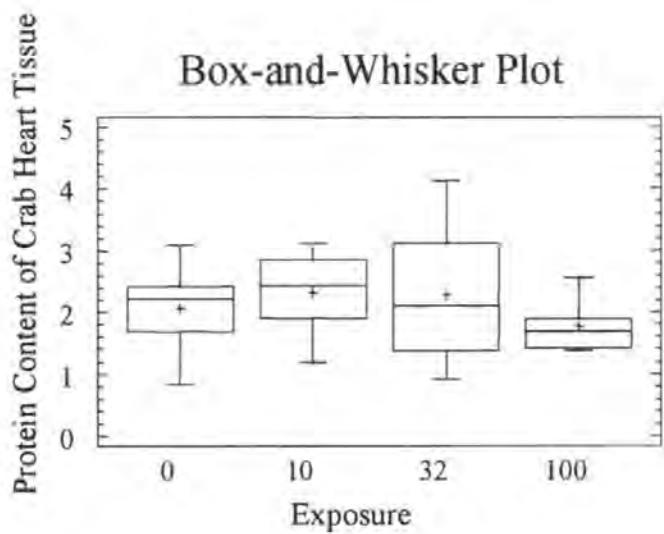


Figure 4.10 The protein content of crab heart tissue exposed to increasing concentrations of mixed effluent.

Protein content is measured as mg protein / g wet weight tissue.

		Total anti oxidant scavenging ability (mmoles / g protein).							
Exposure		0	10		32		100		
Statistics for normally distributed data.	mean	SD	mean	SD	mean	SD	mean	SD	
Muscle	0.445	±1.29	0.36	±0.096	0.397	±0.123	*0.809	±0.216	
Statistics for non-normally distributed data.	median	Min/ Max	median	Min/ Max	median	Min/ Max	median	Min/ Max	
Gill	0.294 0.491	0.215 0.423	0.331	0.210 0.423	0.348	0.263 0.613	0.350	0.281 0.890	
Epidermis	0.271 0.395	0.118 0.796	0.260	0.102 0.796	0.188	0.139 0.383	0.204	0.126 0.375	
MGG	0.247 0.322	0.098 0.291	0.198	0.175 0.291	0.232	0.198 0.629	0.250	0.180 0.325	
Haemolymph	0.045 0.074	0.029 0.147	0.080	0.035 0.147	0.042	0.039 0.194	0.087	0.04 0.163	

Table 4.4

		Total anti-oxidant scavenging ability (μmoles / g ww tissue)							
Exposure		0	10		32		100		
Statistics for normally distributed data.	mean	SD	mean	SD	mean	SD	mean	SD	
Haemolymph	0.84	±0.05	0.86	±0.06	0.89	±0.06	0.89	±0.05	
Muscle	10.5	±1.6	9.4	±1.65	11.4	±2.85	10.3	±1.5	
Epidermis	11	±5.28	6.7	±4.82	8.13	±2.46	*4.13	±1.92	
Statistics for non-normally distributed data.	median	Min/ Max	median	Min/ Max	median	Min/ Max	median	Min/ Max	
MGG	5.86 9.05	3.04 6.33	4.28	3.5 6.33	4.62	2.84 6.8	3.5	3.14 4.76	
Gill	3.99 4.7	3.05 4.6	3.33	2.24 4.6	3.76	2.27 5.9	2.94	2.44 3.3	

Table 4.5

Tables 4.4 & 4.5 Results of the total anti-oxidant scavenging ability of different tissues from the crab (*Carcinus maenas*) exposed to a known mixed effluent during the winter. The mean and standard deviations (SD) are shown for the normally distributed data and the median and the min and max values are shown for the non-normally distributed data. *signifies parameters which are statistically different to at least one of the other exposure levels.

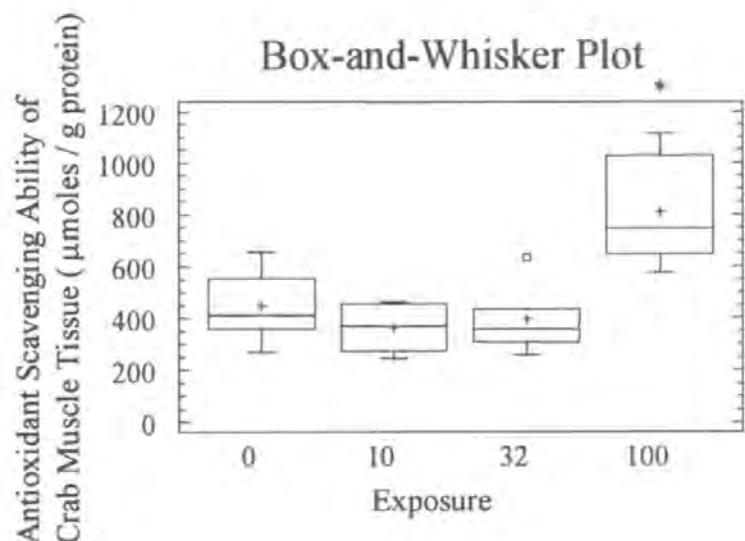


Figure 4.11 The antioxidant scavenging ability of crab muscle tissue. * signifies exposure at which the total antioxidant scavenging ability is significantly different to all other exposure levels. $p = 0.000$

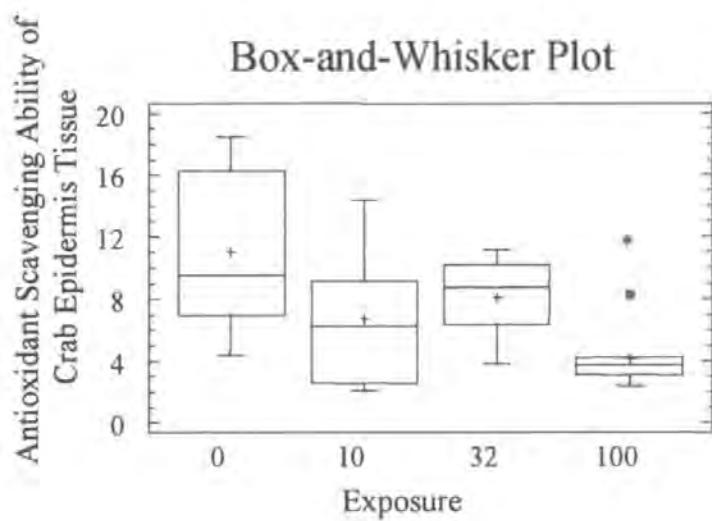


Figure 4.12 The antioxidant scavenging ability of crab epidermis tissue. * signifies exposure at which total antioxidant scavenging ability is significantly different to the control exposure. $p = 0.02$

Total antioxidant scavenging ability is measured as $\mu\text{moles} / \text{g protein}$ for muscle tissue and $\mu\text{moles} / \text{g wet weight}$ tissue for the epidermis tissue.

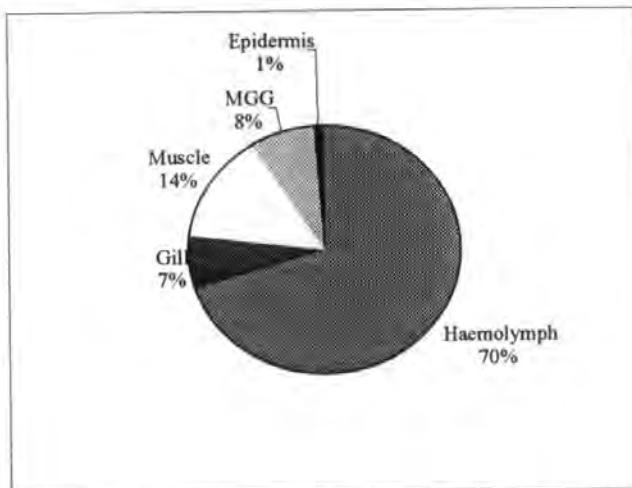


Figure 4.13 The average relative proportions that selected tissues occupy in the crab *Carcinus maenas*.

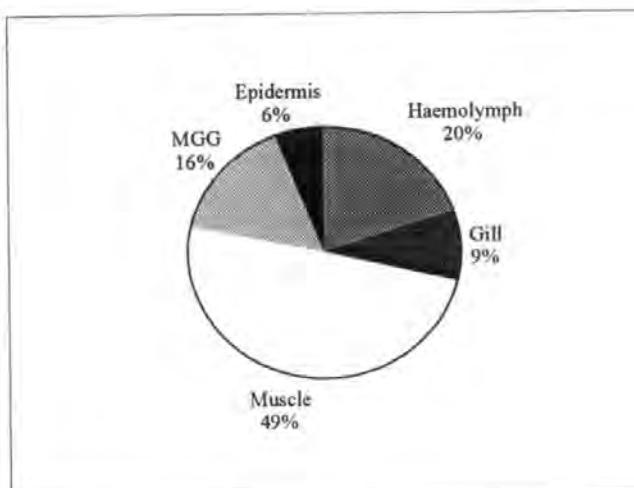


Figure 4.14 The percentage proportions that selected tissues contribute to the total antioxidant scavenging ability of the crab when unchallenged by mixed effluent.

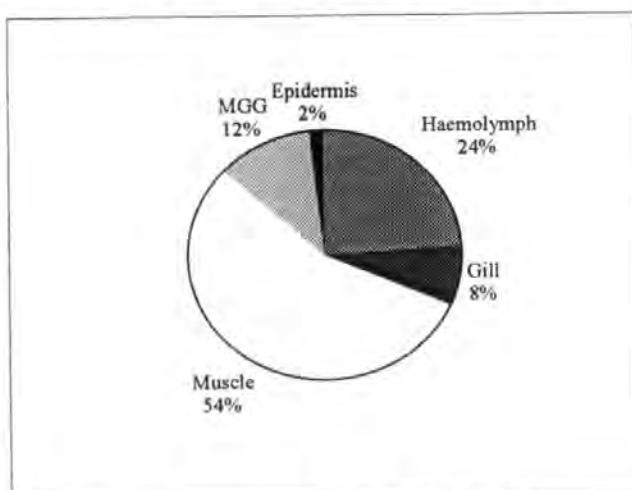


Figure 4.15 The percentage proportions that selected tissues contribute to the total antioxidant scavenging ability of the crab when challenged by 100x mixed effluent exposure.

scavenging ability in a crab that is unchallenged by contamination and challenged by a high mixed effluent exposure are shown in Figures 4.14 and 4.15 respectively.

The average relative proportions that selected tissues occupy in the crab are: -

Haemolymph > Muscle > Mid Gut Gland > Gill > Epidermis

The proportions that the tissues contribute to total antioxidant scavenging ability when unchallenged or challenged by mixed effluent exposures are: -

Muscle > Haemolymph > Mid gut gland > Gill > Epidermis.

The muscle tissue contributes more to the total antioxidant scavenging ability of the crab than the haemolymph, this is despite the haemolymph occupying more space within the crab.

4.3.6 Correlations between data groups.

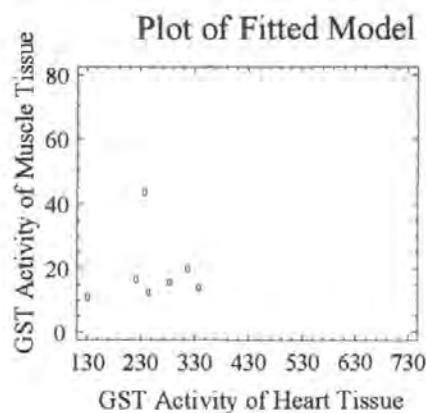
During the winter many positive correlations were made between biomarker parameters. The correlations made between data groups were induced at high levels of contaminant exposure, (Table 4.6).

The GST activities of crab muscle and heart tissue were induced at the highest mixed effluent exposure (Figures 4.16a & 4.16b). The amount by which the GST activities were induced in the two tissues were proportional to one another. The GR activity of crab haemolymph increased with contaminant exposure, however not to a significant level. A correlation was observed between haemolymph GR activity and total glutathione concentrations at the 100x-exposure level (Figures 4.16c & 4.16d). In addition, the total antioxidant scavenging ability of crab and the total glutathione content of haemolymph from crabs exposed to high concentrations of mixed effluent exposure correlate in a positive direction (Figures 4.16e & 4.16f).

<i>Correlations Between;</i>	<i>Statistics</i>	<i>0x Exposure</i>	<i>100x Exposure</i>
GST Heart / GST Muscle	p-value	0.93	0.03
	Correlation coefficient	0.04	0.9
	R-squared	0.2%	67%
GR Haemolymph / total GSH Haemolymph	p-value	0.66	0.02
	Correlation coefficient	-0.2	0.87
	R-squared	4.3%	76%
Antioxidant Scavenging Ability, Haemolymph / total glutathione, Haemolymph	p-value	1.0	0.01
	Correlation coefficient	0.2	0.86
	R-squared	0.02%	75%

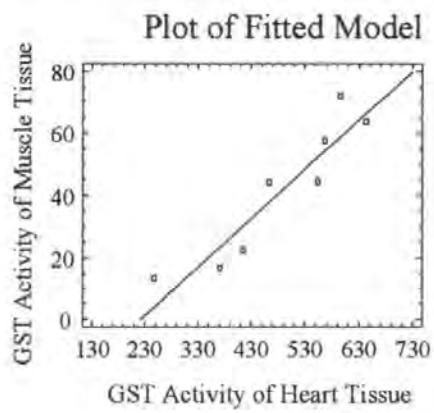
Table 4.6 Statistical results of biomarker parameter correlations at 0 and 100x exposures. The results are from fitting a linear model to describe the relationships between parameters. The R-squared value describes the percentage of the population that the model corresponds to. The p-value describes the significance of the relationship and the correlation coefficient describes how powerful the relationship is.

0x Exposure

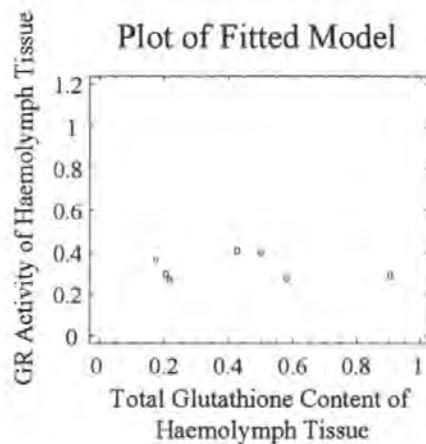


4.16a

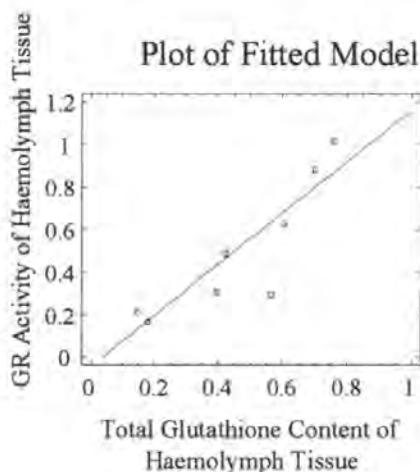
100x Exposure



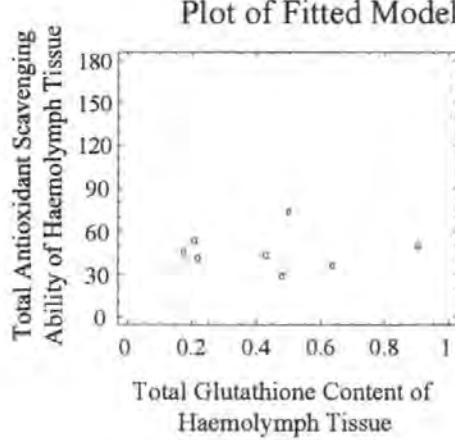
4.16b



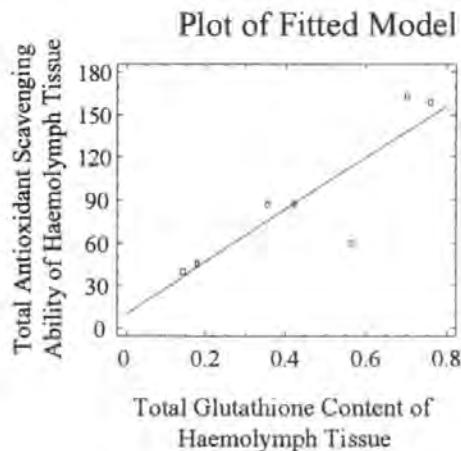
4.16c



4.16d



4.16e



4.16f

Figures 4.16a-f Correlations between different parameters are observed at high levels of mixed effluent exposure. (See tables for units of measure).

Section 4.4

DISCUSSION.

Metal analysis

The accumulation of metals in invertebrate tissues varies from animal to animal (Depledge and Bjerregaard, 1989) and among species (Rainbow and White, 1989; Berge & Brevik, 1996; Bjerregaard and Depledge, 1994). Heavy metals are toxic in excess yet some are essential at lower concentrations, e.g. zinc and copper are essential metabolic requirements. When heavy metal concentrations are excessive, organisms have to protect themselves from the oxidative damage that can result. The measurements of metals in this study were an estimation of levels that accumulated in the crab at 0 and 100x exposures of the mixed effluent. The concentrations of copper and zinc in the control animals were consistent to concentrations measured by Depledge (1989) and cadmium concentrations in crab haemolymph were similar to those measured by Chan *et al.* (1992), and cadmium concentrations in muscle and gill tissues corresponded to those measured by Wright (1977).

The gill tissue is the first membrane barrier to which water comes into contact within the crab. The gill provides the crab with its first line of antioxidant defences against toxicity. As such it is also the tissue that receives the most damage from polluted water (Rtal *et al.*, 1996). Cadmium accumulated in the gill tissue to a greater extent than zinc or copper, which were present in higher concentrations in the exposure medium. The majority of cadmium present in normal conditions within the crab is present in the midgut gland tissues. When crabs are exposed to high concentrations of water-borne cadmium the amount of cadmium within their hepatopancreas increases. Bjerregaard (1990) found that 50 % of cadmium that finds its way into crab haemolymph after cadmium exposure is relocated to the hepatopancreas. Therefore, cadmium accumulates within hepatopancreas and gill tissues. This may explain why an increase of cadmium was not measured within the haemolymph.

Copper and zinc accumulated significantly in the crab gill tissues. The first site of copper toxicity is the crab gill, after which exogenous copper binds to haemocyanin in the crab haemolymph (Rtal *et al.* 1996). In the present study copper did not accumulate in the crabs' haemolymph. Copper is highly regulated in the haemolymph as it has an essential role as the oxidisable element within the crab haemocyanin. Therefore any copper that found its way into the haemolymph may have been redistributed to other crab tissues (Depledge, 1989).

There was a slight increase of zinc concentrations in the haemolymph of exposed crabs. Zinc has been shown to bind to the proteins of haemolymph, of which >80% is hemocyanin (Bryan, 1968). Bryan (1966) also found that crabs exposed to high concentrations of zinc accumulated the metal in the haemolymph but not their muscle tissues. This was also found to be the case in the present study.

Metal regulation and accumulation in invertebrates is still under investigation. Some physiological and environmental factors have been shown to influence the accumulation and distribution of the metals, such as; salinity (Wright, 1977a; Chan *et al.*, 1992); calcium availability (Wright, 1977b); seasonality (Swaileh, 1996) and nutritional status, (Depledge, 1989 and Scott-Fordsmand & Depledge, 1993). The results from this small study demonstrate an increase in metal concentrations in crab gill tissue on exposure to mixed effluent and a maintenance of metal concentrations in the haemolymph and muscle tissues. The gill tissue is also one of the tissues that gave biomarker responses that were most sensitive to the presence of contamination. One of the reasons for this is that the gill has to cope with increased oxidative stress due to accumulation of high levels of heavy metals.

Seasonal variability of biomarkers.

The soluble protein content of haemolymph decreased during the winter (Figure 4.4). Seasonal protein fluctuations have also been observed in the dab, *Limanda Limanda* (Kreeger, 1993) and the mussel, *Mytilus trossulus* (Hutchinson & Manning, 1996). Fluctuations of protein levels in these two species were correlated to reproductive activity. The reproductive activity of the crab, *Carcinus maenas*, occurs during the summer months July-September (Crothers, 1967); haemolymph protein content was at its highest during this period. At this time of the year the crab's foraging behaviour is also more intense compared to the winter when the crab is less active (Attrill & Thomas, 1996; Aagaard *et al.*, 1995, Hunter & Naylor, 1993; and Crothers, 1968). The decrease in haemolymph protein concentrations may be related to sexual activity, foraging behaviour and the extent of migratory activity.

The results of this investigation showed that gill tissue protein content was not affected by seasonality. However, during the winter, crabs exposed to high levels of toxicity (100x mixed effluent exposure) had reduced protein concentrations in their gill tissues (Figure 4.5). As crabs have reduced feeding and foraging behaviour over the winter, the gill tissue protein may have been metabolised for the synthesis of antioxidant proteins. The animals were fed over the winter experimental period, however the crabs may not have assimilated as much protein over this period compared to during the summer. The mussel *Mytilus trossulus* has been shown to have reduced protein assimilation rates during the winter (Kreeger, 1993). The net effect being that protein content of the gill tissue is metabolised for the production of antioxidant enzymes as insufficient protein concentrations are being ingested. During the summer when foraging and feeding behaviour of the crab is higher, the catabolism of gill proteins is not necessary.

The GST activity of crab haemolymph remained unaltered between different seasons; there were also no differences in haemolymph GSH concentrations. However, GST activities of gill tissue were reduced over the winter period compared to the summer. Whereas, GR activity of the gill tissue was higher during the winter months compared to the summer. Seasonal fluctuations of enzyme activities have also been reported in other species. GST activities were lower during the winter in the freshwater bivalve, *Sphaerium corneum* (Looise *et al.*, 1996), and the American red crayfish, *Procambarus clarkii* (Nies *et al.*, 1991). Whereas, the reverse was true for the blue mussel, *Mytilus edulis*; GST activities of gill tissue were highest during the winter (Power & Sheehan, 1996). Antioxidant enzymes measured in the digestive gland of *Mytilus edulis* were highest during the summer months (Viarengo *et al.*, 1991), as were antioxidant enzymes in the digestive glands of *Mytilus galloprovincialis* (Solé *et al.*, 1995).

The seasonal fluctuations of enzyme activities within different tissues from the same animal, as well as between species, are not entirely understood. There have been indications that changes in enzyme activities are related to seasonal fluctuations of contaminant body burdens (Weinstein, 1995; and Solé *et al.*, 1995). Different tissues of the crab, *Carcinus maenas*, accumulate contaminants to different extents depending upon whether they are involved with the detoxification of that compound (Depledge, 1989; Berge & Brevik, 1996; Depledge *et al.*, 1986). The crab also accumulates metals at different rates according to its nutritional status (Depledge, 1989) or changes in haemolymph protein composition (Bjerregaard, 1990; and Depledge & Bjerregaard, 1989). All these factors are likely to influence the seasonal levels of antioxidant enzyme activities observed in different tissue types in the crab, *Carcinus maenas*.

In addition to seasonal variability of enzyme activities, seasonal variability of enzyme responses to contamination were also established from the data. The GST and GR activities of gill tissue during

the summer reduced in response to contaminant exposure, however, this reduction was not reproduced during the winter. (Figures 4.6 and 4.7 respectively). The GST activity fell in a manner that did not follow a conventional dose responsive curve. The activity of gill GST never fell to levels that were lower than those observed during the winter exposures. Decreases in GST activity have been observed in other species in response to contaminant exposure (Regoli *et al.*, 1997; Reddy *et al.*, 1996; Dierickx, 1984; and Fitzpatrick *et al.*, 1995b). The GR activity fell in a dose responsive manner during the summer.

It is not possible to deduce from these results why reduced enzyme activity is not reproduced during the winter. However, it is possible that as with the seasonal variation of enzyme activities, that enzyme responses to pollution are also sensitive to the amount of contamination that accumulates in different tissues during the changing seasons. The contaminant component of the mixed effluent that induces this response may be readily incorporated into the gill tissue during the summer, but not as readily assimilated during the winter. Regoli & Orlando (1993) reported seasonal variability of lead accumulation in the hepatopancreas of the mussel *Mytilus galloprovincialis*. The seasonal differences in lead accumulation were influenced by the gonadal development on digestive gland weight, (Regoli & Orlando, 1993). The period of the reproductive cycle in *Carcinus maenas*, may also affect the accumulation of toxins in crab tissues, which in turn could influence the biomarker measurements made in these tissues.

These results prove that although GST and GR activities are sensitive to contaminant exposures and hence have potential as biomarkers that they are open to misinterpretation if their seasonal activities and responses to contamination are not fully understood.

The multivariate analysis of biomarker data is a relatively new concept, it has been employed in an aquatic biomarker study using fish by Machala *et al.* (1997) and a terrestrial field trial using voles by

Fairbrother *et al.* (1998). Multivariate analysis allows sets of different biomarker data to be analysed simultaneously. Hence, much stronger conclusions can be derived from the data than analysing each set separately.

The cluster analysis programs successfully differentiated between the winter and summer mixed effluent exposure groups (Figure 4.8). The ANOSIM program (analysis of similarities) showed that only the summer mixed effluent exposures, 0, 10 and 32x were statistically similar to one another. All the winter exposures were statistically different to one another and the 100x summer exposure was positioned closer to the winter exposure grouping. Animals are able to tolerate stress to different degrees depending upon factors such as age, fitness, nutritional status and tolerance to pollution. These variables were controlled as far as possible by choosing only male crabs for experimentation with carapace width of approximately 5-7cm, carapace width is an indication of the age of the crab. These differences of ability to tolerate stress can cause biomarker responses to become induced in some animals before others, hence causing an increase in the variation of biomarker data from the mean result (Aagaard & Depledge, 1993; and Depledge & Lundebye, 1996). When all the crabs are relatively healthy and well fed, i.e. during the summer, they all have a similar ability to tolerate contamination exposure. However, when the crabs are exposed to high levels of toxicity, or are of low nutritional status, i.e. during the winter, their responses to toxicity are more varied as some of the animals are able to tolerate stress better than others.

The results of this study show that the crabs that had lower protein levels and antioxidant enzyme levels during the winter were more vulnerable to toxicity. Experimentation by Viarengo *et al.* (1991), supports this theory; in the winter the mussel, *Mytilus edulis*, has lower antioxidant enzyme activities and lower levels of antioxidant compounds compared to during the summer. At this time, the lipid peroxidation levels of the mussels' digestive tissues were at their highest. The reduced

antioxidant scavenging ability of the digestive gland during the winter made the mussel more susceptible to oxidative stress.

The PRIMER program incorporates SIMPER analysis that establishes which parameters contribute the most to dissimilarity between groups formed by the cluster analysis. This enables selection of biomarkers that are most sensitive for distinguishing between contamination levels. During the winter exposure, GST activity of crab haemolymph distinguished the most between groups and GR activity of crab gill distinguished the least. During the summer, GST activity of crab gill tissue and GR activity in crab gill contributed the most to dissimilarity between groups. This analysis allows calculated estimates of which biomarkers will be most successful at highlighting contamination at different times of the year. For example, GR activity would not be a useful biomarker for distinguishing areas of mixed effluent exposure during the winter, however during the summer it could prove to be very efficient.

The use of antioxidant enzymes as biomarkers of toxicity has proved to be more complex than establishing fluctuations in enzyme activity. A thorough understanding of the seasonal variability of enzyme responses to contamination is necessary. Multivariate analysis techniques are a useful method of analysing this type of data as it allows spatial comparisons to be made between exposure groups using a multi-biomarker approach. In addition, it establishes which biomarkers are the most successful to use during different seasons.

Summer exposure biomarker responses.

Additional enzyme activities that were measured during the summer and were not included in the seasonal data analysis include GP activity of gill and haemolymph tissues. GP enzyme activity is decreased in the gill tissue of the freshwater bivalve, *Unio tumidus*, in response to copper

contamination (Doyotte *et al.*, 1997) and also in the red blood cells of the marine fish, *Dicentrarchus Labrax* (Roche and Bogé, 1993). In this study, the GP activities of gill and haemolymph tissues did not alter in response to the mixed effluent. In many other studies the GP activities remained unaltered in response to metal and organic contamination (Tables 1.4-1.6). Therefore, GP activity may not be a reliable biomarker to assess the presence of contamination.

The total glutathione content of gill tissue also remained unaltered. However, a change in thiol status is shown to be a common response to contaminant exposure in many species (Tables 1.4-1.5) and a common initial response to metal exposure (Singhal *et al.* 1987). The exposure period for this experiment was for two weeks. If glutathione had been an initial defence against metal toxicity, it is possible that levels may have returned to normal after other antioxidant mechanisms had been induced.

Metallothionein concentrations of gill tissue increased with increasing contaminant exposure. The animals had to receive high doses of pollution over the two-week period in order to induce significantly higher metallothionein levels compared to the control population. The increase in metallothionein levels corresponded to an increase in metal accumulation within the crab gill tissues (Figures 4.10 and 4.1-4.3). Metallothioneins sequester heavy metals to protect tissues from oxidative damage and or interference with metabolic processes. Experimentation has also shown that metallothionein plays a part in protection against oxidative stress induced by peroxides (Schlenk & Rice, 1998; and Kling *et al.*, 1996). Metallothionein is therefore a useful biomarker of contamination.

Winter exposure biomarker responses.

GR activity of haemolymph did not alter in response to mixed effluent exposure. There was also no change of GR activity in the haemolymph of crabs exposed to increasing cadmium concentrations

(Chapter 3). The results of these two experiments suggest that a change of GR activity in crab haemolymph is not a reliable biomarker of contaminant exposure.

During the summer exposure of crabs to a mixed effluent the GST activities of gill tissues were lowered. In contrast, during the winter exposure of crabs the GST activities of heart and muscle increased at the highest exposure. Metals accumulated in the gill tissues of the crab (Figures 4.1-4.3), however metals did not accumulate in the muscle tissues. The metals may have interfered with the GST enzyme in the gill tissue during the summer exposure, hence reducing its activity; whereas, increased oxidative stress or the organic components of the mixed effluent may have induced GST activities in the muscle tissues. GST activity of muscle tissue from the crab could be applied as a biomarker of organic pollution. However, the disadvantages of using crab muscle tissue for biomonitoring studies are that it is a difficult tissue to remove from the animal and that the tissue is very dense and hence difficult to homogenate to create the enzyme extract.

The protein content of muscle tissue decreased at high levels of contaminant exposure (Figure 4.14). Whereas the protein content of heart tissue which is also a muscle remained unaltered (Figure 4.15). Low nutritional status may account for the fall of muscle protein levels in the crab. Muscle tissue may be metabolised so that antioxidant proteins can be produced to protect the crab tissues from oxidative damage, (this was also hypothesised regarding decreased gill protein as a result of mixed effluent exposure). Of particular interest is the protein levels of the heart muscle did not significantly decrease in the same manner as the muscle and gill tissues. Heart tissue is a modified muscle, and the muscle of this vital organ is not metabolised as quickly as the other muscle tissues. Hence, the health and metabolic status of this vital heart organ is maintained for longer.

The antioxidant scavenging abilities of most crab tissues did not change in response to mixed effluent exposure. However, the distribution of total antioxidant scavenging ability increased with increasing pollution exposure for some of the tissues, for instance, gill and haemolymph (Table 4.5). Increased

data distributions are sometimes indicative of a toxicological response (Forbes & Depledge, 1996). The antioxidant scavenging ability of the crab muscle tissue increased significantly when calculated per gram of protein. This change in total antioxidant scavenging ability was not observed when calculated per g of wet weight tissue. This means that a larger proportion of the muscle protein has antioxidant scavenging ability when the crab is experiencing toxicity exposure. However, the overall amount of antioxidant scavenging ability per amount of tissue remains unchanged. This suggests that enzymes that are involved with other metabolic processes in the crab may be compromised so that the crab can maintain levels of total antioxidant scavenging ability in this tissue.

The total antioxidant scavenging ability of the crab epidermis decreased significantly in response to high concentrations of mixed effluent, when measured per gram of wet weight tissue. The epidermis tissue of the crab is closely associated with the midgut gland of the crab. The total antioxidant scavenging ability of the crab mid gut gland also decreased in response to contaminant exposure, however, not to a significant degree. The total antioxidant scavenging ability for both of these tissues remained unaltered when calculated per gram of protein. Therefore the decreased total antioxidant scavenging ability per gram of wet weight epidermis tissue may have been caused by increased water retention as a result of toxicity exposure.

No other significant differences were observed for the total antioxidant scavenging ability of the other tissues selected. The measurements of the total antioxidant scavenging ability of crab muscle and epidermis tissues have potential for use as biomonitoring tools.

Total Antioxidant Scavenging Ability of Different Tissue Types in the Crab.

The use of total free radical scavenging ability of tissues as a biomarker of pollution exposure is still in the early stages of development (Regoli & Winston, 1998). Figures 4.18-4.20 represent the amount that different tissues occupy within the crab and also how much they contribute to the total

antioxidant scavenging ability of the crab when unchallenged and then challenged by high concentrations of mixed effluent exposure.

The order of the most to the least total antioxidant scavenging ability of the different tissues as calculated per gram of protein is: -

Muscle > Gill > Epidermis > Mid gut gland > Haemolymph

Haemolymph has the least total antioxidant scavenging ability per gram of protein, however its contribution to the total antioxidant scavenging ability of the crab is relatively high, (Figure 4.19 / 4.20). This is because of the large proportion of the crab that the haemolymph occupies. Whereas, the gill tissue which has a relatively high total antioxidant scavenging ability, does not constitute a large proportion of the crab tissue and hence has a relatively small contribution to total antioxidant scavenging ability.

The muscle tissue has both a high total antioxidant scavenging ability and contributes significantly to the total antioxidant scavenging ability of the crab. Hence, it has a large involvement in preventing oxidative damage. The muscle tissue also demonstrated a significant increase of glutathione-s-transferase activity in response to mixed effluent. GST may make a considerable contribution to the antioxidant scavenging activity in this tissue. However, as GST catalyses the conjugation of xenobiotics with glutathione, that have the potential to cause oxidative stress, its involvement with antioxidant scavenging ability is indirect.

The exposure of crabs to a mixed effluent does not affect the order of the tissues contribution to antioxidant scavenging ability. However, the contribution made by both the mid gut gland and epidermis tissues falls. Many scientists measure antioxidant enzymes in digestive tissues because they believe them to be the site of most enzyme activity. These results show that digestive tissue is not the site where most total antioxidant scavenging ability occurs within the crab.

The proportion that haemolymph and muscle tissues contribute to total antioxidant scavenging ability increases slightly with increased mixed effluent exposure. These tissues constitute the largest proportions of the crab so it is biologically favourable that they should be the main sites of oxidative stress control.

Correlations between data groups.

GST activities were induced in both the heart and muscle tissues at high contaminant exposure levels. The GST activities measured for these two tissues were proportional to one another. This suggests that the antioxidant mechanisms of these two tissues are similar. The heart is made of muscle tissue, but it has a more specialised function. The amount of GST induction will have been similar for these two tissues as both of these tissues are types of muscle tissue.

The GR activity of crab haemolymph tissue increased with contaminant exposure, however, not to a significant level. The induction of GR occurs for two reasons, (i) an increase in available enzyme substrates or, (ii) an increase of GR enzyme production. The measurement of enzyme activities necessitated using substrate levels in excess, so that enzyme activities were measured at maximum potential. Therefore, we can conclude that GR activity in this instance was not limited by the availability of glutathione, but that GR activity increased because of an increase of enzyme production. In order to establish conclusively that this was the case, GR protein levels would have to be measured. The analysis of GR protein levels within the crab is still within early stages of development. The possible reasons for glutathione and GR activities having a relationship are discussed in later chapters.

A correlation was also formed between the total antioxidant scavenging ability and the total glutathione content of haemolymph from crabs exposed to high concentrations of mixed effluent. This correlation of parameters suggests, that when the crabs were very stressed, glutathione made a

large contribution to the total antioxidant scavenging ability of haemolymph tissue. Whereas during normal conditions, glutathione did not have a large contribution to total antioxidant scavenging ability (Regoli & Winston, 1998).

The correlations that have been observed between different parameters for this experiment may have potential for application as biomarkers as they indicate when the crab is highly stressed.

Summary

This experimental section has highlighted the importance of understanding seasonal effects upon biomarker responses before they can be successfully employed. Seasonal fluctuations of biomarker responses may be attributed to differences of xenobiotic assimilation over the year or the state of the organism.

This chapter has also highlighted that we cannot assume that the tissues with the highest antioxidant enzyme activities are responsible for the greatest amount of antioxidant scavenging ability in that animal. However, the gills are a first line of defence against contaminant exposure, so efficient function and a high total antioxidant scavenging ability per gram of protein is essential and could prevent later problems.

**The effect of nutritional status on the biomarker
responses of *Carcinus maenas* exposed to a mixed effluent.**

Section 5.1

INTRODUCTION

Shore crabs, *Carcinus maenas* exhibit seasonality of enzyme activities in addition to seasonality of enzyme responses to contamination (Chapter 4). The seasonal fluctuations of antioxidant enzyme activities in the crab are correlated with sexual activity, which in turn is associated with nutritional status. Seasonal fluctuations of MFO activities in the mussel, *Mytilus galloprovincialis*, are also believed to be related to nutritional status (Solé *et al.*, 1995). Similarly, the nutritional status of the Arctic charr, *Salvelinus alpinus*, is shown to influence its biomarker responses to PCBs (Jørgensen *et al.*, 1999) and xenobiotic transforming enzymes are also altered in starved rainbow trout, *Salmo gairdneri*, (Andersson *et al.*, 1985).

The aim of the present study was to determine whether fluctuations of enzyme activity are associated with changes in the nutritional state of crabs. In order to experimentally induce the low nutritional status of crabs during the winter, crabs caught during July were starved for four weeks prior to exposure to a high concentration of mixed effluent. Crabs are able to tolerate longer periods of fasting, however this experiment was not designed to monitor the effects of chronic starvation. The experiment also did not monitor the effects of seasonal temperature changes. A four week starvation period is sufficient to induce physiological, (Uglow, 1969 and Scott-Fordsmand and Depledge, 1993) and metabolic changes, (Uglow, 1969). Starvation of *Carcinus maenas* results in a progressive suppression of metabolism compared to fed crabs (Marsden *et al.*, 1973). The heart rate and oxygen consumption are also depressed following starvation and crabs are less active (Aldrich, 1975 and Depledge, 1985). The net effect was to induce a physiological and metabolic state, which is typical of the crab during the winter season. It was hypothesised that a change in nutritional status

may be responsible for seasonal fluctuations of antioxidant enzymatic responses in different tissue types.

Section 5.2

MATERIALS AND METHODS.

5.2.1 Treatment and exposure of *Carcinus maenas*

Crabs were caught in July (1998). Half of the crabs captured were starved, and the other half were fed with irradiated cockles every second day. After four weeks, eight of the fed and starved animals were exposed to a high concentration of mixed effluent comprising 250 μ g/l Cd, 500 μ g/l Cu, 4mg/l Zn, 2.1 mg/l NH₃ and 500 μ g/l engine oil. A further eight crabs from each of the fed and starved groups were kept in standard seawater. The fed animals continued to be fed during the whole of the exposure period. The water was changed in both the control and exposure tanks of both starved and fed crabs the day after fed animals were given food. The animals were held at 15°C for all experiments, therefore this experiment did not allow for seasonal temperature changes.

After 14 days exposure the animals were sacrificed as described in Section 2.1.3. Gill and haemolymph samples were removed from the animals and GSH levels, GST, GP and GR activities and total antioxidant scavenging ability were measured in both the gill and haemolymph samples. Metallothionein concentrations were measured in the gill tissue and the lysosomal neutral red retention time was determined for the haemocytes.

5.2.2 Statistical analysis.

Non-normally distributed data were transformed where possible to allow for ANOVA statistical testing. GST & GP activities of crab gill tissue were log transformed. Total antioxidant scavenging ability of crab haemolymph was arc-sine transformed in order to normalise the data distribution. P-values for the normally distributed data were evaluated using the ANOVA test for parametric data. Statistical differences between exposures were analysed with the Tukey HSD test. The distribution

of gill GR activities were not normalised by transformation so the p-value was determined using the Kruskal-Wallis test for non-parametric statistical analysis.

Section 5.3

RESULTS.

The results are shown in Table 5.1. The mean and standard deviations are displayed for the normally distributed data and the medians and ranges are given for the non-normally distributed data. Statistically different exposure groups are denoted by different letters above the box and whisker plots.

5.3.1 Protein concentrations.

Mixed effluent exposure reduced haemolymph protein concentrations in the starved crabs ($p = 0.04$, Figure 5.1). There were no differences of haemolymph protein concentrations among treatments.

5.3.2 Glutathione concentrations and enzyme activities.

The glutathione concentrations in the gill tissue of starved and fed crabs in the presence or absence of mixed effluent exposure are shown in Figure 5.2. The glutathione concentration of the fed crabs increased slightly when they were exposed to mixed effluent compared to the unexposed fed crabs, however this small rise of concentration was not statistically significant. When the crabs were starved and not challenged by mixed effluent exposure glutathione concentrations dropped slightly compared to those in unexposed fed crabs. Overall, the net effect was a difference between the fed crabs which had been exposed to mixed effluent and the starved crabs which had not ($p < 0.005$). The glutathione content of the crab haemolymph tissues was unaffected by the different exposures.

Figure 5.3 shows the GST activities for gill tissues from crabs in different nutritional states with or without mixed effluent exposure. GST activities were suppressed in the fed crabs that had been exposed to mixed effluent, but this decrease of activity was not statistically significant as has been

Exposure	Fed		Fed / Mixed Effluent Exposed		Starved		Starved / Mixed Effluent Exposed	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Normally Distributed Data								
GSH Haemolymph	0.35	± 0.2	0.33	± 0.28	0.69	± 0.33	0.6	± 0.4
GSH Gill	2.84	± 1.9	4.28	± 1.35	1.34	± 0.9	4.7	± 2.3
GST Haemolymph	3.4	± 1.3	2.2	± 1.0	2.8	± 0.66	2.16	± 1.3
Total Antioxidant Scavenging Ability Gill	3.26	± 0.78	3.30	± 1.32	2.57	± 0.50	2.93	± 0.86
Metallothionein	60.3	± 20.5	84.1	± 28	48.69	± 13.7	99	± 28
Haemolymph Protein	13.5	± 5.8	10.6	± 7.2	16.5	± 3.8	8.6	± 4.3
Neutral Red	77	± 21.8	28	± 28	34	± 25	17	± 16
Non-Normally Distributed Data	Median	Min / Max	Median	Min / Max	Median	Min / Max	Median	Min / Max
GST Gill	0.16	0.12	0.13	0.07	0.07	0.07	0.05	0.02
		0.29		0.24		0.12		0.10
GR Haemolymph	0.27	0.13	0.53	0.05	0.28	0.12	0.34	0.12
		0.7		1.54		0.8		0.8
GR Gill	1.75	1.4	1.73	0.67	0.98	0.79	0.87	0.43
		2.3		3.37		1.32		2.3
GP Haemolymph	0.09	0.06	0.12	0.03	0.08	0.04	0.09	0.01
		0.1		0.22		0.16		0.1
GP Gill	0.2	0.1	0.27	0.13	0.17	0.04	0.12	0.05
		0.27		0.42		0.23		0.23
Total Antioxidant Scavenging Ability Haemolymph	0.65	0.36	0.85	0.33	0.40	0.36	0.79	0.49
		1.70		2.33		0.48		2.54

Table 5.1 Biomarker parameters measured in crabs with varying nutritional status. The mean and standard deviations are shown for the normally distributed data and the median and min/max values for the non-normally distributed data. GST activity is measured as μ moles of substrate metabolised / min / g protein. GR activity is measured as nmoles of substrate metabolised / min / g protein. GP activity is measured as μ moles of substrate metabolised / min / g protein. [GSH] is measured as μ moles of GSH / g protein. [Metallothionein] is measured as μ g / g wet weight tissue. Neutral red retention time is measured in minutes. Total antioxidant scavenging ability is measured as mmoles of antioxidant scavenging ability / g of protein. Haemolymph protein levels are measured as mg protein / ml of haemolymph tissue

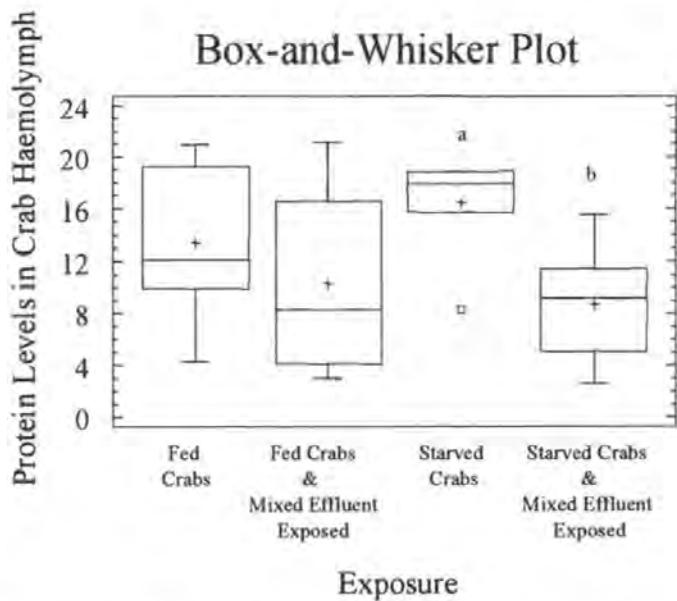


Figure 5.1 Protein levels of haemolymph from crabs unchallenged and exposed to contamination and different nutritional status. Protein levels measured as mg/ml haemolymph. The different letters above the box and whisker plots signify statistically different protein levels between the exposures.

Glutathione Concentration in Gill Tissue.

Box-and-Whisker Plot

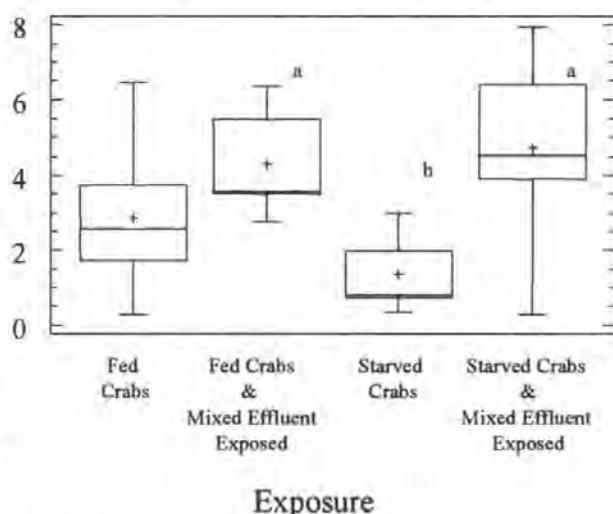


Figure 5.2 Total glutathione content of gill tissue from starved or fed crabs, unchallenged or exposed to contamination. The total glutathione content is measured as μ moles of glutathione / g of protein.

GST Activity in Crab Gill Tissue

Box-and-Whisker Plot

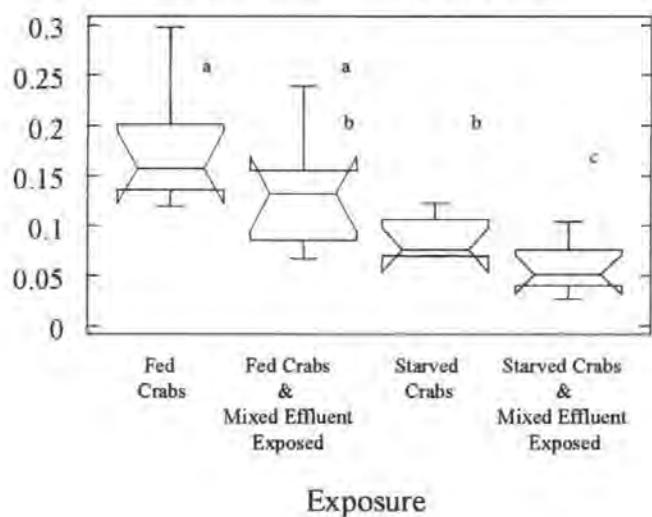


Figure 5.3 GST activities of crab gill tissue from starved or fed crabs, unchallenged or exposed to contamination. The GST activities are measured as μ moles of CDNB metabolised / min / g protein.

The different letters above the box and whisker plots signify statistically different levels between the exposures.

reported in previous experimentation (Section 4.3). Starvation also caused the GST activities to be reduced, ($p < 0.01$). However, lowest GST activities were experienced by crabs that had been both starved and exposed to the mixed effluent, ($p < 0.001$). The GST activities of the haemolymph tissues from the same animals were not statistically different to one another.

The results of glutathione reductase activity in crab gill are shown in Figure 5.4. The starved crabs had significantly lower activities compared to the fed crabs, regardless of mixed effluent exposure ($p = 0$). The fed crabs that had been exposed to mixed effluent had slightly lower gill GR activities than the control group. There was not a statistical difference between exposures for the GR activities in crab haemolymph.

The glutathione peroxidase activities of crab gill are presented in Figure 5.5. Crabs that were starved had lower GP activities compared to their fed counterparts ($p < 0.01$). The lowest GP activities were measured in crabs that had been starved and exposed to mixed effluent. In contrast the GP activities in haemolymph from the same sets of crabs did not show differences among exposure groups.

5.3.3 Total antioxidant scavenging ability

Starved crabs had reduced total antioxidant scavenging ability. However, those that had experienced mixed effluent exposure exhibited enhanced antioxidant scavenging ability ($p = 0.03$, Figure 5.6). This pattern of results is similar to that obtained for glutathione concentrations in gill tissue. The pattern was also similar to the total antioxidant scavenging ability of the crab gill, however in that case there is not a statistical difference between exposures, (Figure 5.7).

Glutathione Reductase Activity in Crab Gill.

Box-and-Whisker Plot

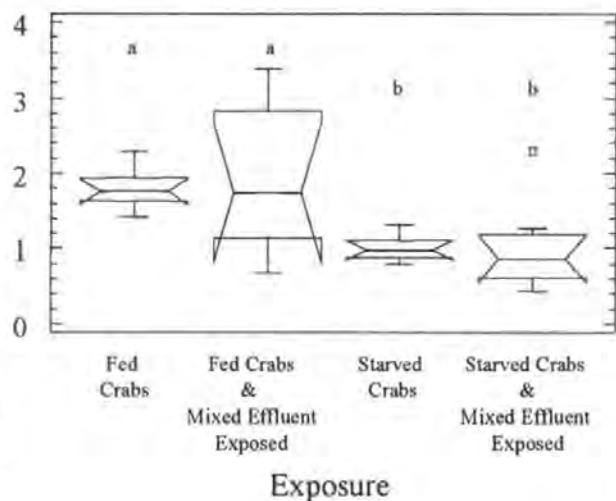


Figure 5.4 GR activities of gill tissue from starved or fed crabs unchallenged or exposed to contamination. GR is measured as nmoles of NADPH oxidised / min / g protein.

Glutathione Peroxidase Activity in Crab Gill.

Box-and-Whisker Plot

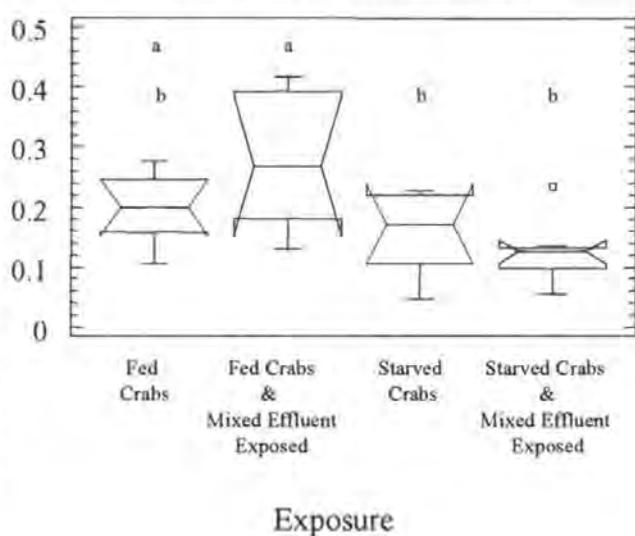


Figure 5.5 GP activities of gill tissue from starved or fed crabs unchallenged or exposed to contamination. GP is measured as mmoles of NADPH oxidised / min / g protein

The different letters above the box and whisker plots signify statistically different exposures.

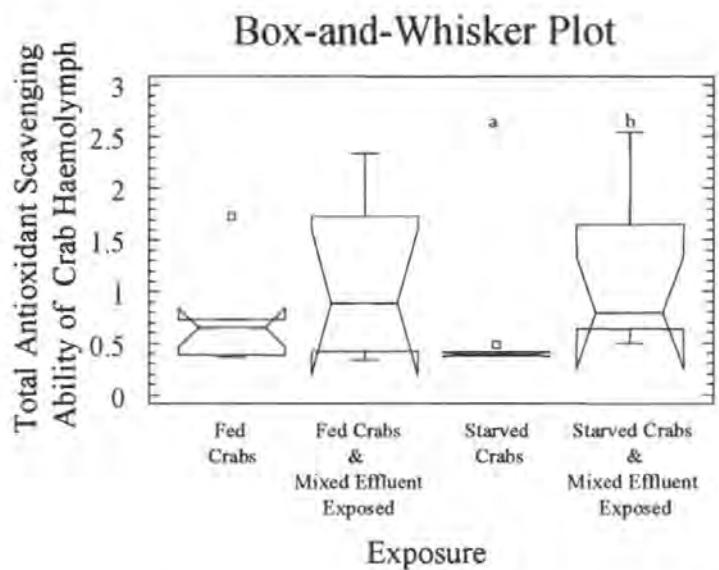


Figure 5.6 Total antioxidant scavenging abilities of crab haemolymph tissue, from starved or fed crabs unchallenged or exposed to contamination.

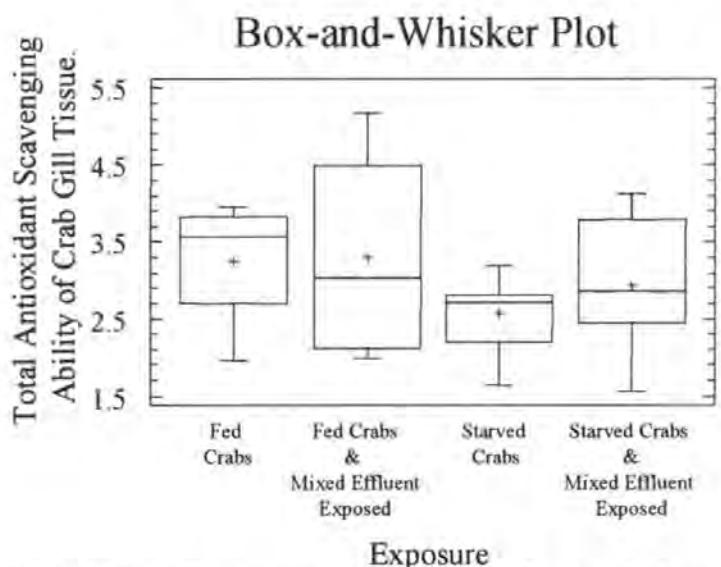


Figure 5.7 Total antioxidant scavenging ability of crab gill tissue, from starved or fed crabs unchallenged or exposed to contamination.

Total antioxidant scavenging ability is measured as $\mu\text{moles} / \text{g protein}$. The different letters above the box and whisker plots signify statistically different levels between the exposures.

5.3.4 Lysosomal neutral red retention time.

Lysosomal neutral red retention time was measured in the crab haemolymph. The fed crabs exhibited longer neutral red retention times compared with all other exposures ($p < 0.001$ Figure 5.8). The starved crabs that were also exposed to mixed effluent exposure had the lowest mean retention time value of all the exposures.

5.3.5 Metallothionein

Metallothionein concentrations for the different exposures are shown in Figure 5.9. The fed crabs that were exposed to mixed effluent had elevated metallothionein concentrations. However, contrary to previous experiments, the increase of metallothionein concentrations was not significantly greater. In contrast, the starved crabs that were exposed to the mixed effluent had significantly higher metallothionein concentrations than the crabs that were only starved, ($p = 0$).

5.3.6 Linear regression analysis between data sets at the different exposures

Linear regression analyses were performed between total glutathione concentration and total antioxidant scavenging ability of crab gill at the different exposure levels. This was done to establish a relationship between these parameters (Figures 5.10a-d). The statistical analyses of these correlations are displayed in Table 5.2. The control animals did not exhibit a positive relationship between the two parameters, however when the animals were exposed to mixed effluent the relationship became stronger ($R^2 = 70\%$ Figure 5.10b). When the animals were starved the relationship between the two parameters was very weak (Figure 5.10c). When the animals were both starved and exposed to the mixed effluent the relationship was moderately weak but re-established (Figure 5.10d).

Neutral Red retention Time in Crab haemolymph.

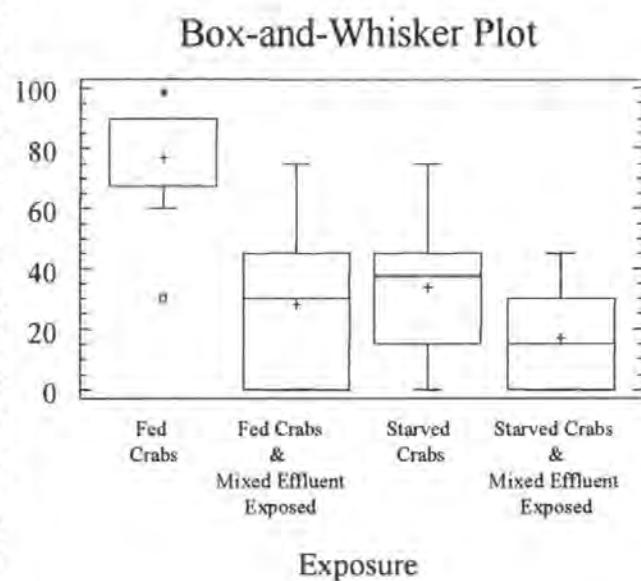


Figure 5.8 Lysosomal neutral red retention time of crab haemolymph from starved and fed crabs unchallenged or exposed to mixed effluent contamination. (Mins).

Metallothionein Levels in Crab Gill Tissue

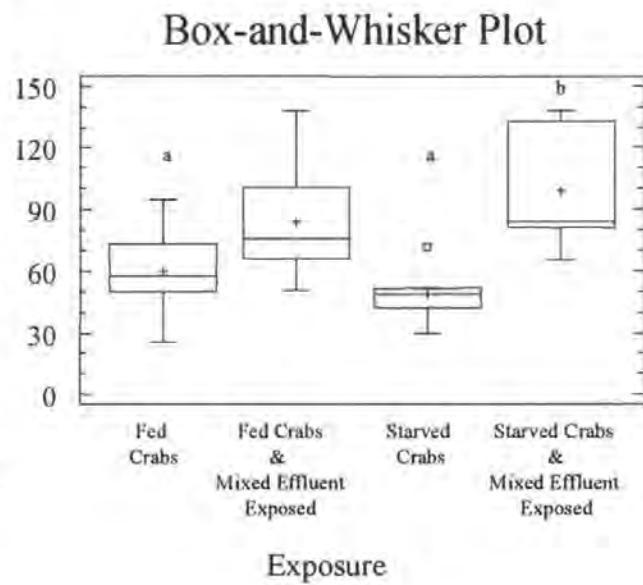
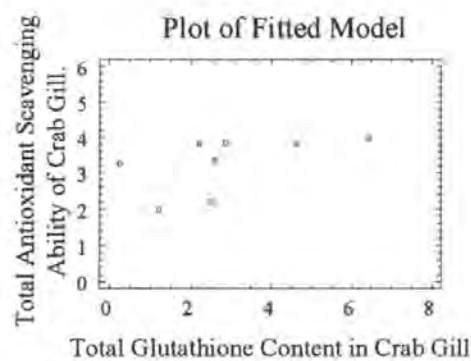
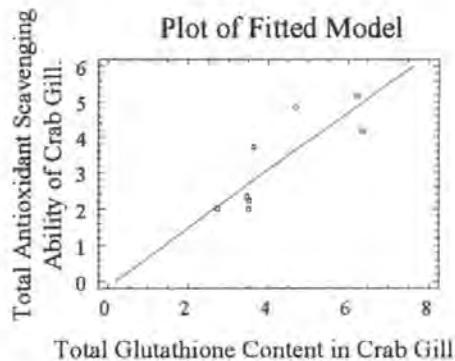


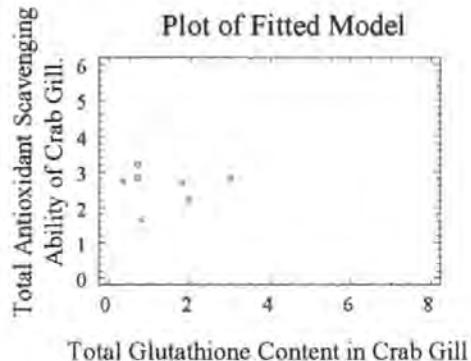
Figure 5.9 Metallothionein levels from crabs fed or starved and unchallenged or exposed to mixed effluent contamination. Metallothionein is measured as μg metallothionein / g wet weight tissue. The different letters above the box and whisker plots signify statistically different levels between the exposures.



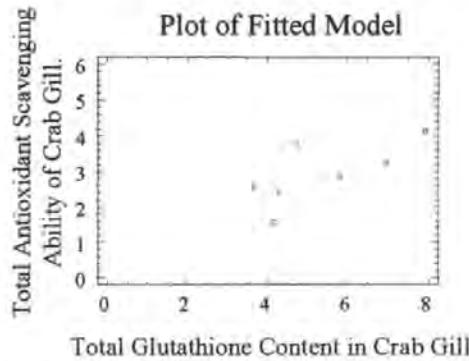
5.10a (Control)



5.10b (Mixed Effluent Exposure)



5.10c (Starved)



5.10d (Starved and Mixed Effluent Exposure)

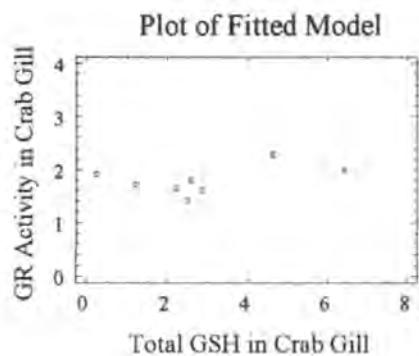
Figures 5.10 a-d Linear regression analysis models between total antioxidant scavenging ability and total glutathione content of gill tissue at different exposures. Total glutathione content is measured as μ moles of glutathione / g protein. Total antioxidant scavenging ability is measured as mmoles of antioxidant scavenging ability / gram of protein. A straight line signifies a significant correlation between parameters.

<i>Correlations Between;</i>	<i>Statistics</i>	<i>Control</i>	<i>Mixed Effluent Exposed</i>	<i>Starved</i>	<i>Starved & Mixed Effluent Exposed</i>
GR Gill / GSH Gill	p-value	0.27	0.64	0.94	0.00*
	Correlation coefficient	0.43	0.19	0.87	0.87
	R-squared.	0.19	0.03	0.00	0.77
TOSCA Gill / GSH Gill	p-value	0.17	0.01*	0.99	0.08
	Correlation coefficient	0.53	0.83	0.003	0.68
	R-squared.	0.28	0.70	0.00	0.47

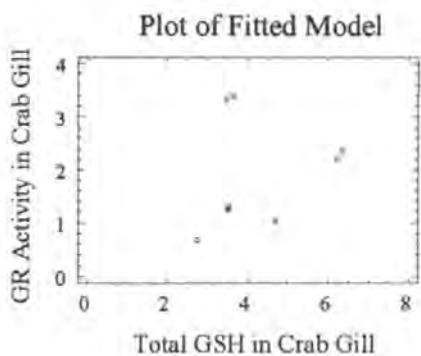
Table 5.2 Statistical results of correlation's made between different exposures and nutritional status and biomarker parameters. The results are made from fitting a linear regression model to describe the relationships between different parameters. The R-squared value describes the percentage of the population that the model corresponds to. The p-value describes the significance of the relationship and the correlation coefficient describes how powerful the relationship is.

A similar pattern of response was also observed for the relationship between total glutathione concentration and total antioxidant scavenging ability in crab haemolymph. However, the relationships were not as strong as for the gill tissue.

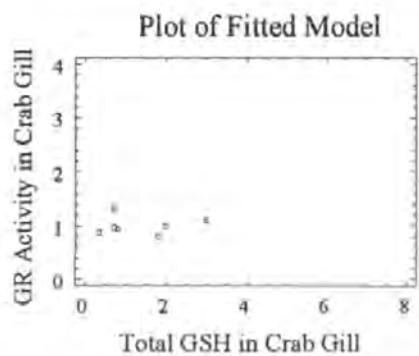
Linear regression analysis was performed between total glutathione content and GR activity in crab gill (Figures 5.11a-d). A positive correlation occurs when the crabs are starved and exposed to mixed effluent (Figure 5.11d & Table 5.2).



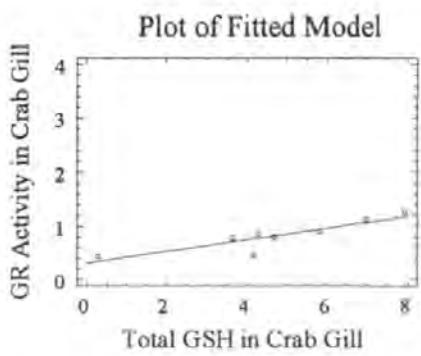
5.11a (Control)



5.11b (Mixed Effluent Exposure)



5.11c (Starved)



5.11d (Starved & Mixed Effluent Exposed)

Figures 5.11a-d Linear regression analysis models between GR activities and total glutathione content of gill tissue at different exposures. Total glutathione content is measured as μ moles of glutathione / g protein. GR activity is measured as μ moles of substrate oxidised / g protein. A straight line signifies a significant correlation between parameters.

Section 5.4

DISCUSSION.

Protein concentrations

Starvation alone did not affect the crab haemolymph protein levels. A significant decrease of haemolymph protein concentrations was reported in fasted *Carcinus maenas* by Uglow *et al.* (1969). An alteration in haemolymph protein constitution was also reported by Busselen (1970). Uglow (1969) achieved his results by repetitively bleeding his crabs throughout the course of his experimentation. He was therefore able to monitor a decreasing of haemolymph protein within each of the crabs. Repetitive bleeding reduced the variability of results and improved observations of statistically significant differences between populations, however the process caused increased stress to the animals which resulted in increased crab mortality (Uglow, 1969). Therefore, this method was not applicable to the present study as repetitive bleeding would have increased stress and may have affected the other biomarker parameters measured.

Crabs that were starved and exposed to the mixed effluent had lower protein status compared to crabs that were only starved. This drop in haemolymph protein levels may be due to protein resources becoming metabolised to produce energy for antioxidant systems to be induced. The starved crabs were not supplemented with exogenous protein sources, so had to rely upon their own protein resources to induce antioxidant defence systems. The haemolymph protein from these crabs may have been metabolised to meet the protein deficit. This could also explain a concomitant increase of total antioxidant scavenging ability in this tissue (see later).

Glutathione concentrations and enzyme activities.

The starved crabs had reduced glutathione concentrations in their gill tissue. Glutathione status also fluctuates in other species depending upon the nutritional status of the animal (see Tables 5.3 & 5.4). In many cases glutathione concentrations are shown to reduce in animals following starvation (Ogasawara *et al.*, 1989; Schimizu & Morita, 1990; Lautermann *et al.*, 1995; Hoffman *et al.*, 1987; Langley & Kelly, 1992). After a starvation period the synthesis of GSH may be reduced due to a reduced intake of amino acids. However, during long fasts or increased metabolic activity, proteins are broken down to fulfil caloric requirements; therefore, considerable amounts of cysteine and methionine are available for GSH synthesis (Rodriguez *et al.*, 1987). The increase in available amino acids can sometimes significantly increase reduced glutathione levels incurred by starvation, (Schinizu & Morita, 1990). In the present study, crabs that were both starved and exposed to pollution had reduced protein status in their haemolymph. This coincided with a concomitant increase of gill glutathione concentrations probably brought on by an increase of available amino acids.

In other species, which have been starved and exposed to chemical stressors, such as the chinchilla (Hoffman *et al.* 1987), the guinea pig (Lautermann *et al.*, 1995) and the rat (Shimizu & Morita, 1990) glutathione status remained lowered and the animals suffer increased toxic effects when compared to the fed controls.

Glutathione status of the haemolymph remained unaltered for all the crab treatments. The glutathione status of haemolymph was also unaffected by seasonality (Chapter 4). These results show that haemolymph glutathione status is not affected by nutritional status.

Fed crabs that were exposed to mixed effluent had reduced gill GST activities. However, this reduction was not significant. The gill GST activities were significantly affected by starvation. The GST activities were reduced by starvation and concomitant exposure to mixed effluent caused the activities to reduce further. Reduced nutritional status is therefore associated with a decline in GST

<i>Author</i>	<i>Animal</i>	<i>Tissue</i>	<i>Starvation Time</i>	<i>Effect of Starvation on Glutathione and Related Enzymes</i>
Ogasawara, <i>et al.</i> (1989)	Rats	Small Intestinal Mucosa	24 Hours	Decreased GSH Increased GP Unchanged GST Unchanged GR
Langley & Kelly, (1992)	Guinea Pigs	Liver	24 Hours	Decreased GSH
		Lung		Decreased GP Unchanged GR Decreased GSH Unchanged GP Unchanged GR
Jung & Henke, (1997)	Rats	Kidney	2-4 Days	Increased GP
		Liver		Increased GST Unchanged GR Decreased GP Unchanged GST Unchanged GR Decreased GSH

Table 5.3 The effect of starvation on the glutathione metabolism in rodents.

The change in glutathione status and enzyme activities are compared to a control population.

<i>Author</i>	<i>Organism</i>	<i>Tissue</i>	<i>Nutritional Amendment</i>	<i>Contaminant</i>	<i>Effect Starvation</i>	<i>of</i>	<i>Effect Contaminant</i>	<i>of</i>	<i>Effect Starvation and Contaminant</i>
Shimizu & Morita, (1990)	Rat	Liver	Starvation for 24hrs	Cadmium	Decreased GSH Unchanged GR Unchanged GP	Decreased GSH Unchanged GR Unchanged GP	Decreased GSH Decreased GP	Decreased GSH Decreased GP	Decreased GSH Decreased GP
Hultberg, (1998)	Rhizobacteria (<i>Pseudomonas fluorescens</i>)		Modified media	Cadmium	Decreased GSH		Decreased GSH		
Lautermann et al. (1995)	Guinea Pig	Cochlear sensory epithelium Liver	Low protein diet	Gentamicin	Decreased GSH	Unchanged GSH	Decreased GSH		
Hoffman et al. (1987)	Chinchilla	Liver	Starved for 48hrs	Ethacrynic acid & Kanamycin	Decreased GSH	Unchanged GSH	Decreased GSH		
Vigano et al. (1993)	Rainbow trout (Oncorhynchus)	Liver	Starved for 21 days	BNF	Decreased GR Decreased GP Unchanged GST	Increased GR Unchanged GP Increased GST	Increased GR Unchanged GP Increased GST		

Table 5.4 The effects of contaminant exposure and altered nutritional status on the glutathione concentrations and glutathione related enzyme activities of different organisms. The altered glutathione status and enzyme activities are compared to a control population.

activities. This may also be the cause of the seasonal variability of crab GST activities. In other studies using different species GST activities have not been affected or have been induced as a result of starvation (Tables 5.3 & 5.4). However, the species of animal, the tissue source of the enzyme and the starvation period are all likely to influence GST activities of these animals.

Previous experiments (Chapter 4) have reported a significant decrease of GST and GR enzyme activities in response to mixed effluent exposure compared to controls. In the present study the observations were not reproduced. These different results may be indicative of a change of season, or due to holding the crabs in the aquarium for a much longer period before the mixed effluent exposure.

The GR activity of gill tissue also decreased in response to starvation. Decreased GST and GR activities as a result of starvation may render the crabs more susceptible to the effects of toxicity. Decreased GR activity is unexpected, as during the winter when the nutritional status of the crab is similar to conditions induced in this experiment, GR activities were higher. In addition, other species have exhibited induced GR activity with starvation (Viganò *et al.* 1993). However reduced GST and GR activities have been reported in the liver of rainbow trout that have been starved for seven days or longer (Blom *et al.* 2000). This effect was thought to be a general way for trout to reduce the metabolic costs of food deprivation and reduced GR was thought to reflect a decrease in the demand for reduced glutathione for both phase II detoxification reactions and oxyradical scavenging caused by reduced metabolic rate (Blom *et al.* 2000). These results may also reflect the metabolic status of the crab during reduced nutritional status.

GR activities may be enhanced during the winter period so that oxidised glutathione can be recycled. This would help to prevent depletion of glutathione due to reduced nutritional status. During the present experiment, the crabs were starved before the GR activity was induced and so glutathione levels declined with decreased nutritional status. An association between GR activities

and total glutathione concentrations has been established during periods of increased stress in crabs. A decline of GR activity may be associated with the decline of glutathione concentrations. Further study is required to establish the mechanisms by which these changes take place.

A positive linear regression analysis between GR and total glutathione levels occurred in the gill tissues of crabs that had been starved and exposed to mixed effluent. This correlation which has also been observed in other experiments (Chapter 4) and may be applied as a measurement of when animals are under severe stress. This correlation suggests that the activity of GR dictates the amount of glutathione that is present within the tissue and not the activities of glutathione synthesising enzymes such as γ -glutamyltranspeptidase. An association between reduced glutathione status and reduced γ -glutamyltranspeptidase activity has been reported for fasting guinea pigs (Langley & Kelly, 1992). This enzyme activity was not measured for crab tissues, however if its activity is also reduced in starved crabs this could account for GR having a greater influence upon the amount of cellular glutathione.

The crabs that were starved and exposed to mixed effluent had lower GP activities compared to the fed crabs that had been exposed to mixed effluent. Starvation prevented GP induction in response to pollution exposure. This could cause crabs to become sensitised to pollution exposure.

It is reported that nutritional status affects the accumulation of xenobiotics within the tissues of *Carcinus maenas*. Copper concentrations increased in the midgut gland and carapace of starved crabs (Scott-Fordsmand & Depledge, 1993), whilst copper concentrations in the haemolymph were depressed and the gill tissue and whole body copper load was increased (Depledge, 1989). An alteration in the accumulation of different xenobiotics within the crab tissues during starvation could also affect the enzyme responses that were measured within them. This has also been observed in other species that suffer reduced nutritional status. In PCB exposed Arctic charr, an increase of PCB levels within the liver of starved fish resulted in a dose-related increase in liver EROD activity (Jørgensen *et al.* 1999). In the rainbow trout the levels of induced cytochrome P-

450-dependent benzo(a)pyrene hydroxylase activities in response to contamination were significantly lower in starved fish compared to fed fish (Andersson *et al.* 1985). The conclusion can be drawn that nutritional status influences the accumulation of xenobiotics in different tissues and this may in turn influence the activities of the detoxification enzymes within them.

Total antioxidant scavenging ability

The starved crabs had diminished haemolymph total antioxidant scavenging ability compared to fed crabs. However, when the starved crabs were simultaneously exposed to mixed effluent, the total antioxidant scavenging ability of the crabs was replenished. The ability to replenish reduced protective antioxidant protein levels when starved crabs are exposed to pollution is advantageous in preventing physiological damage under conditions of low nutritional and metabolic status. The data distributions of the total antioxidant scavenging ability and the glutathione concentrations of crab gill were similar. The two parameters were correlated for gill tissue at each of the different exposure groups. In normal conditions there was no correlation between the two parameters ($R^2=0.28$); however when the crabs were stressed by mixed effluent exposure a positive linear relationship emerged between them ($R^2=0.70$). When the crabs were starved there was no correlation between the two parameters ($R^2=0.00$); however when the crab was starved and mixed effluent exposed the relationship was restored to a less significant degree, ($R^2=0.47$). These results suggest that glutathione contributes significantly to the total antioxidant scavenging ability of gill tissue in fed, mixed effluent exposed crabs. However, when the animal was of low nutritional status its contribution was not as significant. Crabs with reduced nutritional status had reduced antioxidant scavenging ability thus increasing their susceptibility to oxidative damage. Their antioxidant scavenging ability was restored upon exposure to contamination, however until this occurred the crabs were susceptible to increased oxidative stress.

Neutral red retention time

The neutral red retention time decreased in crabs that were exposed to mixed effluent, or starved and exposed to mixed effluent. The lowest mean neutral red retention time was in the crabs that were both starved and exposed to mixed effluent. This proves that starvation effects the physiological health of the crabs and puts them at a disadvantage before they are challenged by mixed effluent exposure. The induction of the total antioxidant scavenging ability may have prevented increased cellular damage in the crab haemolymph.

Metallothionein

The fed crabs that were exposed to mixed effluent had induced metallothionein levels however not to a significant degree. Crabs that were starved and exposed to mixed effluent had significantly higher metallothionein levels compared to the crabs that were only starved. Starvation is shown to increase the levels of metallothionein in rat liver (Shinogi *et al.*, 1999). However, in the present study starvation did not affect metallothionein induction unless the crabs were simultaneously exposed to contamination. However, weight of the crab and the effect of protein concentrations of the gill tissue are shown to affect metallothionein levels (Legras *et al.*, 2000). Despite this a positive correlation has been found between metal accumulation and levels of metallothionein, this gives weight to the potential of using metallothionein levels as a biomarker of metal exposure (Legras *et al.* 2000). The significant increase of metallothionein levels in crabs that are starved and exposed to contamination may have been due to a redistribution of metal loading within the crab tissues in response to starvation (Fordsmand & Depledge, 1993 and Depledge 1989). Depledge *et al.* (1989) reported increased copper loadings in crabs that were starved and exposed to contamination, compared to crabs that were fed and exposed to copper. This supports the theory that increased gill metallothionein levels in starved crabs may be associated with increased metal ion concentrations in these tissues.

Summary

This investigation has shown that enzyme activities are altered in response to nutritional status. The enzyme activities did not always correspond to activities measured during the “winter” exposure (Chapter 4) when the crabs were also of low nutritional status. This may be due to holding the crabs within the aquarium for four weeks prior to experimentation; an abrupt halt to their food intake, which would not have occurred in their natural environment; or the effect of seasonal temperature changes that were not reproduced in this study. Despite this, certain characteristics of crabs suffering high stress levels have remained, e.g. a correlation between GR activity and total glutathione levels and total antioxidant scavenging ability and total glutathione levels. The correlation's made between these parameters when the animals are under severe stress may prove to be useful biomarkers of xenobiotic-induced stress.

This experimentation showed that reduced glutathione and total antioxidant scavenging ability of starved animals are reinstated upon contamination exposure, which could help to prevent increased mortality. However, the initial mixed effluent exposure to these animals may make them more vulnerable than their fed counterparts until their antioxidant mechanisms are induced. Also, reduced activities of GR, GST and GP may increase the possibility of oxidative damage to the gill tissues of starved crabs.

The effect of glutathione depletion on antioxidant enzyme activities and total antioxidant scavenging ability of crabs, (*Carcinus maenas*).

Section 6.1

INTRODUCTION

Many studies have shown that reduced glutathione levels increase the susceptibility of tissues to the toxic effects of pollution (Jones *et al.*, 1995; Cookson & Pentreath, 1996; Kang & Enger, 1988; Kisara *et al.*, 1995; Shimizu *et al.*, 1997; Ringwood *et al.*, 1998; and Masuh *et al.*, 1996). Glutathione is the first line of defence against heavy metal toxicity and its depletion potentiates the toxicity of metals, before metallothionein induction (Singhal *et al.*, 1987; and Chin & Templeton, 1993). Induction of glutathione synthesis decreases the effects of contaminant induced toxicity (Hoffer *et al.*, 1996).

The importance of glutathione in antioxidant pathways is well documented, however a recent paper by Regoli & Winston (1998) states that its contribution towards the total antioxidant scavenging ability of invertebrates is not as great as has been previously assumed. Collectively glutathione, ascorbic acid and uric acid only accounted for 35% of the total antioxidant scavenging ability of marine invertebrates (Regoli & Winston, 1998). In contrast, results from the present study suggest that glutathione makes a greater contribution towards anti-oxidant scavenging ability when animals experience contaminant exposure. This suggests that the conclusions of Regoli & Winston (1998) may not be accurate when invertebrates are highly stressed.

BSO (buthionine sulfoximine) is a potent and specific inhibitor of glutathione synthesis (Griffith, 1982; and Griffith & Meister, 1979). It depletes glutathione synthesis by inhibiting the activity of γ -glutamylcysteine synthetase. Many past experiments using different animal species and cell lines have examined the effect of reducing glutathione status by the administration of BSO. The effects of glutathione depletion have been monitored by establishing lipid peroxidation status (Ringwood *et*

al., 1998; and Palmeira *et al.*, 1995) and the physiological effects of additional toxicity exposure (Kisara, *et al.*, 1995; Masuh *et al.*, 1996; and Singhal, *et al.*, 1987). The conclusions are that glutathione depletion increases physiological damage and increases the effects of toxicity. Some stressors are known to reduce the glutathione status of organisms, for example; starvation (Hultberg, 1998; Hoffman *et al.*, 1987; Gallagher *et al.* 1992; Hum *et al.*, 1990 and Lauterman *et al.*, 1995); seasonality (Viarengo *et al.*, 1991; and Power & Sheehan, 1996; Sheehan & Power 1999); and herbicides such as paraquat (Palmeira *et al.* 1995). Glutathione reduction caused by environmental and anthropogenic stressors can cause animals to become more sensitive to the effects of toxicity.

In the present investigation, glutathione concentrations in the tissues of crabs were depleted using BSO and the crabs were then exposed to high concentrations of mixed effluent. Glutathione related enzyme activities of selected tissues were assayed to monitor the effects of BSO. Also the total antioxidant scavenging ability of the tissues was measured to monitor whether glutathione depletion affects the total antioxidant scavenging ability of the tissues.

In a short preliminary experiment *Daphnia magna* were exposed to BSO via water and simultaneously exposed to cadmium. The mortality of the *Daphnia* was determined to substantiate whether glutathione depletion sensitises *Daphnia magna* to cadmium toxicity.

Section 6.2

MATERIALS AND METHODS.

6.2.1 Preliminary experiment using *Daphnia magna* to establish the toxicity of BSO (0-1.0mM)

The first experiment was designed to establish concentrations of BSO that were toxic to *Daphnia*. 25 juvenile (24 hour old), *Daphnia* were exposed to different levels of D/L - BSO solution; 0; 0.01; 0.05; 0.1; 0.15; and 1.0 mM. The aim was to establish the concentrations at which BSO produced zero mortality. None of the *Daphnia* died at any of the BSO concentration levels. Therefore, 1mM BSO could be used in the chemical exposures without affecting the mortality of the *Daphnia magna*.

50 juvenile *Daphnia* were used at each exposure. The exposures comprised of control; 1mM BSO; 0.5ppm Cd; 0.5ppm Cd & 1mM BSO; 2ppm Cd; and 2ppm Cd & 1mM BSO. *Daphnia* were counted after 12, 18, 21 and 24 hours and their mortality were recorded.

6.2.2 Preliminary exposure of *Carcinus maenas* to BSO

Crabs were either injected with L-BSO into the coxal membrane of their third walking leg (2mmol / kg of crab weight), or exposed to 20 mg L-BSO / l seawater in their exposure medium. Two control groups were used, the first were injected with crab physiological saline (0.5M NaCl, 11mM KCl, 12mM CaCl₂.6H₂O and 45mM Tris/HCl pH 7.4) to establish the stress caused by injection. The second group were kept in filtered seawater. 8 crabs were used at each exposure. 4 crabs were sampled from each exposure after 3 and then 10 days and glutathione concentrations were measured in their haemolymph and gill tissues.

6.2.3 Determination of total antioxidant scavenging ability in glutathione depleted crab tissues

Two groups of crabs had their glutathione levels depleted by an initial injection of 2mmol / kg L-BSO dissolved in physiological saline into the coxal membrane of the third walking leg. Prior to BSO injection a similar volume of haemolymph was removed from the injection point. This ensured that the solution was not rejected from the entry point due to the increase in bodily fluid pressure. Another two groups of control crabs were injected with physiological saline to compensate for the stress imposed by injection. Eight crabs from each of these groups were exposed to mixed effluent or maintained in clean seawater. Maintenance of the crabs is described in Chapter 2.

6.2.4 Statistical Analysis.

The mean and SD have been recorded for the normally distributed data and the median and the minimum and maximum values have been recorded for the non-normally distributed data. Non-normally distributed data were log transformed to reduce variances from the mean and to normalise the distribution of the data. Simple linear regression analysis were performed between GR activity & total glutathione concentrations. Also total antioxidant scavenging ability was examined in relation to glutathione levels for each of the exposure groups.

Section 6.3.

RESULTS.

6.3.1 The effect of BSO on the mortality of *Daphnia magna*

The mortality of *Daphnia* increased with exposure to BSO plus cadmium. The 1mM BSO exposure did not affect mortality of the *Daphnia*. 0.5ppm Cd exposure caused 24% mortality of *Daphnia* after 24 hours. The mortality increased to 60% with simultaneous exposure to 1mM BSO. 2ppm Cd caused 80% mortality after 24 hours. Whereas, 2ppm Cd combined with the additional exposure to BSO increased mortality to 100% (Figure 6.1).

6.3.2 The effect of BSO administration on the glutathione levels of *Carcinus maenas*

Preliminary experiments showed that an initial injection of 2mmol / kg BSO into *Carcinus maenas* was sufficient to reduce glutathione content by approximately 75% in the haemolymph and 95 % in the gill tissues for up to ten days (Figure 6.2). Exposing the crabs to BSO *via* their seawater did not reduce glutathione content in the crabs' tissues. Injecting crabs with BSO was selected as a means of administration, as it was more effective than exposure *via* their tank water.

6.3.3 Glutathione depletion and total antioxidant scavenging ability

The average measurements of glutathione content of the tissues and antioxidant scavenging ability are shown in Tables 6.1 and 6.2 respectively.

BSO injection significantly reduced glutathione content in the crabs' haemolymph and gill tissues ($p = 0.00$). The data is displayed in Figures 6.3 and 6.5 respectively. However, BSO did not reduce glutathione levels in the muscle tissues (Figure 6.7).

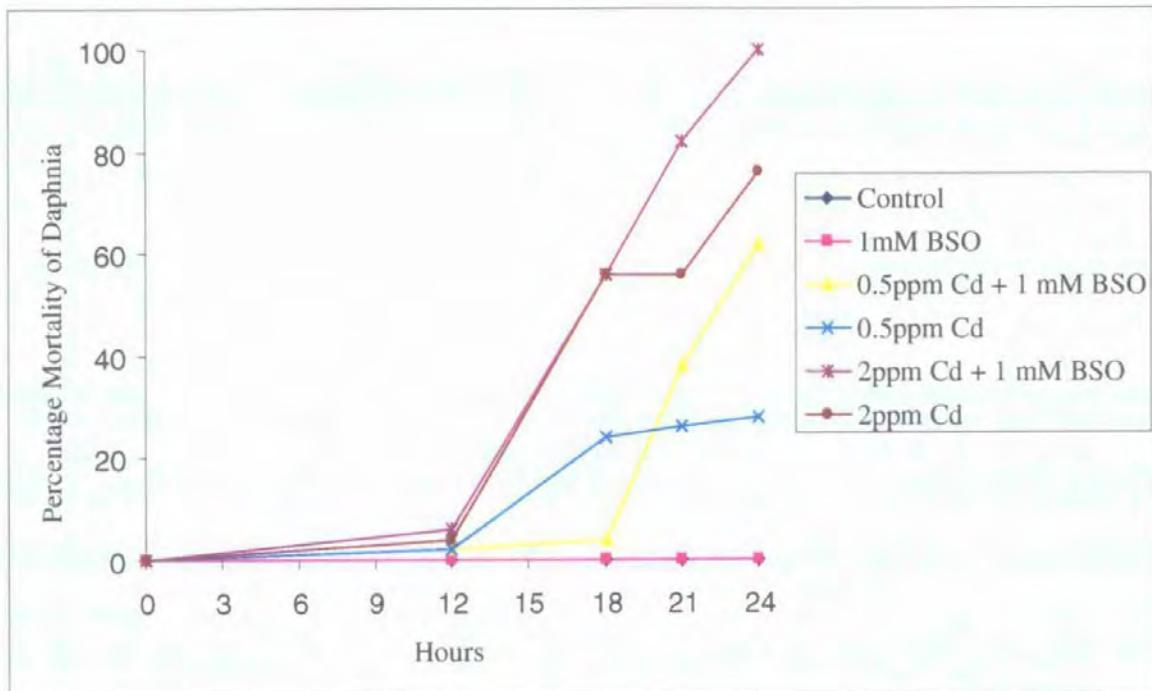


Figure 6.1 The percentage mortality of Daphnia exposed to different concentrations of Cadmium with and without the presence of BSO with respect to time.

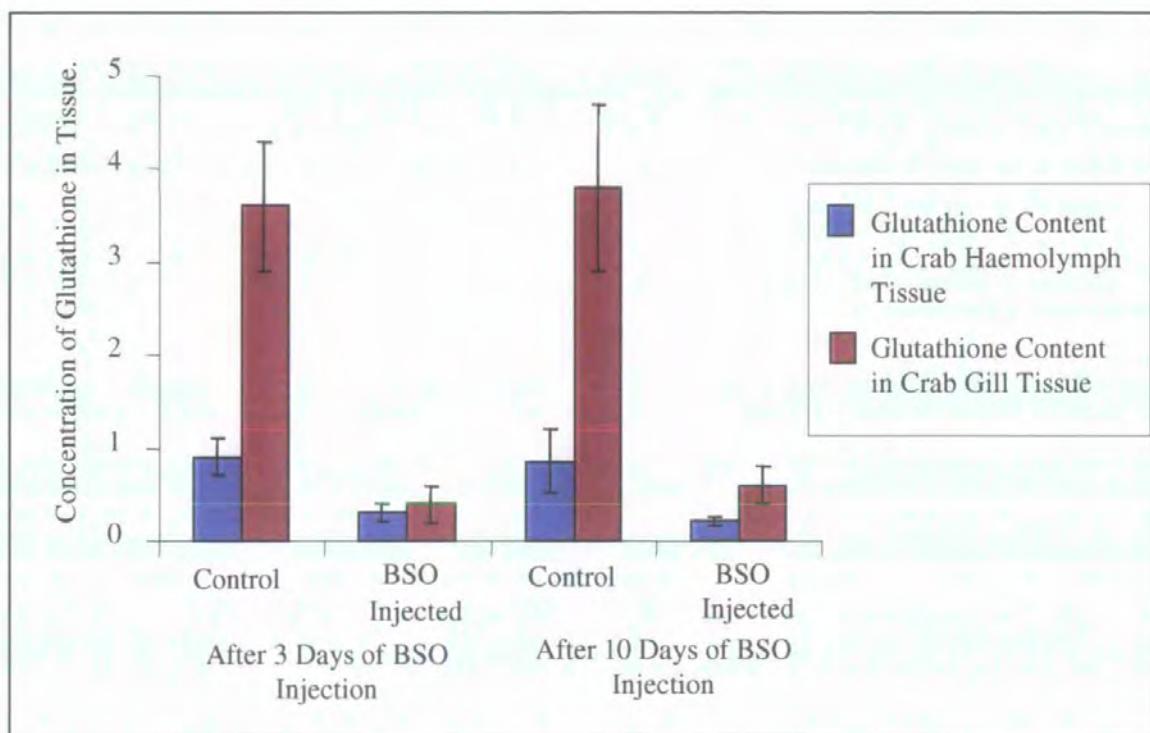


Figure 6.2 Results of a preliminary experiment to establish how effective injection of 2mmol / kg of crab wet weight is in reducing glutathione content of haemolymph and gill tissues. (n=4 for each exposure). Glutathione concentration is measured as μmoles of glutathione / g protein.

Exposure	<i>Control</i>		<i>BSO</i>		<i>Mixed Effluent</i>		<i>BSO & Mixed Effluent.</i>	
Statistics for non-normally distributed data	median	Min/ Max	median	Min/ Max	median	Min/ Max	median	Min/ Max
Glutathione Content of Haemolymph Tissue	0.85 2.0	0.24 0.45	0.23 0.45	0.04 0.45	1.54 4.5	0.26 4.5	0.28 4.5	0.05 0.6
Glutathione Content of Gill Tissue	3.59 26.39	2.46 0.69	0.24 0.69	0.15 0.69	11.98 30.65	5.49 30.65	0.08 30.65	0.019 1.11
Glutathione Content of Muscle Tissue	73.53 1677	36 266	32.85 266	18 266	74.77 187	27 187	48.96 187	13 235

Table 6.1 Glutathione concentrations in different tissue types of the crab after BSO exposure and / or mixed effluent exposure. The total glutathione content is measured as μ moles of glutathione / g protein. The data is not normally distributed, the BSO diminishes glutathione content and hence reduces the variation of the glutathione content data points from the mean in these exposures.

Exposure	<i>Control</i>		<i>BSO</i>		<i>Mixed Effluent</i>		<i>BSO & Mixed Effluent.</i>	
Statistics for non-normally distributed data.	median	Min/ Max	median	Min/ Max	median	Min/ Max	median	Min/ Max
Haemolymph Tissue.	0.45 0.57	0.33 0.65	0.5 0.65	0.37 0.65	0.57 1.7	0.4 1.7	0.4 1.7	0.27 0.6
Gill Tissue.	2.6 7.9	1.5 5.9	4.1 5.9	1.4 5.9	4.3 49	1.3 49	3.0 49	2.0 49
Muscle Tissue.	3.2 5.6	2.2 38.9	4.5 38.9	2.2 38.9	2.5 13	2.7 13	5.8 13	2 13

Table 6.2 Total antioxidant scavenging abilities of crab tissues at different exposures of BSO treatments and / or mixed effluent exposure. Total antioxidant scavenging abilities are measured as μ moles of antioxidant scavenging ability / g protein.

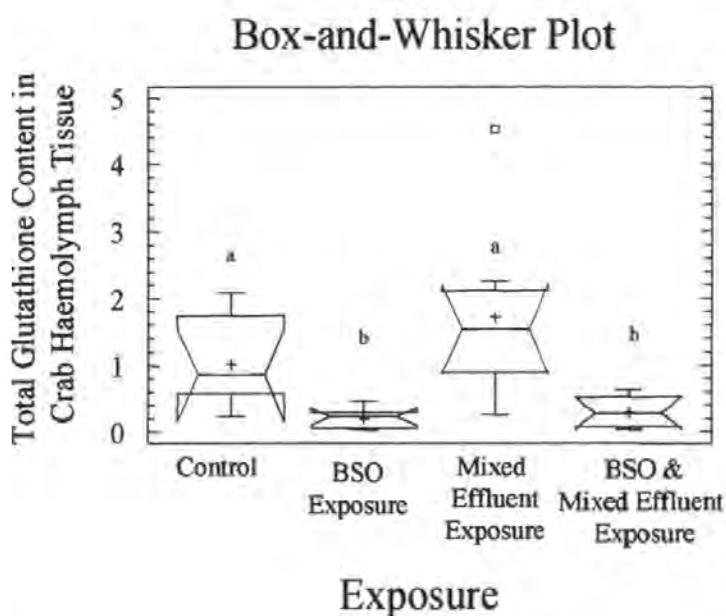


Figure 6.3. Total glutathione content of haemolymph from controls, BSO and or mixed effluent exposed crabs. The total glutathione content is measured as μ moles of glutathione / gram of protein. (The exposures which have the same letter placed above the box and whisker plots are not significantly different to one another.)

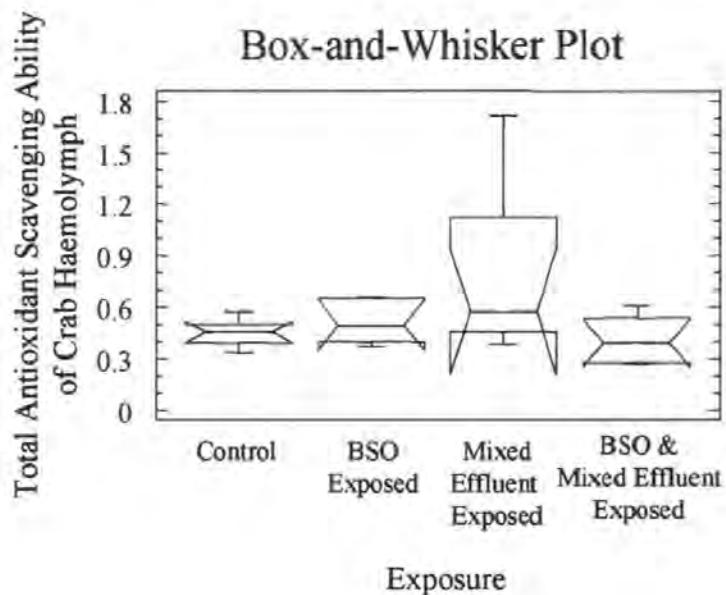


Figure 6.4 Total antioxidant scavenging ability of haemolymph tissue from BSO and or mixed effluent exposed crabs. Units are mmoles of antioxidant scavenging ability / gram protein.

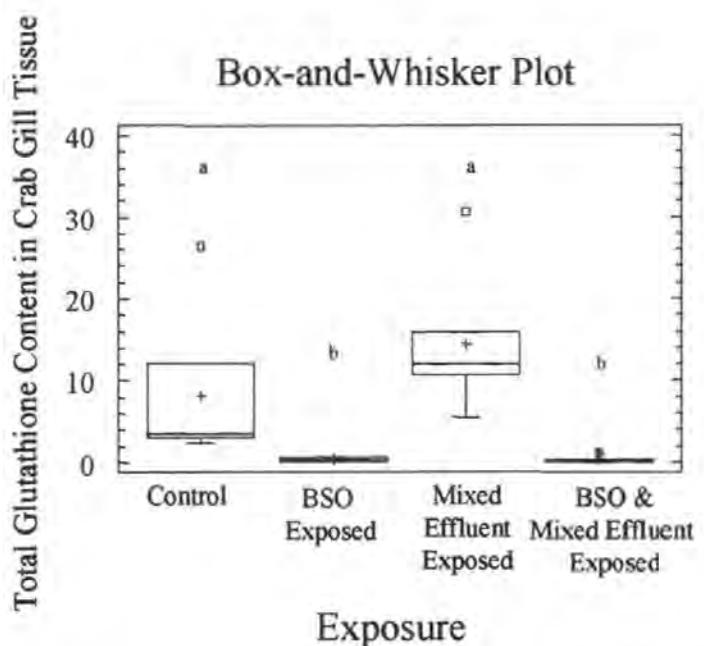


Figure 6.5 Total glutathione content of gill tissue from controls, BSO and or mixed effluent exposed crabs. Units are μ moles of glutathione / gram of protein. The statistically different exposures are denoted with different lower case letters.

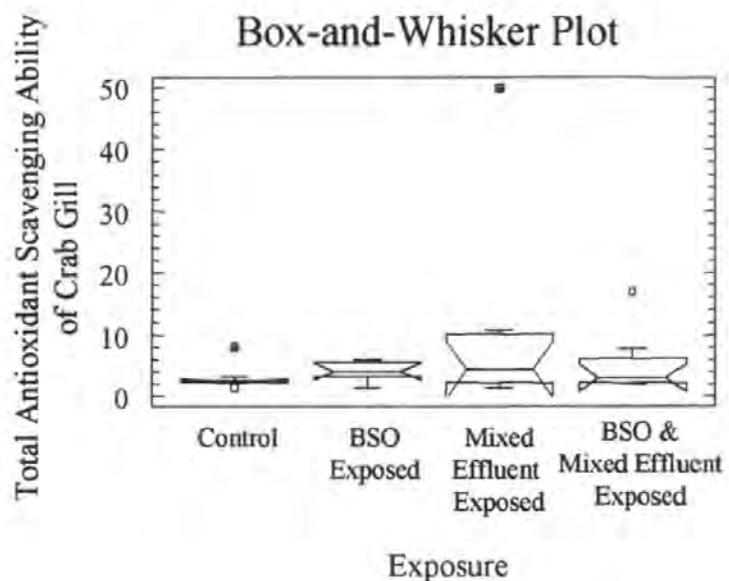


Figure 6.6 Total antioxidant scavenging ability of gill tissue from controls, BSO and or mixed effluent exposed crabs. Units are mmoles of total antioxidant scavenging ability / gram of protein.

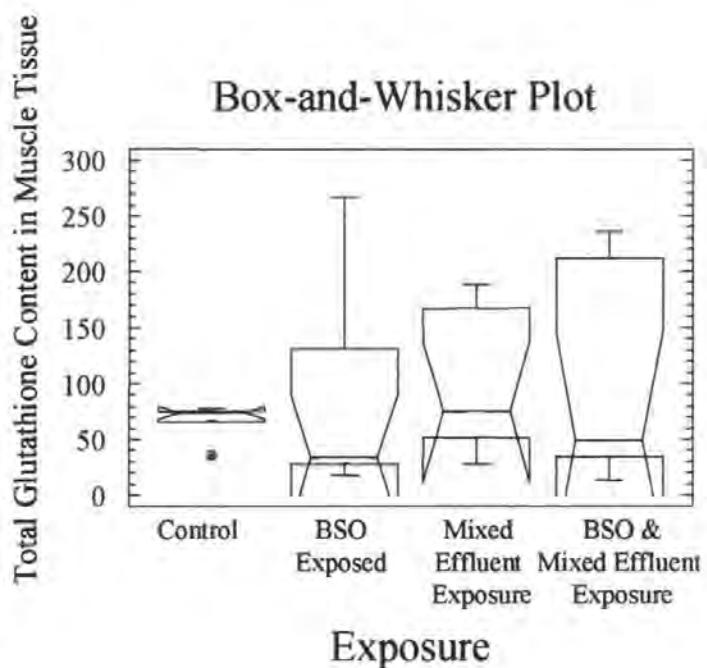


Figure 6.7 Total glutathione content of muscle tissue from controls, BSO and or mixed effluent exposed crabs. Units are μ moles of glutathione / gram of protein.

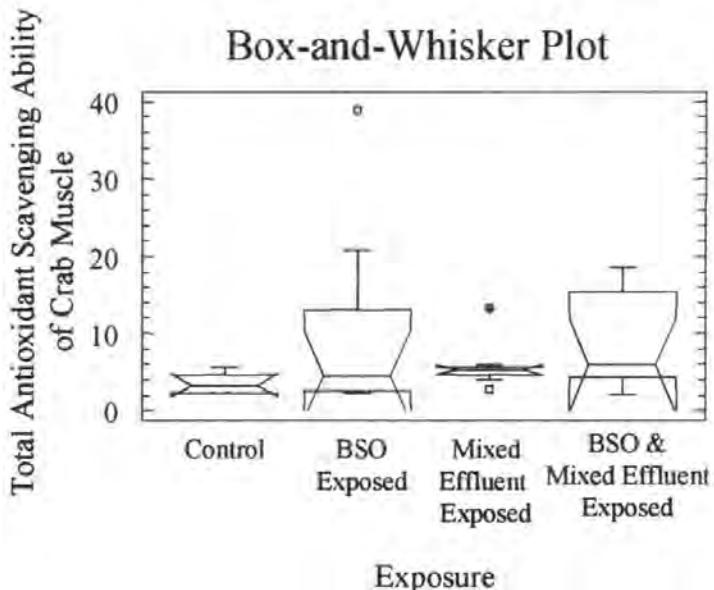


Figure 6.8 Total antioxidant scavenging ability of muscle tissue from controls, BSO and or mixed effluent exposed crabs. Units are mmoles of total antioxidant scavenging ability / gram of protein.

The values of the total antioxidant scavenging ability of the crab tissues after BSO injection and or mixed effluent exposure are displayed in Figures 6.4, 6.6 and 6.8. Glutathione depletion did not affect the total antioxidant scavenging ability of any of these tissues.

BSO and mixed effluent altered the data distributions of the total antioxidant scavenging abilities for the different groups by increasing the variability of the results from the mean value. BSO exposure increased the variability of the total antioxidant scavenging ability by increasing the spread of data values in the direction of the positive y-axis. Mixed effluent exposure further increased the data distribution in a positive direction. These results are indicative of a biological response to toxicity.

6.3.4 Glutathione depletion and antioxidant enzyme activities

The effect of BSO on the enzyme activities of crab tissues is shown in Table 6.3. BSO did not induce changes in most of the enzyme activities measured; however, in most instances it increased the variance of data points from the mean. GST activity was the only enzyme that was significantly affected. GST activity of gill tissue was increased in response to BSO exposure (Figure 6.9), whereas exposure to mixed effluent decreased it. GST may have increased for conjugation reactions of GSH with BSO metabolites that had been formed. This resulted in a significant difference of GST activities between the BSO exposure and the mixed effluent exposure, $P = 0.05$. The net effect of both BSO and mixed effluent exposure caused a wide distribution in the data, which spanned all activities measured for the other groups.

6.3.5 Metallothionein levels and neutral red retention time of glutathione depleted tissues

The different exposures affected the metallothionein levels of gill tissue and the neutral red retention time of haemolymph (Figures 6.10 and 6.11 respectively). The lysosomal neutral red

<i>Exposure</i>	<i>Control</i>		<i>BSO</i>		<i>Mixed Effluent</i>		<i>BSO & Mixed Effluent.</i>	
	median	Min/ Max	median	Min/ Max	median	Min/ Max	median	Min/ Max
Statistics for non-normally distributed data								
GST Activity in Haemolymph Tissue	3.58	2.46 8.71	6.25	3.94 11.36	5.21	1.66 12.95	3.66	2.3 8.66
GST Activity in Gill Tissue	0.30	0.0 0.4	0.72	0.08 1.2	0.17	0.0 0.4	0.4	0.16 1.59
GST Activity in Muscle Tissue	0.02	0.0 0.041	0.024	0.005 0.146	0.042	0.006 0.23	0.036	0.019 0.101
GR Activity in Haemolymph Tissue	0.69	0.17 1.14	1.13	0.22 3.01	1.23	0.71 1.88	0.68	0.04 0.95
GR Activity in Muscle Tissue	8.9	5.67 37.4	19	7.16 124.7	14	4.8 96	20.7	7.5 42.4
GP Activity in Haemolymph Tissue	0.75	0.59 1.2	0.8	0.57 1.49	0.7	0.35 2.4	0.67	0.42 1.82
Statistics for normally distributed data.	mean	SD	mean	SD	mean	SD	mean	SD
GP Activity in Gill Tissue	0.14	± 0.08	0.17	± 0.06	0.1	± 0.1	0.15	± 0.1
Metallothionein Content in Gill Tissue	32.8	± 8.15	30.2	± 13.2	70.8	± 35	67	± 40
GR Activity in Gill Tissue	8.5	± 5.23	13.9	± 5.8	7.4	± 3.7	10.7	± 8.9
Neutral Red Retention Time.	56	± 22	33	± 11	28	± 19	15	± 11

Table 6.3 Biomarker values from crabs exposed to BSO and / or mixed effluent exposure. The mean and standard deviations are shown for the normally distributed data and the median and min/max values are shown for the non-normally distributed data. GST activity is measured as μ moles of substrate metabolised / min / g protein. GR activity is measured as nmoles of substrate metabolised / min / g protein. GP activity is measured as μ moles of substrate metabolised / min / g protein. [Metallothionein] is measured as μ g / g wet weight tissue. Neutral red retention time is measured in minutes.

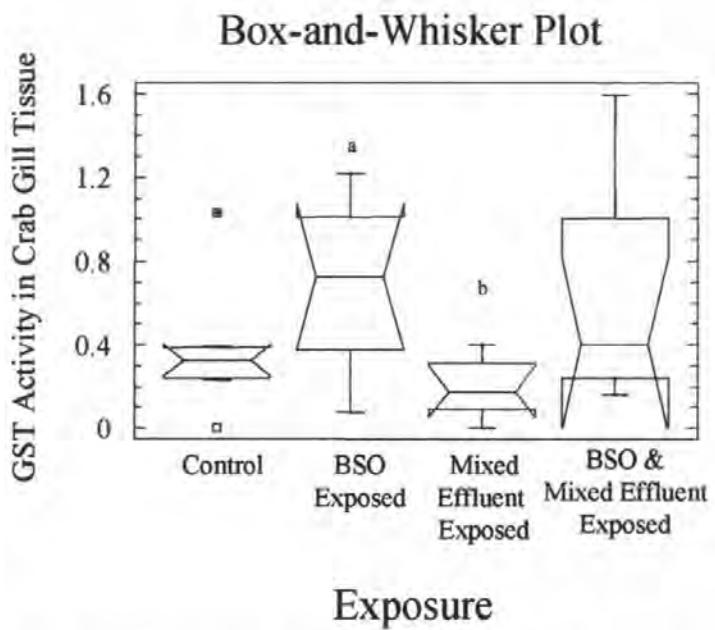


Figure 6.9 The GST activity of crab gill tissue from control, BSO and or mixed effluent exposed crabs. GST is measured as μ moles of substrate oxidised / min / g of protein.

Different letters above the box and whisker plots denote statistically significant differences.

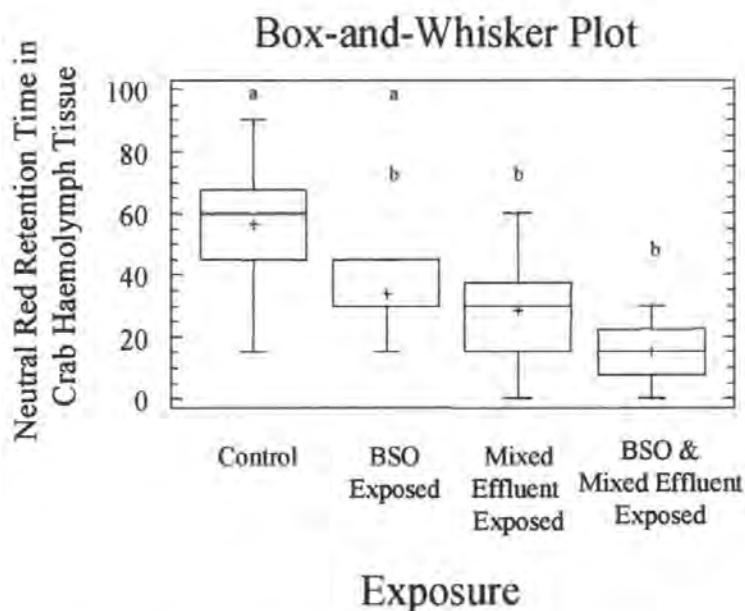


Figure 6.10 Lysosomal neutral red retention time of crab haemolymph from control, BSO and or mixed effluent exposed crabs. Neutral red retention time is measured in minutes.

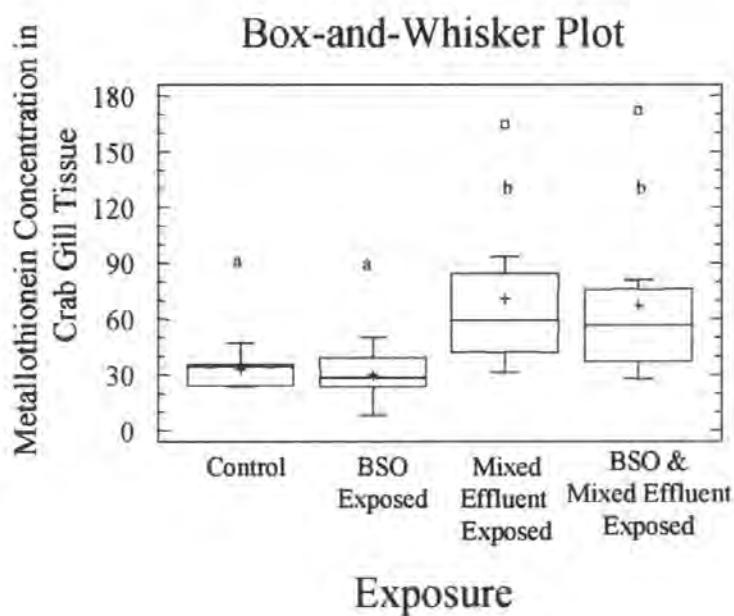
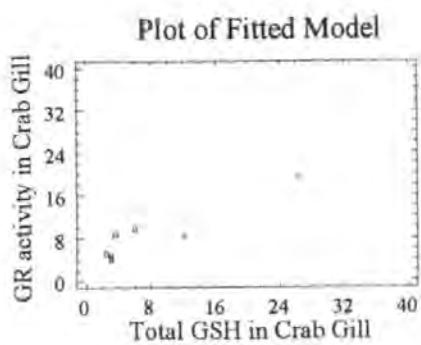


Figure 6.11 Metallothionein concentrations of gill from control, BSO and or mixed effluent exposed crabs. The metallothionein concentrations are measured in $\mu\text{g} / \text{g}$ wet weight tissue.

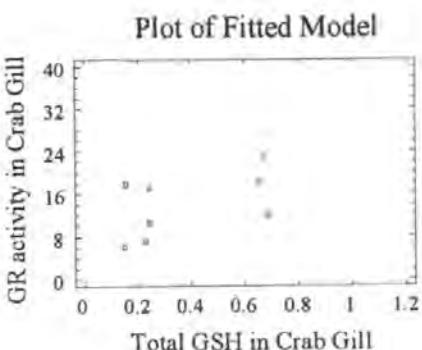
Different letters above the box and whisker boxes denote statistically significant differences.

<i>Correlations Between:</i>	<i>Statistics</i>	<i>Control</i>	<i>BSO Exposed</i>	<i>Mixed Effluent Exposed</i>	<i>BSO & Mixed Effluent Exposed</i>
GSH gill / GR gill	p-value	0.25	0.19	0.00*	0.89
	correlation coefficient	0.55	0.5	0.98	-0.06
	R-squared	0.31	0.26	0.97	0.00
GSH muscle / GR muscle	p-value	0.57	0.01*	0.02	0.00*
	correlation coefficient	0.29	0.82	0.76	0.95
	R-squared	0.00	0.68	0.58	0.91
TOSCA gill / GSH gill	p-value	0.31	0.22	0.00*	0.61
	correlation coefficient	0.49	0.48	0.99	-0.23
	R-squared	0.24	0.23	0.99	0.00
TOSCA muscle / GSH muscle	p-value	0.36	0.00*	0.07	0.00*
	correlation coefficient	0.45	0.97	0.66	0.93
	R-squared	0.21	0.94	0.44	0.88

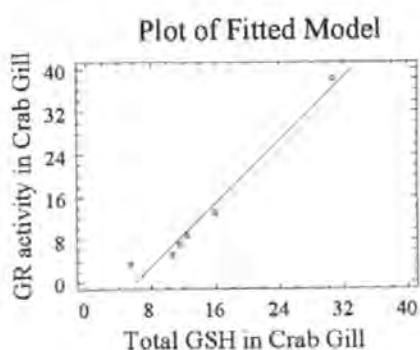
Table 6.4 Statistical results of linear regression analysis made from different exposures and biomarker parameters. * indicates significant linear regression. The R-squared value describes the percentage of the population that the model corresponds to. The p-value describes the significance of the relationship and the correlation coefficient describes how powerful the relationship is.



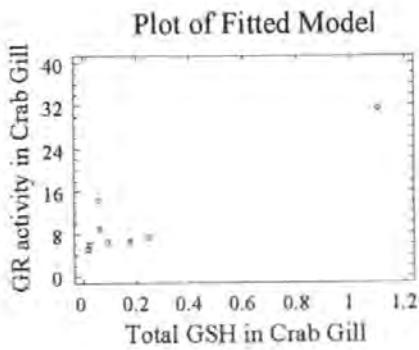
6.12a (control)



6.12b (BSO Exposure)

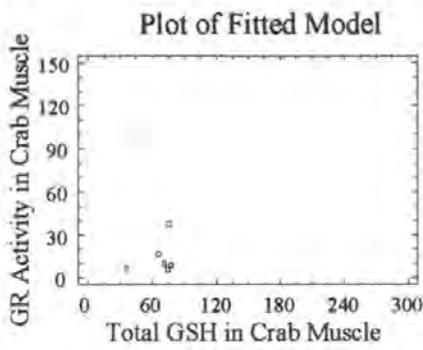


6.12c (Mixed Effluent Exposure)

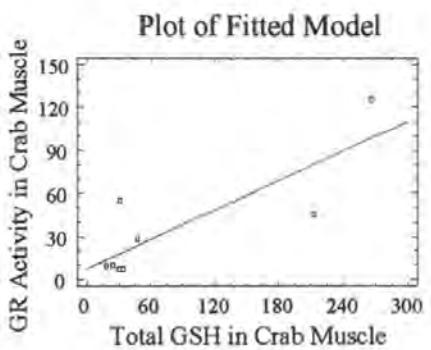


6.12d (BSO & Mixed Effluent Exposure)

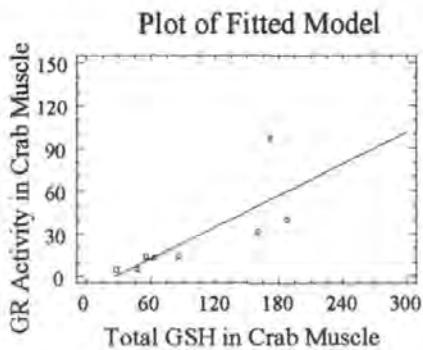
Figures 6.12 a-b Linear regression models between total glutathione content and glutathione reductase activity of crab gill tissue at the different exposures. Total glutathione is measured as μ moles of glutathione / g protein. Glutathione reductase activity is measured in μ moles / min / g protein. A straight line donates significant linear regressions.



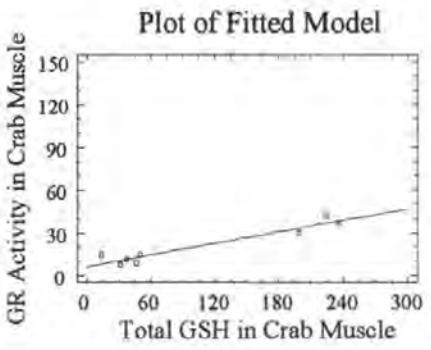
6.13a (Control)



6.13b (BSO Exposure)



6.13c (Mixed Effluent Exposure)



6.13d (BSO & Mixed Effluent Exposure)

Figures 6.13 a-d Linear regression models between total glutathione content and glutathione reductase activity of crab muscle tissue at the different exposures. Total glutathione is measured as μ moles of glutathione / g protein. Glutathione reductase activity is measured in μ moles / min / g protein.

Significant linear regressions are denoted by a straight line.

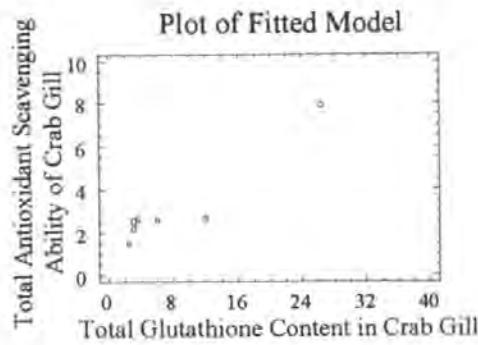
retention times of haemocytes significantly decreased in the two groups of crabs that were exposed to mixed effluent, $p < 0.001$ (Figure 6.10). Metallothionein concentrations increased in gill tissue in response to the mixed effluent exposures, $p < 0.01$ (Figure 6.11).

6.3.6 Linear regression model statistics

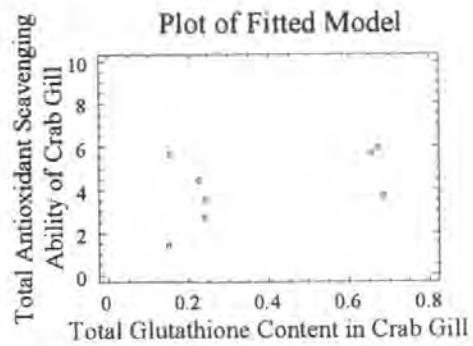
Statistical analyses of linear regression models performed between different parameters are shown in Table 6.4. A positive linear correlation existed between GR and total glutathione concentrations in crab gill and muscle tissues with some treatments (Table 6.4). Linear correlations also existed between total antioxidant scavenging ability and total glutathione concentrations (Table 6.4).

Great care had to be taken when analysing the linear correlation data for the different parameters. The number of data points at each exposure was relatively small, so some points that were numerically high influenced the data set such that a positive correlation appeared where in fact one did not exist between the remaining points. The studentised residual test plots the studentised residual against the predicted value (Sokal and Rohlf, 1995). This test allows statistical determination of influential points that may affect interpretation of the linear regression models. This test was applied in the following analysis.

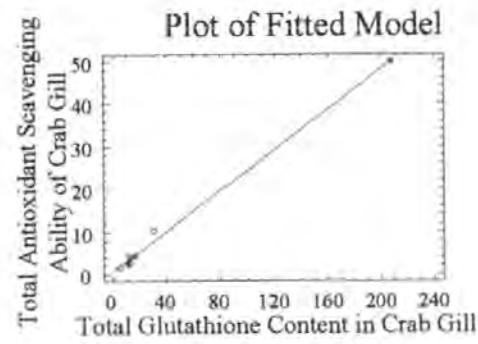
In evaluation of the statistics for Figure 6.12d, (the BSO injected and the mixed effluent exposed crabs), the linear regression model for all data points between GR activity and GSH concentration gave a statistical analysis of p -value = 0.00 and R^2 value = 85%. When the most influential point of the analysis was removed, (the point with the highest GR activity and GSH concentration), there was no relationship between the remaining data points. The results of the latter analysis were included in Table 6.4 as it shows a true representation of the whole data set.



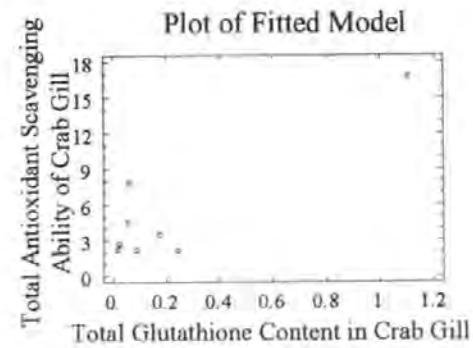
6.14a (control)



6.14b (BSO Exposure)

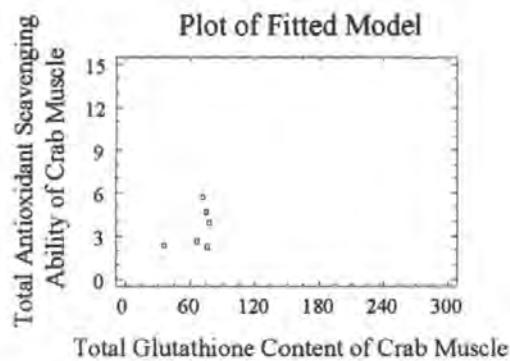


6.14c (Mixed Effluent Exposure)

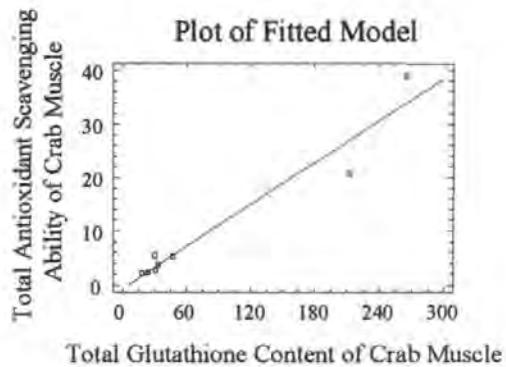


6.14d (BSO & Mixed Effluent Exposure)

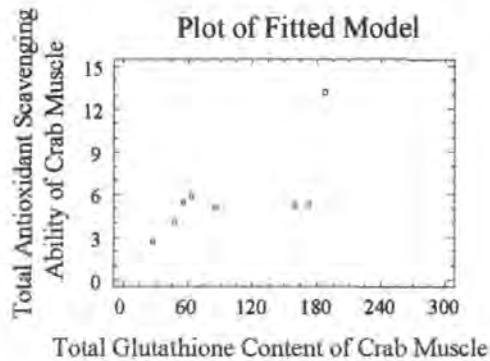
Figures 6.14 a-d Linear regression models made between total antioxidant scavenging ability and total glutathione content of crab gill tissue at the different exposures. Total glutathione is measured as μ moles of glutathione / g protein. Total antioxidant scavenging ability is measured as mmoles of antioxidant scavenging ability / gram of protein. A straight line denotes a significant linear regression.



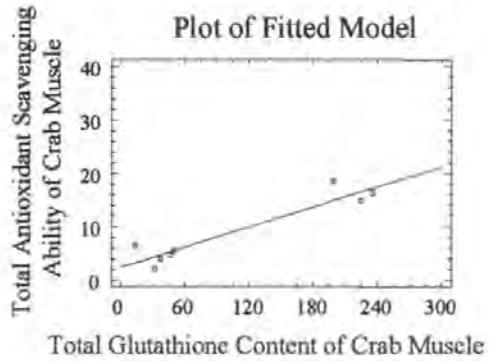
6.15a (control)



6.15b (BSO Exposure)



6.15c (Mixed Effluent Exposure)



6.15d (BSO & Mixed Effluent Exposure)

Figures 6.15a-d Linear regression models between total antioxidant scavenging ability and total glutathione content of crab muscle tissue at the different exposures. Total glutathione is measured as μ moles of glutathione / g protein. Total antioxidant scavenging ability is measured as mmoles of antioxidant scavenging ability / gram of protein.

Significant regression analysis is denoted by a straight line.

The linear regression analysis of GR activity and total glutathione concentrations for muscle tissue posed similar problems. When all data points were used for the analysis of Figure 6.13c (crabs that had been exposed to mixed effluent alone) the relationship represented 58% of the population. When the unusual residual was removed (the point with the highest GR value), the linear regression correlation increased to represent 97% of the population. The statistics using all the data points have been recorded in Table 6.4 even though the relationship is less strong. Including the unusual residual in the regression analysis did not significantly alter interpretation of the data. The recorded statistics are considered to be a fair representation of the data obtained and the lower R^2 value is still accepted as signifying a good relationship between the two parameters.

A significant relationship also existed between total antioxidant scavenging ability and total glutathione content of muscle and gill tissues. When all data points obtained for the gill tissues of the control animals were considered the linear regression model described 89% of the data (Figure 6.14a). When the point which shows the highest total antioxidant scavenging ability and glutathione concentration was removed from the data, the R-squared value fell to 24% and the p-value showed that a relationship did not exist. For this reason the relationship between the two parameters for the control crabs was not considered significant.

Section 6.4

DISCUSSION.

The effect of cadmium toxicity on glutathione depleted Daphnia magna

The aim of this experiment was as a preliminary study to establish whether glutathione depletion, in invertebrates, increases the animals' sensitivity to contamination. *Daphnia magna* are commonly used for toxicological purposes in both acute and chronic tests of chemical toxicity (Adema, 1978). Cadmium is toxic to *Daphnia magna*, it reduces the growth rate, possibly by the reduction in food intake, and also affects the swimming velocity of the animal (Baillieul and Blust, 1999).

In this experiment the *Daphnia* were too small for it to be possible to obtain enough tissue samples to measure glutathione concentrations. Therefore, this experiment was used as a study to monitor the effects of BSO and cadmium toxicity on mortality.

BSO did not affect the mortality of the *Daphnia*. However, BSO increased the sensitivity of *Daphnia* to cadmium toxicity. The mechanism by which BSO most likely sensitised *Daphnia* to cadmium is by diminishing their glutathione levels. Glutathione is shown to be the first line of defence against cadmium toxicity and its reduction by BSO has shown to potentiate the effects of cadmium toxicity (Singhal *et al.*, 1987). Topical application of the butyl ester derived from BSO, applied to the exoskeleton of *Triatoma infestans* nymph V stage, increased their mortality in response to pesticides by decreasing glutathione levels (Masuh *et al.*, 1996). (The butyl ester of BSO was administered, as it is less ionic than BSO and can hence penetrate the waxy exoskeleton of the larvae). In addition, the LC-50 of cadmium toxicity in a cell line of human tumour cells decreased in cells which were exposed to BSO (Kang & Enger, 1988). These investigations prove that BSO exposure can augment the effects of chemical toxicity by decreasing glutathione concentrations.

Glutathione depletion and total antioxidant scavenging ability

The initial aim of this study was to assess whether glutathione depletion significantly reduces total antioxidant scavenging ability of crab tissues. Results showed that BSO injection resulted in reduced glutathione status of gill tissue and haemolymph, however it had no effect on the total antioxidant scavenging ability of these tissues. Correlations were made between total antioxidant scavenging ability and glutathione levels and these results complied with observations made in previous studies; glutathione positively correlated with total antioxidant scavenging ability in the gill tissue of crabs that were exposed to mixed effluent (Figure 6.14c). This result suggests that under these circumstances glutathione plays a significant role in antioxidant mechanisms of gill tissue. When crabs were treated with BSO prior to mixed effluent exposure the relationship between total antioxidant scavenging ability and glutathione levels was destroyed (Figure 6.14d). However, as this did not affect the overall antioxidant scavenging ability of the gill tissue, other antioxidant mechanisms may have been induced to compensate for diminished glutathione status; such as ascorbic acid, carotenoids and uric acid. These results show how the crabs has the ability to initiate alternative antioxidant mechanisms when glutathione levels are depleted. This ability may help to prevent physiological damage that can result from reduced glutathione status and the presence of toxic compounds and may account for the crabs ability to populate highly industrialised areas.

BSO had no effect on either the glutathione content or the total antioxidant scavenging ability of the muscle tissue. However, BSO was shown to increase the variability of the data distribution from the mean. Increased variability of biomarker responses is a typical toxicological response to contamination exposure (Depledge & Lundebye, 1996; Aargaard & Depledge, 1993 and Forbes & Depledge, 1996). BSO had a toxicological effect on the muscle tissues, however this effect was not shown to be significant in the parameters that were measured. If BSO is detoxified within the crab muscle tissue this could account for it not having an affect on glutathione status. The BSO may

have been metabolised such that it was no longer a potent inhibitor of λ -glutamylcysteinyl synthetase.

Glutathione and total antioxidant scavenging ability correlated positively in the muscle of crabs that were exposed to BSO and the BSO plus mixed effluent. This correlation has also been associated with induced stress (Chapters 4 & 5). Therefore it can be assumed that BSO causes induced stress in the muscle tissues. It is necessary to understand more thoroughly which types of toxins cause biological effects in different tissues so that eventually these effects can be related to a particular chemical species. For example, metal toxicity is associated with increased metallothionein levels (Pedersen *et al.*, 1997) and the inhibition of butyryl cholinesterase is associated with organophosphorus (Mineau, 1991).

The Effect of Glutathione Depletion upon Enzyme Activities

In most instances glutathione depletion did not affect enzyme activities. The exception was GST activity, which was induced in gill tissues in response to BSO exposure, whereas mixed effluent exposure decreased it. This result shows that BSO and mixed effluent toxicity have an antagonistic effect upon GST activities. When crabs are exposed to a combination of the two, the distribution of GST activity increased and the bias was in a positive direction. Hence, BSO had a stronger inducing effect on it than mixed effluent has an inhibiting effect. This experiment provides evidence that BSO induced GST activity in crab gill tissues. It is useful to understand the type of chemicals that induce or suppress enzyme activities as it can help to identify the types of toxins that are causing these effects *in situ*.

There were no other significant differences among exposure levels for the other enzyme activity measurements. In most instances the presence of BSO and mixed effluent induced large variations from the mean enzyme activities, but did not significantly increase or decrease them. GST activity

of crab muscle increased in response to mixed effluent and BSO exposure, but not to significant levels. GP and GR activities did not significantly differentiate between the exposure groups.

Linear regression analysis of GR activity and total glutathione content also showed a significant relationship in some of the exposure groups. A strong relationship between the two parameters occurred in the gill tissue of crabs exposed to mixed effluent (6.12c). The presence of BSO in addition to mixed effluent exposure destroyed the relationship by depleting glutathione levels (6.12d). A correlation between the two parameters also occurred in the muscle tissue from crabs at all exposures apart from the control group (Figures 6.13 b&d). These observations indicate that BSO exposure and mixed effluent induces a toxicological stress response in the muscle tissues, whilst only mixed effluent exposure induces toxicological responses from the gill tissues, (Chapter 5 & 6). The accumulative effects of both BSO and mixed effluent caused the most significant correlation between GR and GSH in the muscle tissue. This result indicates that with increasing stress exposure the relationship between these two parameters becomes more profound. Therefore this correlation could be a useful biomarker of crabs suffering from highly levels of pollution exposure.

Lysosomal Neutral Red Retention Time

BSO alone did not significantly affect lysosomal destabilisation rates in crabs. However, in past experiments using oysters lysosomal destabilisation rates increased upon BSO exposure (Ringwood *et al.*, 1998). Mixed effluent exposure decreased the neutral red retention time and additional BSO reduced it further.

The crab lysosomes in the haemocytes were therefore physiologically impaired due to mixed effluent exposure. The influence of BSO did not incur as much damage to the physiology of the haemocytes as the presence of mixed effluent exposure. However, crabs that were exposed to both

BSO and mixed effluent had the lowest lysosomal neutral red retention times. In laboratory based experiments, cellular glutathione levels can briefly rise and can bind to metal directly, providing the cells with resistance to the toxic substances until metallothioneins are induced (Wahba *et al.*, 1990). When glutathione levels are reduced animals do not have this initial protective mechanism. Therefore crabs that have reduced glutathione status are more likely to suffer the initial toxicity of metal exposure until metallothioneins are induced. Other species that have been exposed to anthropogenic waste have reduced glutathione status and this has shown to be correlated with increased lysosomal destabilisation rates (Ringwood *et al.*, 1999).

Metallothionein

Mixed effluent exposure induced metallothionein in the crab gill tissue. The reduction of glutathione levels in the gill tissue did not affect the induction of metallothionein despite previous reports of strong relations between them (Kawata & Suzuki, 1983; Freedman *et al.*, 1989; and Da Costa Ferreira *et al.*, 1993). BSO treatment also had no influence upon the induction of metallothionein in other organisms (Ringwood *et al.* 1998, Ochi, 1988 and Shimizu *et al.* 1997). These results show that not all tissues have mechanisms that incorporate glutathione into the induction of metallothionein. However, a lot of evidence suggests that glutathione depletion increases sensitivity to metal toxicity, until the time at which metallothionein is induced (Singhal *et al.* 1987, Ochi, 1988, Chan & Cherian, 1992). This is because glutathione acts as a first line of defence against toxicity until metallothionein induction. In some instances metallothionein levels are found to increase in animals that are starved (Shinogi *et al.*, 1999). Glutathione status has also been reported to be reduced in starved animals (Hultberg, 1998; Hoffman *et al.*, 1988 and Lauterman *et al.*, 1995). This may be a protective function of some animals to prevent the possibility of short-term metal toxicity when glutathione levels are low. Further experimentation is needed to verify this.

Summary

The results of this study suggest that different toxicants are distributed and detoxified in different tissue types. The mixed effluent induced responses principally in the gill and haemolymph, whereas BSO induced responses in the muscle tissues.

Glutathione depletion did not significantly affect the total antioxidant scavenging ability of the crab tissues. Results suggest that this is not because glutathione has an insignificant function in antioxidant scavenging mechanisms, but other antioxidants are stimulated and compensate for the absence of glutathione. However, glutathione depletion still resulted in increased lysosomal destabilisation of crab haemolymph in response to mixed effluent exposure. This suggests that compensatory antioxidant scavenging mechanisms that are induced in its absence may not be as efficient in preventing physiological damage from exposure to pollution.

**Field application of antioxidant enzyme analysis at the Tees Estuary
and a novel analysis of biomarker data.**

Section 7.1

INTRODUCTION.

Biomarkers have been applied extensively in laboratory experiments to study the biological effects of contamination (Tables 1.4 & 1.5). Laboratory based experiments are particularly effective in establishing cause and effect mechanisms, however they do not realistically reflect responses that may occur in the complex and fluctuating natural environment. There are several reviews of the usefulness of biomarkers in risk assessment (Decaprio, 1997; and Lange & Lange, 1997) and in human health assessment (Timbrell *et al.*, 1996). Before this becomes routine practice it is necessary to establish whether biomarkers are successful in highlighting areas of contamination when applied *in situ*.

Animals are exposed to a variety of natural stressors in their environment, including, salinity changes, hypoxia, age, sex, thermal stress and predation. These factors together with phenotypic and ontogenetic differences in susceptibility to stress result in a high degree of variability of biomarker responses (Depledge & Lundebye, 1996; and Forbes & Depledge, 1996). Consequently the data collected in field trials may be more difficult to interpret than data from laboratory based studies. To address this problem attempts have been made to see if biomarker responses can be analysed collectively rather than singularly in order to gain a balanced overview of the results. For example, Van der Oost *et al.* (1998) developed a “biotransformation index” (BTI) in which the activities of Phase I and Phase II enzymes are expressed as a function of one another. The highest BTI values were associated with the most contaminated sites (Van der Oost *et al.*, 1998). Although this is a

useful way of assessing the activation of several antioxidant enzymes, additional physiological data cannot be incorporated with it.

The data collected in this experiment was processed using the computer program Plymouth Routines in Marine Environmental Research software, (PRIMER). The program uses multi-dimensional scaling (MDS) techniques and is applied in this instance to analyse complex biomarker data. Previously, the PRIMER technique has been applied to the statistical analysis of community data (Tapp *et al.*, 1993; Olsgard & Gray, 1995; Hall *et al.*, 1996 and Olsgaard *et al.*, 1997). However, more recently MDS has also been applied in an aquatic biomarker study using fish (Machala *et al.*, 1997) and to a terrestrial field trial of voles (Fairbrother *et al.*, 1998). MDS analysis is beneficial in that all types of biomarker data may be considered simultaneously and there is potential for correlating biomarker responses with the chemical profile of the environment in which they are measured. MDS provides a spatial arrangement of the results and allows contaminated sites that are similar, in respect of the biomarker responses produced at them, to be grouped together.

The present study was carried out at the Tees Estuary in the Northeast of England. The Tees sites represented a well-established toxicity gradient. Crabs were caught on site from the Tees sites and are referred to from now on as the 'indigenous crabs'. A second set of crabs were caught from the Southwest of Britain and taken to the Tees estuary where they were deployed in cages. This populations of crabs are referred to as the 'deployed crabs'. In addition, control sites were selected, one in the North East of Britain, (Robin Hoods Bay) and two others in South West of Britain, (the River Avon and the River Erme). The deployed animals were collected from the River Avon and deployed at all the sites. Also a control group were returned to the River Avon from the North East in order to assess the effect that transportation had upon the biomarker responses.

The deployed and indigenous animals were sacrificed as described in the methods section and GP, GST, GR activities and metallothionein levels were established for the gill tissues. In addition, GP,

GST activities and lysosomal neutral red retention time were measured in the haemolymph. Biomarker responses were analysed individually in the first instance and then subsequently using MDS techniques to gain an overview of all the results.

Section 7.2

MATERIALS AND METHODS.

7.2.1 Experimental design.

Crabs (*Carcinus maenas*) were caught in the south west of Britain (see Section 2.1) and deployed at selected sites in the south west and north east of Britain (See Figure 7.1 for the selected sites). Where possible crabs from these sites were also caught. Biomarker responses were compared in both the “deployed” and “indigenous” sets of animals and the results compared using multivariate analysis techniques.

7.2.2 Sampling and Deployment.

Green inter-moult males of carapace width between 5 and 7 cm were selected. Crabs used for the deployment experiment were caught from Bantham on the River Avon. They were transported to the Tees estuary in buckets held within large containers filled with ice to keep them cool. The crabs were covered with tissue paper to prevent them from becoming too stressed. The crabs were fed to satiation with squid prior to deployment and fed weekly for the rest of the deployment time. A group of crabs that had travelled to the North were transported back to the Avon and deployed there to assess the effect that transportation had upon the biomarker responses.

The deployed animals were held intertidally for two weeks in large stainless steel mesh cages. The cages were compartmentalised into eight sections. A crab was held in each separate compartment and provided with a terracotta flowerpot for shelter. The lid was made from Perspex and contained holes large enough to allow the animals to be fed. Each cage had four legs that extended from its base in order to wedge it securely into the estuary mud. The cages were positioned to be accessible for 1.5 hours either side of low tide.

7.2.3 Biomarker analyses.

The crabs were sacrificed as described (Section 2.1). GP and GST analyses were established for both the gill tissue and haemolymph. GR activity and metallothionein levels were measured in gill tissue. Lysosomal neutral red retention times were determined in haemocytes.

7.2.4 Choice of field sites

This study was performed near the mouth of the Tees estuary on the Northeast coast of England. The Tees was chosen for this study as it is a highly industrialised area and includes Teesport, the third busiest port in England (Office for National Statistics). For over 50 years, untreated sewage and industrial effluents have given rise to problems of deoxygenation and fish toxicity (Brady *et al.*, 1983). Since the 1970's there have been restrictions on pollution loads into the estuary. However, it remains highly polluted, and comprises extensive petrochemical storage and production facilities and a nuclear power station. An improvement in the benthic fauna has been observed from 1979 to 1990 (Tapp *et al.*, 1993) but, the area is still subject to localised accidental spills in addition to effluent discharge. For example, in 1998 there was a localised hydrochloric acid spill from ICI Wilton, which caused distress to the local fauna (Wedderburn *et al.*, 2000).

Crabs were collected from a relatively clean site on the River Avon to use in the deployment experiments. Three control sites were selected, two in the south west of Britain, and one in the north east of England, where chemical and biological water quality were graded a or b (very good or good, respectively) by the Environment Agency (1995 and 1996a).

7.2.5 Description of the sites.

The sites chosen for experimentation lay along a presumed pollution gradient along the length of the estuary (Figure 7.1). Crabs from the River Avon at the south west of England were sampled at both sides of the mouth of the estuary, at the Royal National Lifeboat Institute at South Gare (NZ 555 275) and at the breakwater at North Gare (NZ 543 286). Crabs were also deployed at the South Gare site but not at North Gare as there were no secure places to attach the cage. Other crabs were deployed in the Seal Sands nature reserve by Greatham Creek (NZ 522 255), where indigenous crabs were also sampled, and opposite Hartlepool power Station (NZ 537 263). The final deployment site was opposite Dab Holm Gut, on a site owned by Tees Storage Ltd (NZ 543 243), no indigenous populations could be caught from this site.

Control sites were at Robin Hoods Bay (NZ 958 049) on the east Yorkshire coast in the north east of England, at Bantham (SX 663 441) on the River Avon estuary and also at Mothercombe (SX 620 477) on the River Erme estuary in Devon. Crabs were deployed and sampled from all of these sites.

7.2.6 Tees estuary sites

The Gares.

At North Gare, crabs were sampled beyond the estuary on the North Side of the pier. No animals were deployed here as bathers frequent the area and there were no secure areas available for the crab cages. At South Gare the animals were deployed and indigenous animals caught off the lifeboat station. The crab cage was securely chained to the lifeboat station on the northern side of the headland within the estuary.

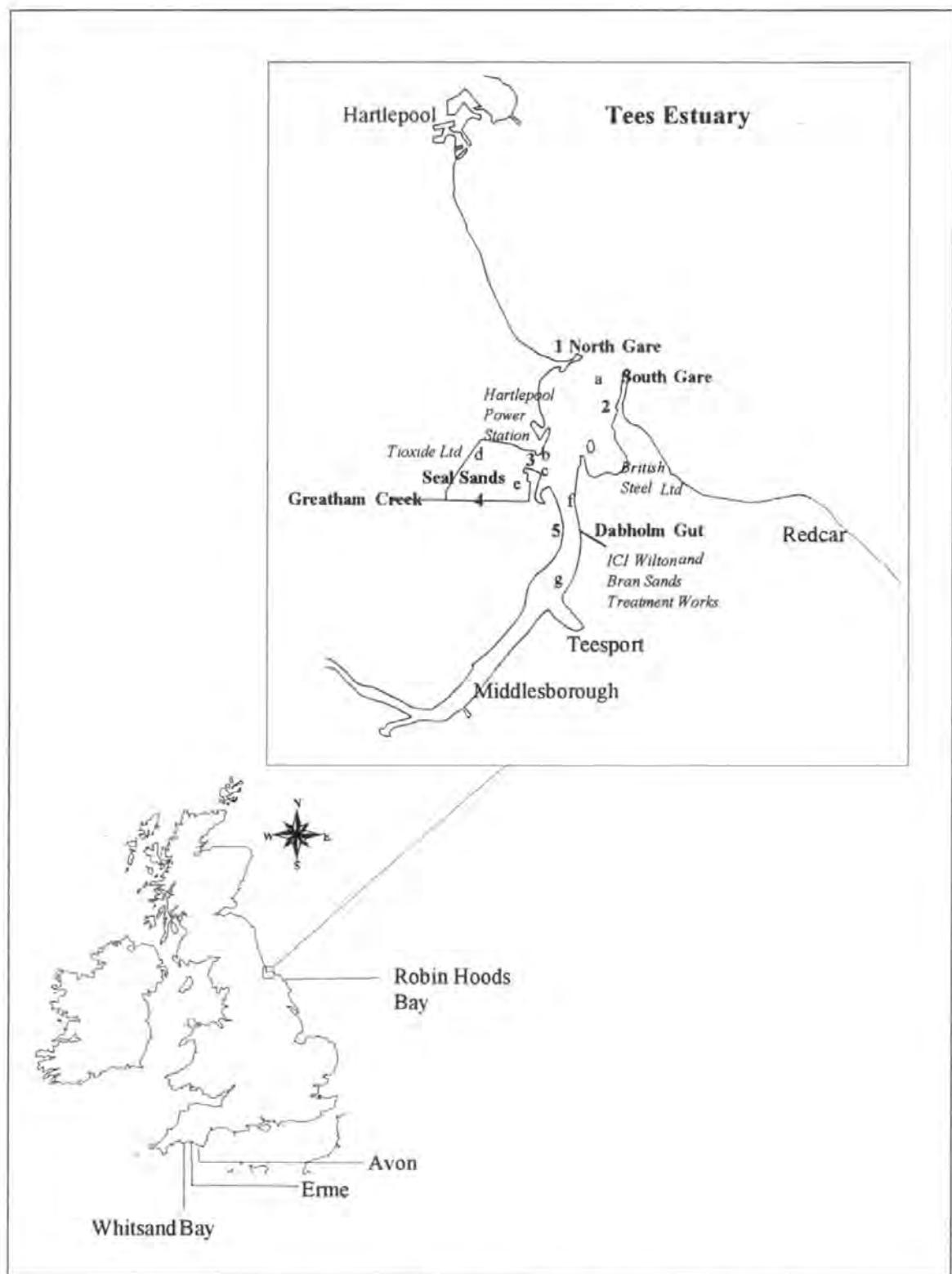


Figure 7.1 Map of sampling and deployment sites, with detailed map of the Tees Estuary. The numbered sites are the deployment sites upon the Tees estuary and the lettered sites are those used by the Environmental Agency. Major industrial sites are labelled in italics and the deployment areas are named in bold print. Field sites: **1**, North Gare, **2**, South Gare, **3** Hartlepool Power Station, **4**, Greatham Creek, **5**, Tees Storage. Environmental Agency Sampling Sites: a, the Gares, b Phillips Approach North, c, Phillips Approach South, d, Seaton Snook light North, e, Tees control Site middle, f, Redcar jetty, g, Smiths docks.

Hartlepool Power Station and Greatham Creek sites.

Both of these sites are situated upon Seal Sands. Seal Sands is a mud flat and nature reserve situated on the Northern side of the Estuary where Greatham Creek enters the River Tees. Within the locality are two main industrial activities, Tioxide UK Ltd and Hartlepool Power station (a nuclear power plant). Tioxide UK Ltd has one outflow pipe which enters the Seaton channel, which runs through Seal Sands. Details of the effluent entering the area from the outflow pipe surrounding the experimental period are shown in Table 7.1. The crabs were deployed and indigenous populations caught where Greatham Creek enters Seal Sands; this area is referred to as the Greatham Creek site. Crabs were also deployed opposite Hartlepool Power Station on a reach of land that separates the Seal Sands mud flats from the main river. This site is referred to as the Hartlepool Power Station site. Access to Seal Sands nature reserve was granted by English Nature.

Tees storage site

This site was on the northern side of the river opposite Dabholm Gut, which is where the main outflow from the Bran Sands Treatment Plant (the treatment works for ICI Wilton Chemicals and Polymer Ltd) enters the Tees. Access to the area was granted by Tees Storage Limited. Details of the effluent that was expelled from the outflow pipe of Bran Sands Treatment Plant during the experimental period are given in Table 7.2. No indigenous populations were caught from this site despite attempts to do so. One of the crabs deployed at this site died during the exposure period.

Control sites

The control sites were judged to be pristine according to water quality reports (Environment Agency, 1996a). They are all in rural areas away from areas of industrial activity, although as in all rivers in the South of England, there are sewage treatment works on both the River Erme and the

Date	Sus solids	Acidity $\text{CaCO}_3 \text{mg l}^{-1}$	Fe	V	Cr	Cd	Hg	Zn	Pb	Cu	TOC mg l^{-1}
30/04/97	38	435	3600	85.4	<1	<0.1	0.06	102	1.23	<1	3.35
29/05/97	25	1205	2840	133	125	0.436	0.04	103	2.9	76.7	2.76
27/06/97	18	130	4400	125	64.1	0.877	0.06	555	10	65.1	4.92
13/07/97	54	138	4400	32.8	31.4	0.393	0.1	113	5.46	67	7.55
29/08/97	118		1250	14.3	54	0.628	0.2	219	12.7	52.7	

Table 7.1 Tioxide

Date	Sus solids	BOD Mg l^{-1}	CHCl_3	Cd	Hg	Cu	Cr	Zn	phenol	CN mg l^{-1}	Benzene	Toluene	Xylene ortho	Xylene Meta+ Para	1,2 dichloro ethane	Pb	$\text{NH}_3\text{-N}$ mg l^{-1}	COD mg l^{-1}	Mtx %	Flow m^3hr^{-1}
05/06/97	174	500	28	0.458	<0.3	53.3	12.7	73	220	0.053	9300	12000	3900	7900	81	Na	6.08	1735	0.4	Na
06/06/97	94	581	16	0.869	0.98	93.5	208	263	280	0.015	340	200	120	480	<15	Na	4.36	1045	8	Na
17/06/96	228	409	37	0.704	<0.3	36.8	17.8	121		<0.01	660	320	200	480	<15	Na	4.55	750	Na	Na
07/07/97	190	370	31	0.64	0.39	53.8	12.8	116	620	0.1	347	188	113	320	<15	Na	2.03	683	8	3625
10/07/97	168	338	25	0.863	0.44	65.1	36.1	152	470	0.19	239	177	150	369	<15	45.7	0.8	795	Na	3781
13/07/97	360	291	590	2.14	2.78	230	70.9	734	502	0.014	760	250	140	420	<15	Na	3.05	940	6	6996
18/07/97	230	552	30	0.72	<0.3	128	68.7	138	<0.5	0.012	Na	Na	Na	Na	<10	Na	1.34	1075	8	5475
28/07/97	84	343	Na	1	2.96	98.8	52.4	286	21	0.215	Na	Na	Na	Na	Na	Na	5.83	880	12	4814
31/07/97	200	502	Na	0.716	<0.3	259	23.3	166	<0.5	0.43	Na	Na	Na	Na	Na	Na	5.16	965	6	5410
06/08/97	186	485	31	0.785	<0.3	52.7	25.6	165	<0.5	0.025	440	140	76	230	<15	Na	4.26	1070	1	Na
16/08/97	252	407	78	965		124	13.1	128	420	0.037	550	Na	Na	<15	Na	2.88	810	6	Na	
20/08/97	292	397	38	0.484	<0.3	75.9	17.1	103	300	0.03	1900	Na	Na	<15	Na	3.67	984	17	Na	
29/08/97	228	413	56	0.675	0.17	121	83	241	<0.5	0.015	310	120	110	310	<15	51.3	2.36	871	Na	Na
30/09/97	162	416	na	0.443	0.06	59.5	12.3	150	<2	0.03	na	na	na	na	na	21.6	3.9	792	na	na

Table 7.2 Bran Sands treatment works.

Table 7.1 and 7.2 Contaminants entering the Tees, from the outfall pipes of Tioxide Ltd and the Bran Sands treatment works, in the months surrounding the field trial. All concentrations are in $\mu\text{g l}^{-1}$ unless otherwise stated; sus solids, suspended solids; TOC, total organic carbon; BOD, biochemical oxygen demand; COD, chemical oxygen demand; Mtx, Microtox 15 minute EC-50 value given as percentage of outflow.

River Avon. However, the River Avon hosts a successful oyster farm at the top of the estuary that supplies the south west of Britain, hence an excellent water quality is essential for this to be successful. Crabs were deployed at the mouth of the River Avon and the River Erme. Unfortunately, the crabs deployed at the River Erme escaped due to vandalism of the cage.

7.2.7 Water quality at the field sites

Water samples at the field sites were not analysed as it was decided that a satisfactory assessment of the water could not be attained. The water would have had to be sampled at different times of the tidal period and it would be necessary to choose a selection of pollutants for analyses out of the many contaminants and by-products that are present in the water. The water quality of the sites was gauged from the substantial amount of information available from literature and the Environment Agency. The aim of the experiment was to establish whether biomarkers could successfully distinguish between contaminated and polluted sites and not to correlate the biomarker responses with specific components of the effluents that are present.

The Environmental Agency regularly samples water from sites along the Tees estuary. Water samples are also obtained from the control sites, though, less frequently. The values of contaminant concentrations obtained by the Environmental Agency, which were sampled along the Tees Estuary between May and October, are shown in Table 7.3. Table 7.3 shows that large fluctuations in contaminant concentrations occur over time in the Tees estuary. The Environmental Agency sampling sites are shown in Figure 7.1.

Most contaminants appear to be entering the estuary at Dabholm Gut, and Smiths docks, which is opposite the Tees Storage site. In particular, large amounts of ammonia are present in this area as well as high concentrations of Zn. After these two sites the Greatham Creek sites show the next

Sampling Site	Pb	Cd	Cu	Zn	Hg	NH ₃ -N	TBT	C ₂ Cl ₄	CHCl ₃	1,2-DCE	DO(%)
Gares											
The Tees at the Gares sites	<2.5-3.61	<0.25-0.508	<0.5-2.02	5.72-28.6	<0.02-0.02	<20-70	<0.005	<0.1	<0.1-0.18	<0.1	49-82
Greatham Creek											
Philips approach North	<2.5-2.58	<0.25-0.121	2.02-3.02	11.6-15.8	<0.02	na	na	na	na	na	35
Philips Approach South	2.5-53.6	0.147-<0.25	0.647-5.33	7.9-39.3	0.02	<20-85	0.008	0.14	0.17-0.43	0.24	43-113
Seaton Nook light north	<2.5-2.39	<0.25-0.164	1.17-2.43	9.17-26.3	<0.02	na	na	na	na	na	48-62
Tees control site middle	2-5.07	0.116-<0.25	1.22-3.19	7.84-23.7	0.06	<20-130	na	na	<0.1-0.99	na	21-78
u/s Dabholm gut											
Redcar jetty	2.57-6.85	0.094-0.311	1.42-7.41	10.1-163	<0.02-0.02	50-2480	<0.005-0.012	<0.1-0.68	<0.1-2.1	<0.1	25-110
d/s Dabholm Gut											
Smiths docks	3.3-21.6	0.105-0.503	2.36-7.41	23.7-64.8	<0.02-0.07	2300-13400	<0.005-0.013	<0.1-0.19	1.0-1.6	<0.01-0.77	68-109

Table 7.3 The range of contaminant concentrations found by the Environment Agency in water samples taken along the Tees estuary between May and October 1997. Measurements are $\mu\text{g l}^{-1}$. 2-4 samples were taken at each site during this period. DO, dissolved oxygen as a percentage; na-not analysed.

highest contaminant loading, whereas the sites at the Gares were the least contaminated. In summary there was clearly a pollution gradient down the estuary extending towards the sea.

The contaminants, which entered the estuary from the Tioxide outfall and the Bran Sands treatment works, are shown in Tables 7.1 and 7.2. The results are from the Environment Agency analyses of samples taken at the mouth of the outflow pipes.

The Environmental Agency carries out general assessments of the water quality of British rivers and streams. The rivers entering the Tees were graded C, satisfactory (Environment Agency, 1996b). The Environment Agency also assesses beaches, the beach at the Avon met the guideline coliform and faecal streptococci standards; the Erme, Robin Hoods Bay and North Gare met the lower mandatory coliform standards, (Environmental Agency, 1995).

Further information regarding the Tees Estuary water quality can be obtained from literature. Results of the contaminants found in or near sites in this study are displayed in Table 7.4. Redcar jetty and Smiths Docks at the top of the estuary and close to the Tees Storage site receive high loadings of PAHs. Dawes and Waldock (1994) and Law (1997), both found increased volatile compounds towards the inner estuary and a pollution gradient which decreased towards the mouth of the estuary. They also found that other areas, which are considered to be highly polluted, had much lower concentrations of PAHs, for example Plymouth Sound and the Bristol Channel. A recent study by Blackburn *et al.* (1999), showed that the Tees estuary had significantly high levels of weakly oestrogenic degradation products of alkylphenol polyethoxylate (APE) surfactants, which exceed the levels that are associated with the induction of vitellogenin in caged trout. The highest concentrations were reported at Redcar Jetty and Phillips approach.

In addition to the contaminant data obtained there has been considerable research on the community structure of species at the Tees sites. Since restrictions were enforced upon the amount of effluent

<i>Reference</i>	<i>Contaminant</i> $\mu\text{g l}^{-1}$	<i>Tees Estuary or Offshore Tees(*)</i>	<i>South Gare</i>	<i>Philips Approach</i>	<i>Redcar Jetty</i>	<i>Smiths Docks</i>
Blackburn and Waldeck, 1995	nonylphenol				3100 (D) 5200 (TE)	1300(D), 2000(TE)
	octylphenol				1300(TE)	
Dawes and Waldeck, 1994 (sampled September 1992)	Chloroform		286		1490	11 500
	1,1,1-Trichloroethane		29.8		94.2	602
	1,2-Dichloroethane		1210		1410	4020
	Bromodichloroethane		<10		151	1160
	Dibromochloromethane		10		67	172
Laslett, 1995 (sampled in 1991 or 92)	Cadmium	20-42 (1991) 15-97 (1992)				
	Copper	1000-1300(1991) 449-4000(1992)				
	Lead	96-920(1991) 54-820(1992)				
	Total PAH's	*61 (U,HW), 21 (U,LW)	2193 (U,HW), 4220 (U,LW) 2403 (D,HW)	1120 (U,HW) 2537 (U,LW) 1730 (D,LW) 3408 (D,HW) 9404 (U, 7/7/95) 3888(D,7/7/95)	2689 (U,HW) 2364 (U,LW) 3312 (D,HW) 713 (D,LW) 816 (U,7/7/95) 548 (D,7/7/95)	
Law, 1997 (samples taken 15/6/94) unless otherwise stated.	nonylphenol		5.3 (TE) 1.4 (D)	5.8 (TE) 2.6 (D)		
	NPEO+NP2EO		7.8 (TE) <0.6(D)	76 (TE) 15 (D)		
Blackburn <i>et al</i> , 1999 (samples taken 1994 and 1995)						

Table 7.4 Range of concentrations of selected contaminants in the Tees Estuary detected by various authors in the 1990's. D- dissolved, TE-total extractable, U water sample unfiltered, HW- high water, LW-low water, (DATE)-date water sample was taken.

entering the Tees Estuary in 1970, there appears to have been an improvement in the benthic biology of the Tees estuary, from 1979 to the mid-eighties (Shillabeer and Tapp, 1990). Continued observations of the benthic fauna up to 1990 confirmed that this was the case, however an impacted area beside Dabholm Gut was identified where there was sparse marine fauna populations. This was related to discharge from the Gut. Further monitoring of populations over the ten-year period showed slight improvement in this area (Tapp *et al.*, 1993). Benthic fauna diversity has at least improved up to 1992 and a reduction in contamination over this period has also been observed (Hall *et al.*, 1996). Changes in the macroalgae of the Tees have also been monitored. Hardy *et al.* (1993) found an increase in macroalgae red/brown and blue/green algae colonisation at the mouth of the Tees estuary, the south Gare site, compared to a site at British steel Redcar Works.

The information of the water quality at the control sites is less detailed. However, as the water graded by the Environment Agency was listed as an A or B grade, excellent or very good, they can be considered as sites which are much less contaminated compared to the Tees sites.

Section 7.3

STATISTICAL ANALYSIS OF THE DATA

7.3.1 Statistical analyses techniques

The mean and SD values are displayed for the normally distributed data and the median and the minimum and maximum values are shown for the non-normally distributed data. The GP activity of gill tissue and the neutral red retention time for the indigenous animals were log transformed to make the data distribution normal so that parametric analyses could be performed on it.

All of the data obtained from the deployed crabs were non-normally distributed. GP activity data for gill and haemolymph and GST activity data for haemolymph were log transformed so that standard ANOVA analysis of normally distributed data could be performed. GST activities of crab gill tissue were square root transformed.

The metallothionein data were analysed using non-parametric analysis techniques. The Kruskal-Wallis test was used to establish the p-value for statistical differences among the groups.

7.3.2 Non-parametric multivariate analysis of the data.

All data obtained were analysed using a non-parametric multivariate approach. The software package was PRIMER, developed at the Plymouth Marine Laboratory, United Kingdom. It was developed for the study of community structure. Not all analysis techniques of the program were utilised, as they were not applicable to the analyses of biomarker data. Multivariate analysis methods are characterised by basing their comparisons upon two or more samples, which share particular species, at comparable levels of abundance, (Clarke & Warwick, 1994). In this instance the “samples” were classified as the test sites and the “species” as the results of the biomarker responses. Analytical tests utilised for analyses of the biomarker data included, CLUSTERING, MDS,

ANOSIM and SIMPER programs, details of the computational techniques are explained in the user manual.

Transformation of the data.

When community data are analysed by PRIMER any species that has a high population density will dominate the analysis. Likewise when using biomarker data any measurement which provided a high numerical figure, for instance the neutral red retention time of crab haemolymph, would similarly dominate the analyses. The multivariate analyses would therefore not be a true representation of the biomarker responses. To prevent this from occurring all the data was expressed as a percentage of the highest value for that test, such that for every biomarker assay 100% is the highest possible attainable figure.

CLUSTER ANALYSIS.

All multivariate techniques are founded on *similarity coefficients*, calculated between every pair of samples. They then facilitate a *classification* or *clustering* of samples into groups that are mutually similar (Clarke & Warwick, 1994). The techniques used in this program represent a method of hierachial agglomerative clustering (e.g. Everitt, 1980), in which groups are successfully fused into larger groups, as the criterion for the similarity level is gradually relaxed. The cluster program computed a similarity matrix based upon the Bray-Curtis similarity coefficient (Bray & Curtis, 1957). The similarity matrix was then subject to hierachial agglomerative classification using group averaged sorting (Lance & Williams, 1967) in which the sites were classified upon biomarker responses measured at them. This clustering technique is represented by a dendrogram.

MDS

The data are also represented by an ordination plot in which sites are mapped out in two or three dimensions so that distances between pairs of plots represent their relative dissimilarity of species composition, or in this case, biomarker responses. Non-metric multi-dimensional scaling (MDS) achieves this type of data analysis (Kruskal & Wish, 1978).

MDS is generally applicable to many types of situations as it relies on the rank order of similarities, although this still relies on any transformations that may have been used when constructing the similarity matrix or dendrogram. The transformation facility is present to prevent species from dominating the analysis. The rarer species, which are lower in population, may be more significant to the analysis than the common species. As the biomarker data has already been transformed in order to prevent high figures from dominating the analysis, a “real distance” matrix can be constructed which has not been subject to transformation processes. The MDS analysis provides us with a “stress coefficient” which reflects the extent to which the ranking matrix is reproducible. The MDS algorithm, (which uses the Bray-Curtis similarity coefficient), utilised in this program, is an iterative procedure. It successively refines the positions of the points until they satisfy, as closely as possible, the dissimilarity of relations between samples (Clarke & Warwick, 1994). At least 15 random starts were used in the analysis of the biomarker data. The stress increases with reducing size of the ordination, a stress of less than 0.05 gives an excellent representation of the data. Stress less than 0.1 corresponds to a good ordination with minimal possibility of misinterpretation of the results, stress less than 0.2 is still a potentially useful plot whereas, stress less than 0.03 indicates that points are close to being arbitrarily placed in the ordination space.

ANOSIM

Other useful statistics can be obtained from the ANOSIM program (analysis of similarities), which provides a p-value. This statistic reflects the observed differences of the biomarker data between sites. This is achieved by comparing the differences between groups or sites with the differences among replicates within a group or site. This means that we are using much more information to highlight differences among the sites than analysis of separate biomarker responses.

The ANOSIM program makes very few assumptions about the data, for instance it does not assume that the data are normally distributed which makes it very useful for the analysis of biomarker data. In this instance it is based upon the Bray-Curtis similarities between samples.

SIMPER

SIMPER analysis, (similarity percentages) also provides some useful statistics, it provides a means to monitor which biomarker is responsible for the greatest contribution towards the Bray-Curtis similarity matrix and hence dissimilarity among sites. Therefore, it is possible to select which biomarkers are the most sensitive for distinguishing between sites. It lists the biomarkers that contribute the most through to the least for each pair of groups (or sites) measured. The results to this analysis have been presented such that the number of times that a biomarker has ranked first, second, third *etc.*, for dissimilarity between two pairs of data, is listed below the rank.

Section 7.4

RESULTS

7.4.1 Biomarker parameters measured from indigenous animals.

The results obtained from biomarker responses from the indigenous animals are shown in Table 7.5. The majority of the data were non-parametrically distributed.

Enzyme activities

Statistical analysis of the GP activity of gill tissue highlighted North Gare and South Gare, which had significantly lower GP activities, $p=0.006$, (Figure 7.2). Greatham Creek was not differentiated from the control sites in this analysis. However, GST activities of haemolymph were inhibited at the Greatham Creek site, compared to Robin Hoods Bay and South Gare sites that had comparatively increased activities $p<0.01$ (Figure 7.3).

Lysosomal neutral red retention time

Finally, the neutral red retention time assays highlighted all the Tees sites to be statistically different to the control sites, $p=0.00$ (Figure 7.4). The Tees sites had lower neutral red retention times.

The other biomarker responses measured were not successful in distinguishing differences between the Tees and the control sites; that is GST and GR activity of crab gill tissue, the metallothionein levels in the crab gill tissue and the GP activities of the haemolymph tissue.

Site	Tissue;	Gill												Haemolymph					
		No. of samples n	*GST		*GP		*GR		*MT		GST		GP		*NR				
			median	Min Max	median	Min Max	median	Min Max	median	Min Max	Mean	SD	Mean	SD	median	Min Max			
River Avon		8	0.30	0.21 0.8	0.21	0.16 0.35	5.3	2.8 9.2	35	20 52	1.9	±0.9	0.32	±0.09	90	30 120			
River Erme		8	0.23	0.12 0.4	0.17	0.09 0.22	3.9	0.0 4.7	31	14 41	2.7	±1.0	0.44	±0.15	60	15 90			
Robin Hoods Bay		8	0.16	0.14 0.53	0.19	0.11 0.34	3.5	0.0 7.5	33	24 37	3.0	±1.3	0.35	±0.12	75	30 120			
Greetham Creek		8	0.19	0.15 0.25	0.16	0.13 0.22	5.4	4.3 13.0	25	22 34	1.1•	±0.72	0.49	±0.11	0•	0 15			
North Gare		7	0.19	0.16 0.83	0.15•	0.09 0.17	6.0	2.8 7.5	36	21 66	2.7	±1.0	0.39	±0.23	15•	0 30			
South Gare		8	0.18	0.13 0.31	0.13•	0.11 0.2	3.3	2.9 5.1	27	17 61	3.4	±1.7	0.31	±0.15	15•	0 45			

Table 7.5 The biomarker results of crabs indigenous to the Tees Estuary and control sites. The mean and standard deviations are shown for the normally distributed data and the median and minimum and maximum values are shown for the non-normally distributed data. GP activity is measured as µmoles/min/mg protein. GST activity is measured as µmoles/min/mg protein in gill tissue and as µmoles/min/g tissue in the haemolymph tissue. GR activity is measured as µmoles/min/g protein. MT is measured as µg/g wet weight gill tissue and NR (Neutral red retention time) as minutes. (* Represents the non-normally distributed data, • represents significant difference in biomarker result with two or more other sites)

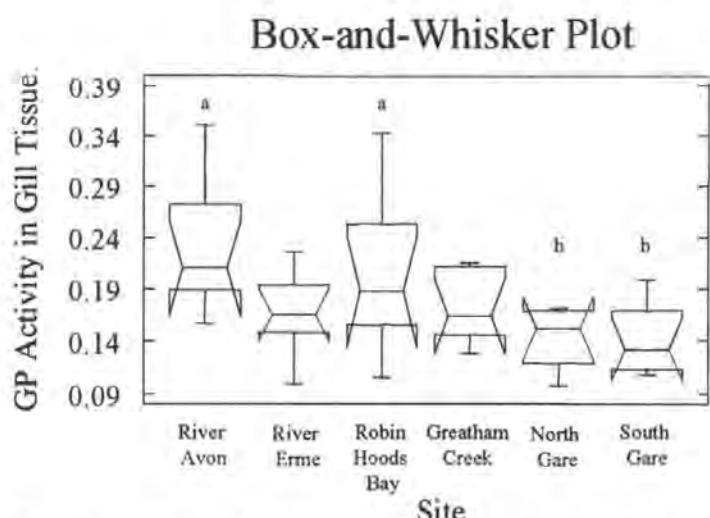


Figure 7.2 The GP activity of crab haemolymph, from crabs indigenous to the sites. The GP activity is measured as μ moles of substrate oxidised / min / mg protein.

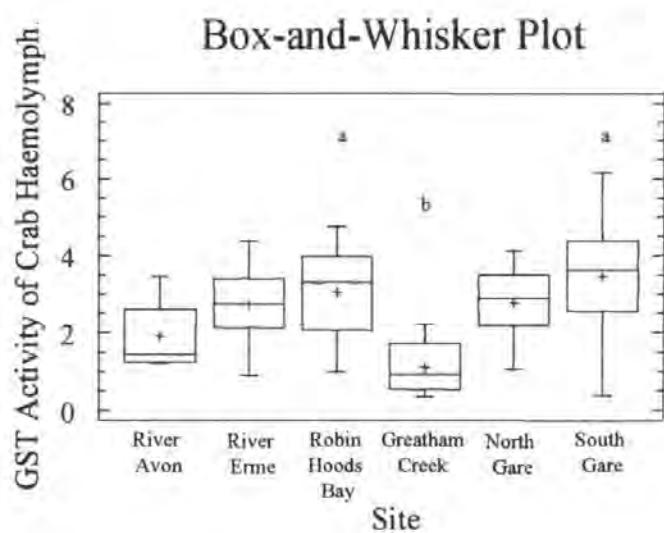


Figure 7.3 The GST activity of crab haemolymph from crabs indigenous to the sites. The GST activity is measured as μ moles of substrate metabolised / min / g protein.

The different letters above the box and whisker plots indicate which sites are statistically different from one another.

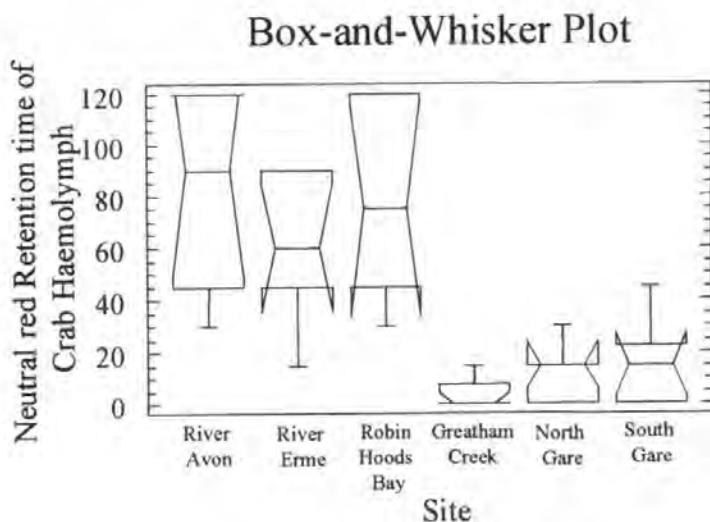


Figure 7.4 The neutral red retention time from crabs indigenous to the sites. Neutral red retention time is measured in minutes.

7.4.2 Biomarker parameters measured from deployed animals

The values of the biomarker responses from the deployed crabs are shown in Table 7.6.

Enzyme activities

The Tees Storage site induced statistically significant enzyme activities for several of those measured. In most instances the activities measured at this site were different to at least one or more of the other sites. Results showed that GP and GST activities of gill were decreased at the Tees Storage site, $p=0.00$, (Figure 7.5 and Figure 7.7, respectively). Whereas, GP and GST activities of haemolymph were increased, $p = 0.00$ for each, (Figures 7.6 and Figure 7.8, respectively).

GR activities of crab gill tissue were not successful in distinguishing between the sites.

Metallothionein levels

Metallothionein levels of gill tissue did not generally differentiate between sites. However, the animals deployed at the River Avon had statistically higher metallothionein levels than at all other sites. They were also higher than the levels measured from the indigenous crabs at this site, $p = 0.00$ (Figure 7.9).

Lysosomal neutral red retention time.

The neutral red retention time of the crabs deployed at the different sites was successful at distinguishing the contaminated sites from the control sites, $p = 0.00$, (Figure 7.10).

	<i>Tissue;</i>	<i>Gill</i>						<i>Haemolymph</i>							
Site	No. of Samples	GST		GP		GR		MT		GST		GP		NR	
Statistics	n	median	Min Max	median	Min Max	median	Min Max	median	Min Max	median	Min Max	median	Min Max	median	Min Max
River Avon	8	0.19	0.12 0.24	0.09	0.06 0.22	3.5	1.8 4.8	117*	27 240	4.1	0.6 6.1	0.5	0.34 0.77	90	60 120
River Avon (Travelled)	8	0.18	0.1 0.25	0.1	0.07 0.15	2.3	1.5 6.8	71	17 171	2.7	0.0 6.4	0.52	0.42 0.85	90	15 120
Robin Hoods Bay	5	0.28	0.18 0.5	0.15*	0.10 0.49	2.3	1.9 6.0	29	25 32	4.9	3.2 7.0	0.4	0.31 0.58	90	90 120
Greetham Creek	8	0.22	0.16 0.3	0.15	0.1 0.26	2.8	1.7 5.4	29	18 44	3.0*	1.4 6.0	0.35	0.24 0.55	30*	0 45
Hartlepool Power Station	8	0.26	0.15 0.5	0.16*	0.1 0.23	2.8	0.0 5.3	31	22 40	5.4	2.6 6.7	0.31*	0.21 0.37	30*	0 45
South Gare	5	0.21	0.04 0.24	0.14	0.12 0.21	3.7	2.9 7.8	24	20 29	2.9	2.6 6.3	0.35	0.25 0.74	45*	0 90
Tees Storage	8	0.08*	0.04 0.11	0.09*	0.04 0.14	3.8	0.0 11.0	42	25 61	5.7*	4.3 18.4	0.58*	0.35 1.3	15*	0 30

Table 7.6 Biomarker results of crabs deployed at the Tees Estuary and control sites. The median and minimum and maximum values are shown as the data is non-normally distributed. GP activity is measured as $\mu\text{moles}/\text{min}/\text{mg protein}$. GST activity is measured as $\text{mmoles}/\text{min}/\text{g protein}$ in gill tissue and as $\mu\text{moles}/\text{min}/\text{g tissue}$ in the haemolymph tissue. GR activity is measured as $\mu\text{moles}/\text{min}/\text{g protein}$. MT is measured as $\mu\text{g}/\text{g wet weight}$ gill tissue and NR (Neutral red retention time) as minutes. (* indicates biomarker response at that site is significantly different to two or more other sites).

Box-and-Whisker Plot

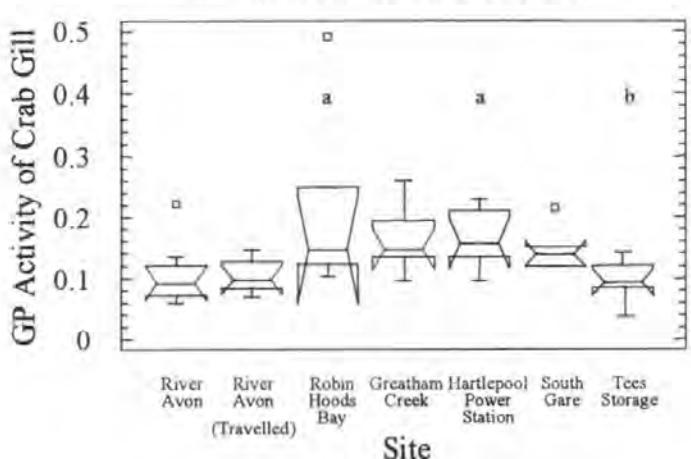


Figure 7.5 GP activities of crab gill from crabs deployed at the different sites.

Box-and-Whisker Plot

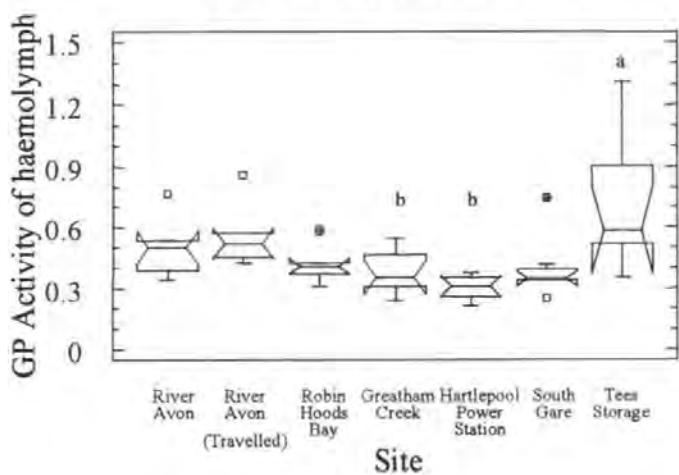


Figure 7.6 GP activities of crab haemolymph from crabs deployed at the different sites.

GP activity is measured as $\mu\text{moles} / \text{min} / \text{mg protein}$. Different letters above the box and whisker plots signify which sites are statistically different from one another

Box-and-Whisker Plot

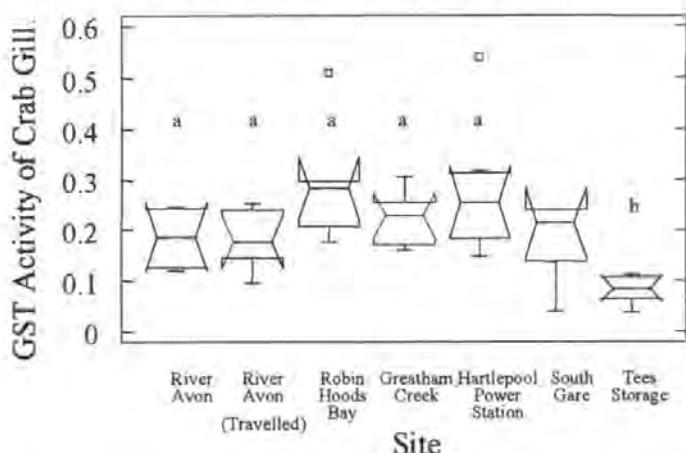


Figure 7.7 The GST activities of crab gill tissue from crabs deployed at the different sites. GST activity is measured as mmoles of substrate metabolised / min / g protein.

Box-and-Whisker Plot

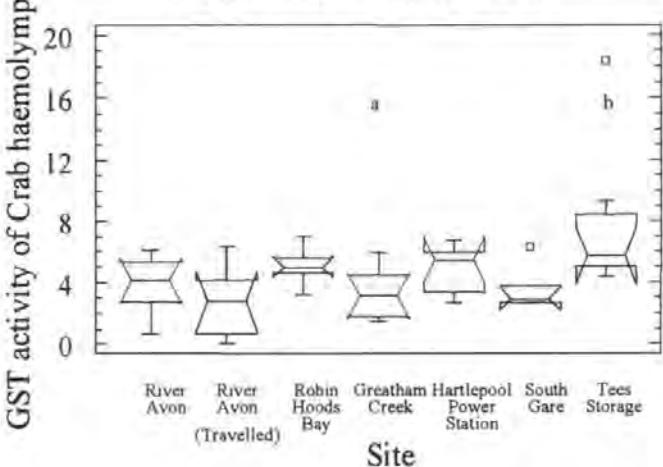


Figure 7.8 The GST activities of crab haemolymph tissue from crabs deployed at the different sites. GST activity is measured as μmoles of substrate metabolised / min / g protein.

The different letters above the box and whisker plots represent which sites are statistically different from one another.

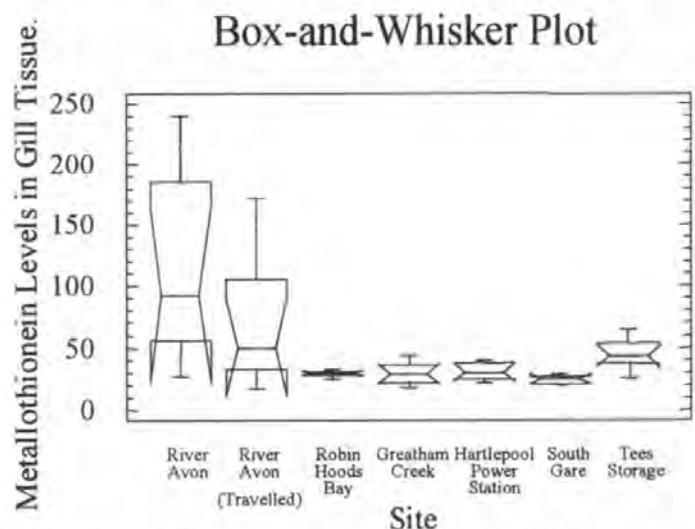


Figure 7.9 Metallothionein concentrations in gill tissue from crabs deployed at the different sites. Metallothionein levels are measured as $\mu\text{g/g}$ wet weight gill tissue

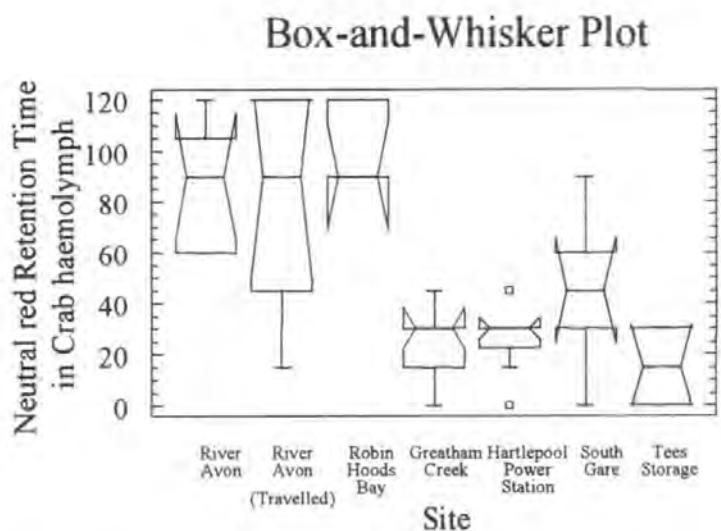


Figure 7.10 Lysosomal neutral red retention time of haemolymph tissue from crabs deployed at the different sites. Neutral red retention time is measured in minutes.

7.4.3 Comparison of parameters obtained from the indigenous and deployed crab populations

The biomarker values for the indigenous and deployed populations were compared for each of the sites where both techniques were successfully completed. The biomarker responses from the deployed and indigenous populations were generally the same. However, the exceptions are displayed in Figures 7.11 to 7.14. The River Avon deployed crabs, including those that had travelled to the Tees Estuary and back, had significantly different GP activities in both their gill tissue and haemolymph compared with the indigenous populations at this site, ($p = 0.01$ for both activities, Figures 7.11 & 7.13). In addition, the River Avon deployed crabs had significantly higher metallothionein levels compared to the indigenous population, ($p=0.002$, Figure 7.14). The only other site which produced a significant difference between deployed and indigenous crabs was at Greatham Creek, where gill GR activities were higher for the indigenous population, $p = 0.01$. (Figure 7.12).

7.4.4 Multivariate analysis of biomarker data from the indigenous crab populations

Cluster analysis of the group averaged data placed the sites into two clusters as shown in Figure 7.15. The Tees sites were placed into one cluster and the control sites into another. ANOSIM analysis was performed upon the data, which analyses the percentage of similarities of biomarker responses. Crabs from the River Avon and Robin Hoods Bay showed the greatest similarity of biomarker responses among the control sites (52%). North Gare and South Gare showed the greatest similarity of biomarker responses among the Tees sites (62%). The MDS plot as shown in Figure 7.16, demonstrates the spatial arrangement of the sites which is determined by the similarity of their biomarker responses. The sites that are encompassed within the different coloured circles are not statistically different from one another (as calculated from the ANOSIM program). The

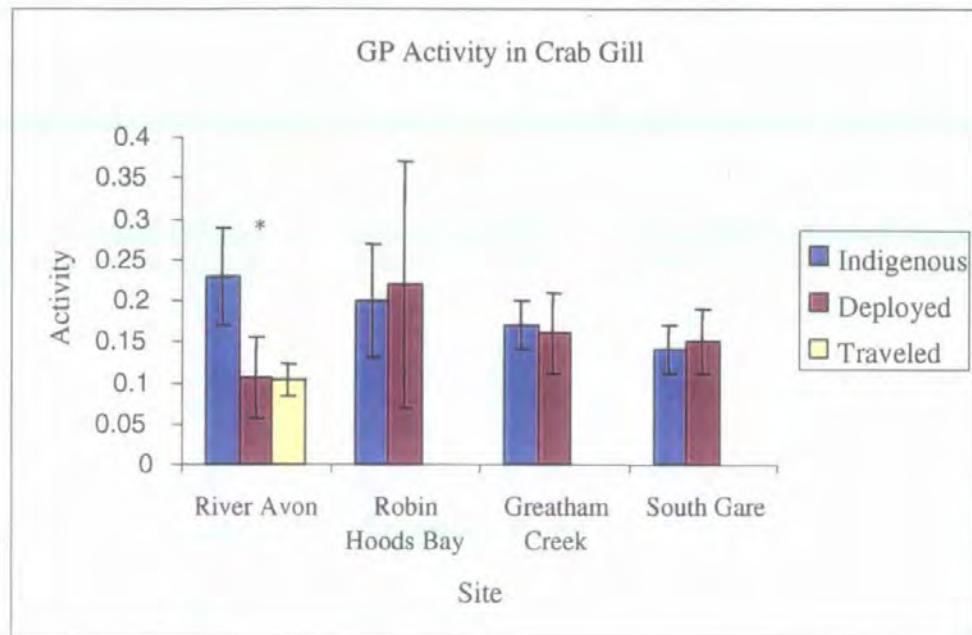


Figure 7.11 The mean and SD values of the GP activities of crab gill.

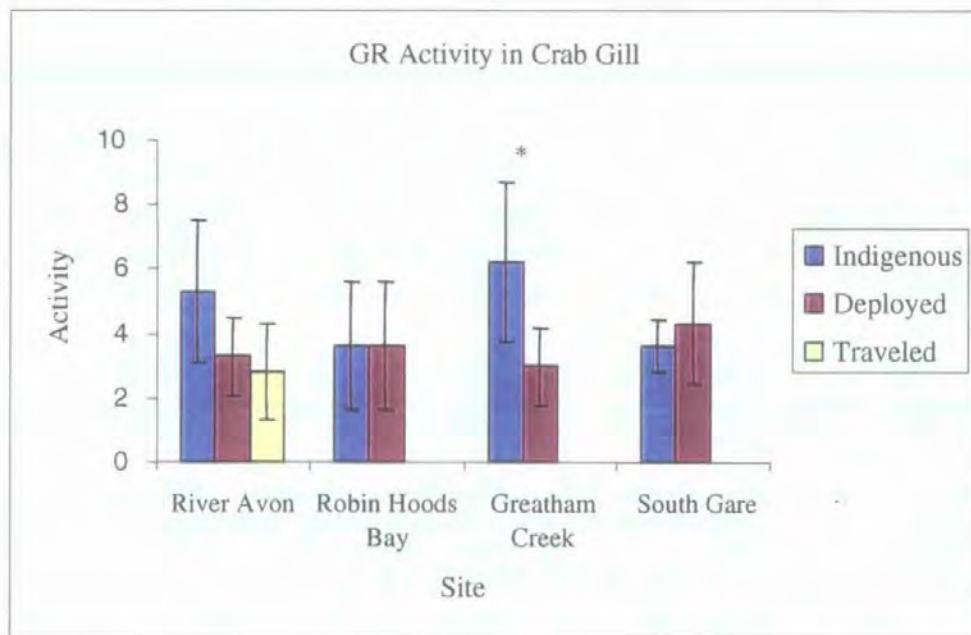


Figure 7.12 The mean and SD values of the GR activities of crab gill tissue.

Indigenous and deployed crabs activities are shown beside each other so that comparisons of the two sets of data can be made. (* represents a statistically significant difference between the enzyme activities of the deployed and indigenous crabs). See table 7.1 and & 7.2 for enzyme activity values.

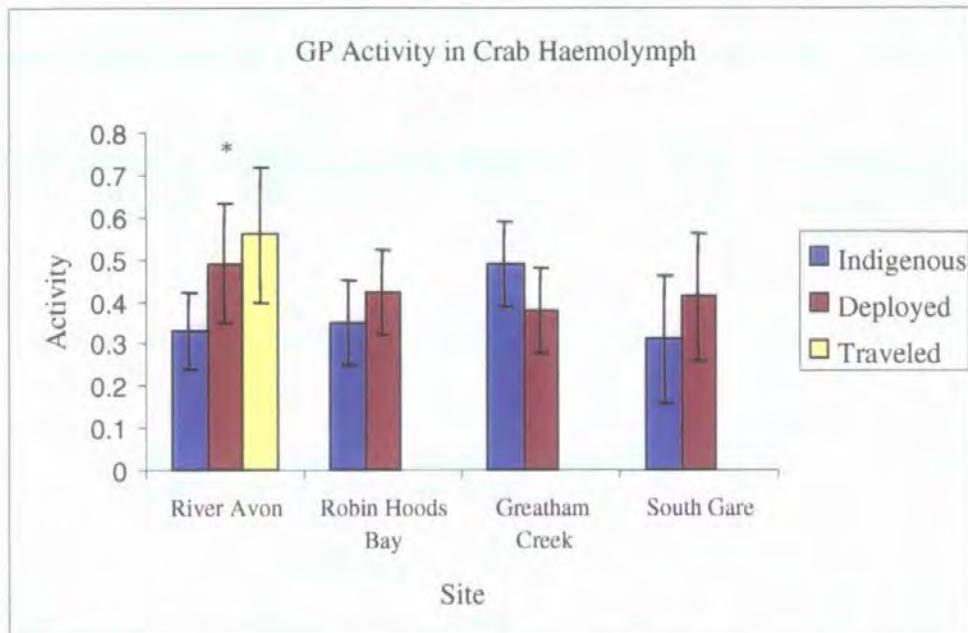


Figure 7.13 The mean and SD values of the GP activity of crab haemolymph .

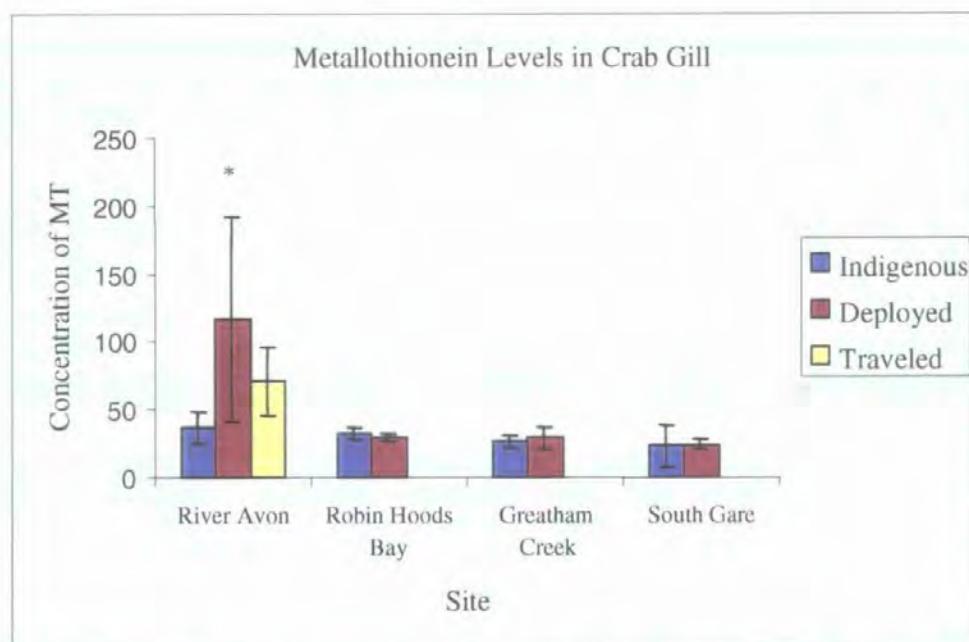
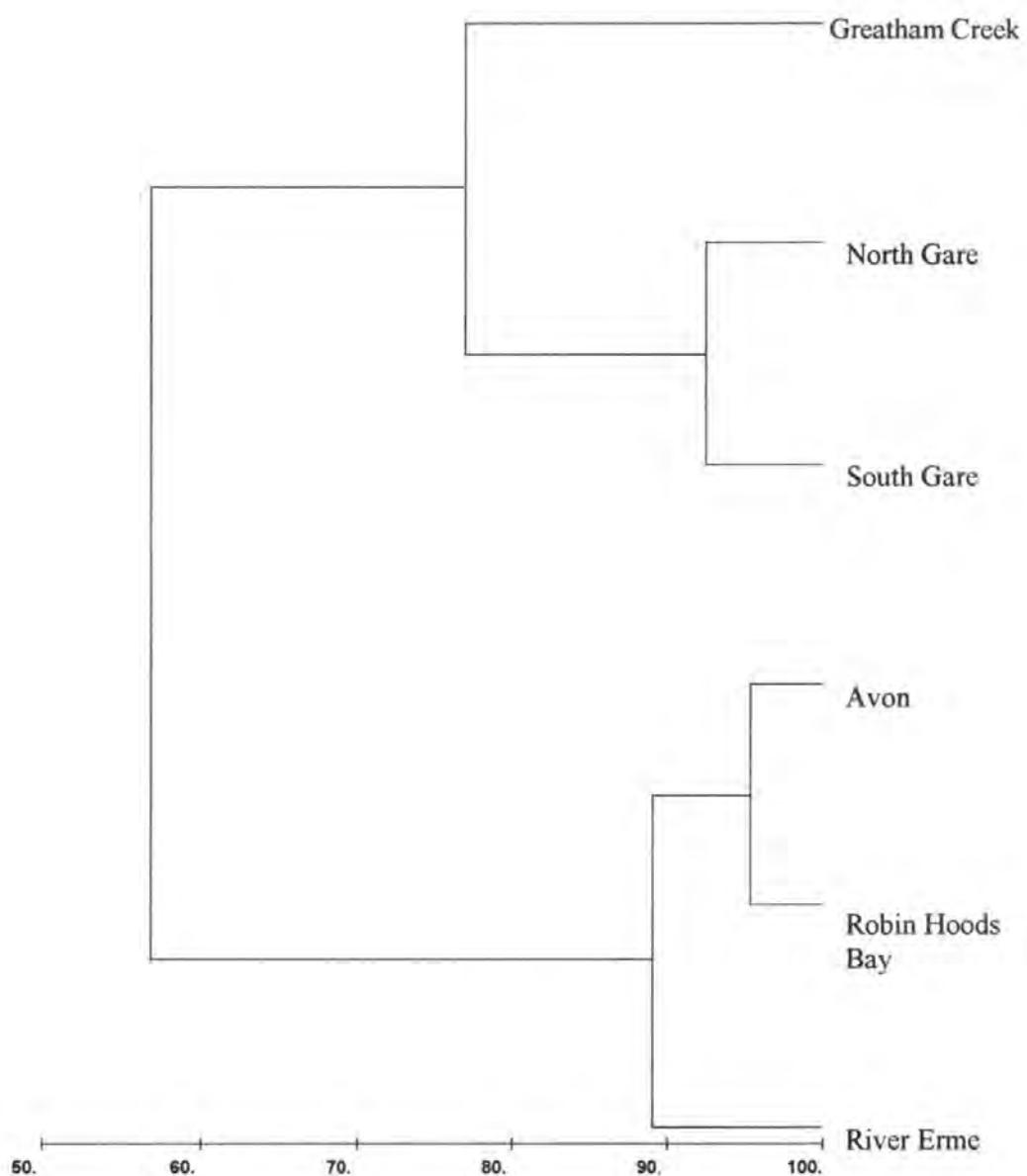


Figure 7.14 The mean and SD values of metallothionein levels from crab gill tissue.

The results from indigenous and deployed crabs are shown beside each other so that comparisons of the two sets of data can be made. (* represents a statistically significant difference between the enzyme activities or metallothionein levels of the deployed and indigenous crabs). See table 7.1 and & 7.2 for enzyme activity and metallothionein level units.

Indigenous Crabs



BRAY-CURTIS SIMILARITY

Figure 7.15 Dendrogram of the averaged biomarker responses in animals indigenous to the field sites. Biomarkers used in the cluster analysis were crab gill metallothionein, GST, GP and GR and crab haemolymph neutral red retention time, GP and GST.

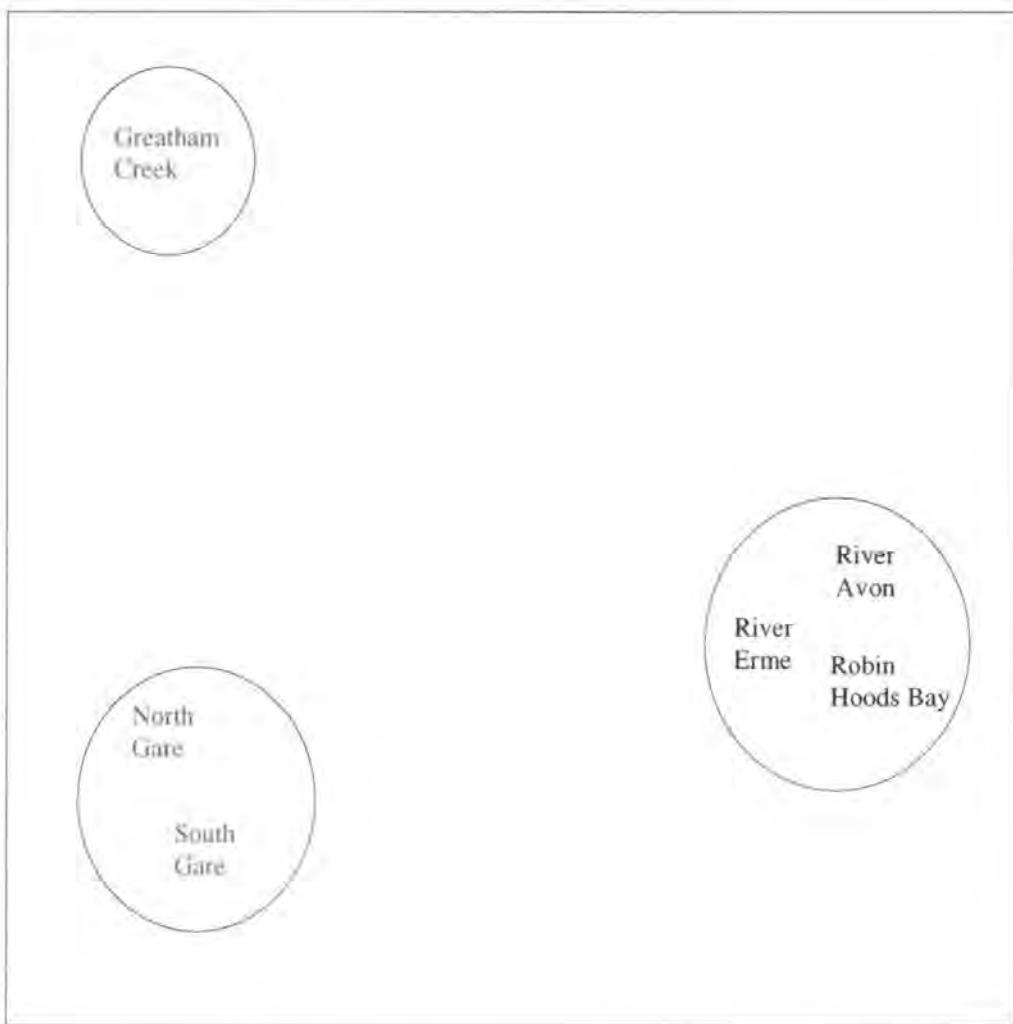


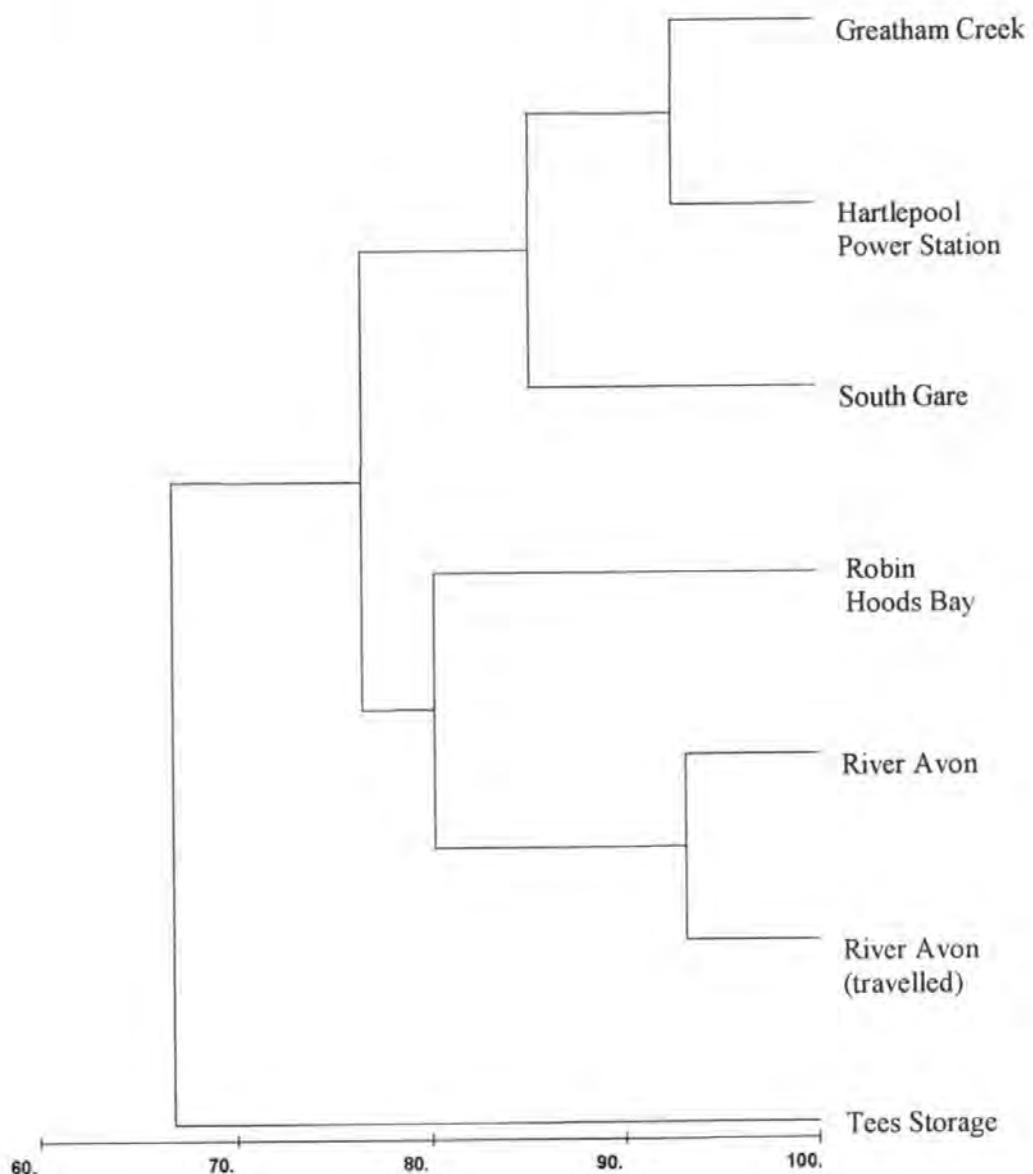
Figure 7.16 MDS plot, stress = .01, based on a similarity matrix using the averaged individual biomarker responses in crabs indigenous to the different sites, sites within the same circles are not significantly different from each other. Control sites are in blue lettering, Tees sites are in red lettering. Biomarker responses used in the analysis are gill metallothionein levels, GR GP and GST and haemolymph neutral red retention time, GP and GST.

Greatham Creek site was found to be statistically distinct from both the North and South Gare sites and statistically different to the control sites. The MDS plot provides more information than the dendrogram as the sites have been arranged in two dimensions instead of just one. The stress value for the MDS analysis was 0.01, which is indicative of an excellent representation of the data.

7.4.5 Multivariate analysis of biomarker data from the deployed crabs

Multivariate analysis of the group averaged biomarker data for the deployed crabs placed the data into three clusters, (Figure 7.17). The Tees storage site was placed aside from the rest of the Tees sites and the control sites. This grouping was also confirmed by the MDS plot, Figure 7.18. ANOSIM analysis of the data showed that the sites at Hartlepool Power Station and Greatham Creek had the most similar biomarker responses, 73% similarity. Among the control sites the River Avon crabs that had travelled and were deployed back at the site and the River Avon crabs that had not travelled had biomarker responses that were most similar out of the control crabs, 67%. The circles on the MDS plot encompass the sites that were statistically similar to one another. The Avon crabs that had travelled to the North and had been deployed back in the south were found to possess biomarker responses that were not statistically different to South Gare. Otherwise the Tees and control sites were grouped together with the exception of the Tees Storage site which was positioned furthest away.

Deployed Crabs



BRAY-CURTIS SIMILARITY

Figure 7.17 Dendrogram of the averaged biomarker responses in animals deployed at the field sites. Biomarkers used in the cluster analysis were crab gill metallothionein, GST, GP and GR and crab haemolymph neutral red retention time, GP and GST.

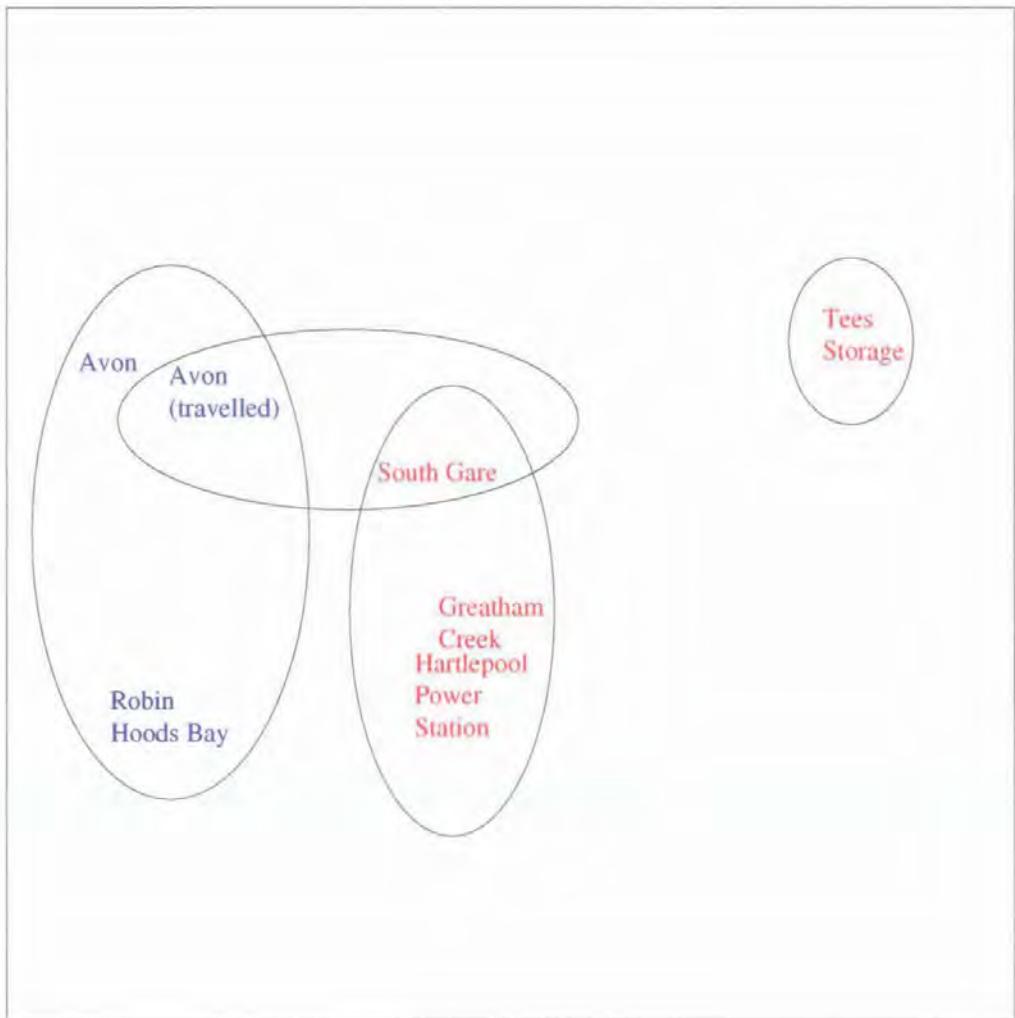


Figure 7.18 MDS plot, stress = .01, based on a similarity matrix using the averaged individual biomarker responses in crabs deployed at the different sites, sites within the same circles are not significantly different from each other. Control sites are in blue lettering, Tees sites are in red lettering Biomarker responses used in the analysis are gill metallothionein levels, GR GP and GST and haemolymph neutral red retention time, GP and GST.

Section 7.5

DISCUSSION

Enzyme responses from indigenous crab populations

Crabs indigenous to North and South Gare had reduced gill GP activities. The geographical positioning of these two sites suggest that they are likely to be exposed to similar anthropogenic discharges. However, relative to the other Tees sites they are exposed to less contamination probably due to a dilution effect, (Table 7.3). GP activities are shown to reduce after exposure to contamination in other invertebrates used in field trials (Doyotte, *et al.*, 1997; Cossu, *et al.*, 1997 and Regoli *et al.*, 1998b). In all of these instances GP was found to be a sensitive biomarker of toxicity. Cossu *et al.* (1997), reported a reduction of GP activity that was associated with an increase in contamination of sediments with a high level of polycyclic aromatic hydrocarbons and polychlorinated biphenyls. In contrast, GP activities were increased in the arctic bivalve, *Macoma balthica*. These increased GP activities were correlated with increases of metal contamination within its tissues (Regoli *et al.*, 1998a). Whereas, shorthorn sculpin (*Myoxocephalus scorpius*) showed increased liver cytosol GP activities in a field trial using animals caught in four harbours on the southwest coast of Iceland (Stephensen *et al.* 2000). However, the increased GP activities were not correlated with contaminant body burdens. The results from the present study suggest that GP activities have potential to be used as a sensitive biomarker of toxicity.

The crabs indigenous to the Greatham Creek site had reduced GST activities compared to the control site, Robin Hoods Bay and the Tees site, South Gare. The other sites showed varying responses but were not significantly different to one another. The Greatham Creek site receives increased metal contaminant loading (Table 7.3). It is also within close proximity to the Tioxide Ltd outflow pipe, which has a particularly high iron discharge. GST levels have also decreased in other

field experiments using invertebrate species (Regoli & Principato, 1995). However, in most instances GST activity has been induced in field studies when animals are exposed to anthropogenic waste (Lee, 1988; Sheehan *et al.*, 1991; Fitzpatrick *et al.*, 1997; Kaaya *et al.*, 1999 and Van der Oost *et al.*, 1998). GST activities may prove to be a useful biomarker because of the selectivity of enzyme activity in response to contaminated sites. In this study and the study by Regoli & Principato (1995) its activity reduced in sites with high metal contamination. Whereas, GST was induced in response to organic contamination in another field study performed by Van der Oost *et al.* (1998). GST activities from a field trial using *Macoma balthica* gave heterogenous responses at the different field sites (Regoli *et al.*, 1998a). Also, the activities from red mullet livers only highlighted one contaminated site in a field study in the northwestern Mediterranean Sea (Burgeot *et al.*, 1996). Although, GST is not a universal biomarker of the presence of all types of contamination, understanding its mechanistic responses to different types and mixes of pollution may make it useful in distinguishing which pollutants are causing the effect.

The GR activities of gill tissue did not differentiate between sites, however other field trials have shown GR to be a strong indicator of prooxidative compounds (Stephensen, 2000). Although GR did not prove to be a good indicator of toxicity exposure in this data analysis, the results gave more insight when used for the MDS analysis (see later).

Lysosomal neutral red retention time of indigenous crabs

The neutral red retention time was successful in highlighting the Tees sites as significantly different to control sites. The neutral red retention time is a useful biomarker of toxicity, it is reliable in distinguishing animals that have been exposed to contaminant exposure from those that have not.

Lysosomal destabilisation times of other species have also been successful in identifying contaminated sites (Lowe *et al.*, 1992; Lowe *et al.*, 1995b; Regoli, 1992; and Wedderburn, 1998).

Metallothionein levels measured in indigenous crab populations

Metallothionein levels did not significantly differentiate between the contaminated and control sites, although undoubtedly some sites were subject to higher metal ion loading than others. In past field experimentation metallothionein levels have been successfully induced in response to metal toxicity. The crab, *Carcinus maenas*, had induced metallothionein concentrations in response to metal exposure along the Fal Estuary (Pedersen *et al.*, 1997). Metallothionein levels may not have been induced in this investigation because the levels of metal contamination were not sufficiently high. Much higher concentrations of cadmium and copper were required to induce metallothionein in laboratory experiments, than were present in the water at the Tees Estuary (See Chapter 4 & Table 7.3). Metallothionein may therefore only be a useful biomarker for establishing chronic levels of metal exposure.

Enzyme responses from deployed crab populations.

The enzyme responses from the deployed crabs highlighted the Tees Storage site as highly contaminated. The enzyme responses measured at this site were significantly different to at least one or more of the other sites. The contamination at Tees Storage caused increased haemolymph GP and GST activities, whereas these activities were decreased at the Greatham Creek site. In addition, gill GST activities were reduced at the Tees Storage site, whereas at the Greatham Creek site they were increased. The chemical composition of the water at these two sites must be quite different in order to induce these varying responses. The Tees Storage site is positioned further up the estuary

compared to the Greatham Creek site, and receives contamination from the outflow at Dabholm Gut. In comparison the Greatham Creek site is positioned inside seal sands which, is more likely to be affected by waste from Tioxide Ltd. The changes of enzyme activities in response to different types of mixed effluent exposure could provide an indication of the type of pollution that the crabs are exposed to.

The GST activity of gill tissue only highlighted the Tees Storage site as contaminated. This shows the selectivity of GST activity in highlighting contaminated sites. Most of the other enzyme responses showed which sites had the most opposing enzyme activities, but did not show a definite response for the presence of contamination. GST activity of crab haemolymph tissue was less selective; the Tees Storage site was only significantly different to the Greatham Creek site and all other enzyme activities fell between the two.

The enzyme activity responses varied depending upon the tissue in which they were measured; gill GST activity was suppressed and haemolymph activities were induced at the Tees Storage site. Whereas, gill GP activities were reduced and haemolymph activities were induced at the Tees Storage site. Different effects of contamination in separate tissues from within the same animal has also been observed during other field trials (Regoli & Principato, 1995 and Cossu *et al.*, 1997). This demonstrates the necessity of understanding antioxidant mechanisms, as antioxidant enzyme responses to contamination are not the same in each of the animal's tissues.

Metallothionein levels

Metallothionein levels were induced in the crabs deployed at the River Avon, but were not in the crabs that were indigenous. The reasons for this are explained later. Otherwise there was no significant difference in metallothionein levels among the other sites.

Lysosomal neutral red retention time

The lysosomal neutral red retention time was successful in highlighting significant differences between the control and Tees populations. The lysosomal neutral red retention time significantly decreased at the Tees sites compared to the control sites. This result shows the potential that this technique has for highlighting areas that receive anthropogenic waste. The method is also useful for proving that the contamination is causing physiological damage.

Comparison of parameters obtained from the indigenous and deployed crab populations

There was not a significant difference between biomarker values obtained for deployed and indigenous populations for the majority of the data, except at the River Avon which showed several discrepancies. The deployed River Avon crabs had significantly decreased GP activity of gill tissue; and significantly increased GP activities of haemolymph and metallothionein levels in gill tissue, compared with the indigenous population at this site. There had been a gap of a fortnight between the indigenous crabs being collected from the River Avon and the deployed crabs being retrieved from the River Avon. The enzymatic and metallothionein changes must have occurred during this time. These changes were not likely to have been caused by industrial effluent, as there is no industry within the vicinity. Neither was it likely to be caused by caging stress as an induction of metallothionein would have been observed in the other deployed crabs. The inconsistent results may be due to a period of heavy rain prior to deployment, (an average of 10.12 mm of rain and 1.7 hours of sunshine per day); which followed three weeks of sunny weather with very little rain (1.83 mm of rain and 7.7 hours of sunshine per day), (Meteorology department, IMS, University of Plymouth, 1997). This period of heavy rainfall may have resulted in an increase of concentrations of nitrate and

phosphates from agricultural run off; effluent loading and storm run-off from local sewage treatment works; from increased road run-off; agricultural pollution (halogenated hydrocarbons); or from increased concentrations of naturally occurring phenols from Dartmoor. Water drains from Dartmoor to the River Avon. Concentrations of phenols above the EQS concentrations are frequently detected in the Avon and as such the Avon is no longer expected to conform to the set environmental standards (Environment Agency, 1998). The enzyme responses that were triggered in the deployed crabs from the River Avon, show that GP and metallothionein may prove to be sensitive, effective biomarkers of short term environmental changes and highlights the potential that biomarkers have in continual environmental monitoring.

The other discrepancy that was observed between indigenous and deployed crabs was at the Greatham Creek site, this was where gill GR activities were significantly decreased in the deployed crabs compared to the indigenous crabs. This is the only parameter out of the seven measured that shows a significant difference between the indigenous and deployed population. For this reason it is possible that an adaptive response has occurred in the indigenous crabs of this site, rather than a change in the chemical profile of the environment. Or there may have been a genetic difference between the deployed crabs from the River Avon in Devon and the indigenous crabs of the River Tees. If a change of chemical constitution had occurred it is likely that other biomarkers also would have highlighted the difference. Other species indigenous to an area have also shown adaptation to their environment; the freshwater bivalve, *Unio tumidus*, showed selenium dependent GP adaptation to its contaminated environment (Cossu *et al.*, 1997). In the mussel, *Mytilus galloprovincialis*, GP and GR also showed adaptation to their polluted environment (Regoli & Principato, 1995). These results show that adaptation of a species to its environment must be considered especially if enzyme activities of indigenous and deployed animals are to be compared.

MDS analysis of biomarker parameters measured in indigenous crabs

The dendrogram analysis of the indigenous crab populations grouped the sites into control and Tees sites. MDS analysis showed a distinct separation of the sites into three groups; the control sites; the north and south Gare sites; and the Greatham Creek site. ANOSIM analysis of the data showed that all three groups were statistically distinct from one another. The two groups that comprised the Tees sites were equally removed from the control sites however, they were spatially arranged in separate directions on the MDS plot. The spatial arrangement of the sites depends upon which biomarker responses are most influential in distinguishing between them. SIMPER analysis lists the contribution of each of the biomarkers to dissimilarity between sites. Table 7.7 displays the rank order that each of the biomarkers contributes from the most, (position 1) to the least (position 7) towards the dissimilarity matrix. Overall the lysosomal neutral red retention time contributed the most towards distinguishing between the sites, this is because it was the most effective biomarker at highlighting contaminated sites. However, GST and GP activities of haemolymph contributed the most for distinguishing between the Tees sites and neutral red retention time contributed the least. Therefore, the enzyme activities contributed more towards the spatial arrangement of the Tees sites on the MDS plot than the lysosomal neutral red retention time.

Crabs that inhabit waters with similar chemical composition will exhibit enzyme responses that are also similar. This type of statistical analysis is effective in grouping together sites that have similar chemical profiles to one another. We can assume that the Gares receive similar anthropogenic waste because they are both positioned on the opposite sides of the mouth of the estuary. Whereas, Greatham Creek is positioned within the estuary opposite the outflow of Tioxide Ltd so will be exposed to a different chemical composition, this is verified by water chemical analysis results from these sites (Table 7.3).

<i>Biomarker Parameter</i>	<i>Position</i>	1	2	3	4	5	6	7
Crab Lysosomal RT		12	3					
GST Haenolymph			7	5	2		1	
GP Haemolymph			1	4	5		3	2
GST Gill			2	2	1	6	3	1
GP Gill		1	1	3	4	3	3	
GR Gill		2	1	1	3	6	2	
MT Gill						3	12	

Table 7.7

<i>Biomarker Parameter</i>	<i>Position</i>	1	2	3	4	5	6	7
Crab lysosomal RT		16			3			2
GST Haemolymph		1	1	3	5	3	2	6
GP Haemolymph		1	3		6	4	6	1
GST Gill		1	3	8	1	2	5	1
GP Gill			2	4	5	5	5	
GR Gill		2	6	4	1	4	3	1
MT Gill			6	2		3		10

Table 7.8

Table 7.7 & 7.8 Results of SIMPER analysis. The rank order of the number of times each biomarker contributes the most, (Position 1) to the least (Position 7) towards dissimilarity between sites. MT = metallothionein Levels. RT = retention time.

MDS analysis of biomarker parameters from the deployed crabs

The dendrogram analysis of the biomarker parameters from the deployed crabs separated the sites into three clusters; the Tees Storage site; the rest of the Tees sites; and the control sites. These groupings were also shown by the MDS analysis of the data. The Tees Storage site was the furthest removed from the control sites and the rest of the Tees sites were positioned between them. ANOSIM analysis of the data showed that the Tees Storage site was not statistically similar to any of the other groups. The control sites were similar to one another, as were the rest of the Tees sites. However the South Gare site was not statistically different to the River Avon crabs that had travelled to the Tees and back. Greatham Creek and Hartlepool Power Station were positioned very close to one another, this is not surprising as they are also geographically close. However, in this instance the Greatham Creek site was not statistically different to the South Gare site. This was a different result to the responses obtained using the indigenous crabs. The crabs deployed there may not have left for long enough to elicit biomarker responses to distinguish it from the South Gare site.

SIMPER analysis of all the data showed that neutral red retention time distinguished the most among the sites and metallothionein levels distinguished the least. However, it was found that GST activity of crab gill tissue distinguished the most between the Tees Storage site and the rest of the Tees sites, followed by GP activity of haemolymph tissue. Once again this demonstrates the usefulness of enzyme activities in distinguishing between sites that are contaminated and which always induce increased lysosomal destabilisation. The gill GST activity from the deployed crabs was more influential in separating the Tees sites, whereas for the indigenous populations it was haemolymph GST activity. This may be due to the gill being the first line of defence against water-borne contamination, so is more likely to elicit biomarker changes in short term toxicity tests.

The Tees Storage site was positioned furthest along the estuary opposite Dabholm Gut which receives anthropogenic waste from both ICI Wilton and Bran Sands Treatment works. The pollution

from this area was sufficient to induce significant biomarker responses during the deployment time to distinguish it effectively from the other Tees sites.

Summary

MDS has proved to be an effective means of analysing biomarker data. The combinative effects of all the biomarker data make interpretation of the results more effective than analysing biomarkers individually. The multi-biomarker approach is also particularly effective in creating profiles of biomarker responses, which with further research could lead to establishing the types of toxicity causing the effect.

Water samples from the sites used in this field experiment were also tested using conventional toxicity tests, MicrotoxTM and *Tisbe battagliai* LC₅₀. The MicrotoxTM assay did not indicate toxicity in water samples from any of the sites; the *T.battagliai* test highlighted Greatham Creek and Tees Storage as toxic (Astley *et al.*, 1999). MDS analysis of the biomarker data was effective in highlighting all of the Tees sites as toxic and in this instance proved to be more sensitive than using conventional toxicity tests in establishing contaminated sites.

ANOSIM statistical analysis is particularly effective because it incorporates data distributions of the biomarker responses. Toxicity exposure increases the distribution of biomarker responses and this can frequently mask significant results when using ANOVA analysis techniques (Depledge & Lundebye, 1996 and Forbes & Depledge, 1996). ANOSIM utilises similarities between individuals of a group, in addition to between group similarities; hence the distributions of the data for each biomarker response are incorporated as part of the analysis. This makes it sensitive for analysing biomarker data, as increased data distributions are an important indicator of an early toxicity response.

The SIMPER analysis also allows us to establish which measurements are sensitive biomarkers of contamination exposure, so that only the highly selective biomarkers need to be employed for future field trials. This can decrease the cost and man-hours required for carrying out biomarker analysis.

The use of multi-biomarker approaches to toxicity testing and for environmental assessment has had growing impetus (Bresler *et al.*, 1999; Burgeot *et al.*, 1996; Fossi *et al.*, 1996; Livingstone *et al.*, 1995; Porte *et al.*, 1991 and Van der Oost, 1998). The general consent is that far more can be ascertained using a multi-biomarker approach than the use of singular biomarkers. MDS is an effective method by which multi-biomarker data can be analysed and the method as such could be useful in Direct Toxicity Assessment, if used in conjunction with established toxicity testing.

DISCUSSION

Section 8

The present study was designed to investigate mechanisms of oxidative stress with particular focus upon the role of glutathione and its related enzymes. Preliminary experiments showed that the metabolic responses of the crab, *Carcinus maenas*, to xenobiotic exposure could be used as a suitable model for this investigation. The crab's metabolic responses to contamination also had potential to be applied in ecotoxicological studies.

The crab is a useful sentinel organism of contamination in Britain as it is widely distributed around the coastline and is relatively resilient to pollution. Indigenous populations of crabs inhabit highly polluted areas such as harbours and estuary based industrial sites. They are therefore a potentially useful bio-indicator species for areas at risk from pollution. They can also be successfully deployed in these areas without the process of deployment significantly influencing mortality.

The initial part of the investigation was laboratory based so that any exogenous factors that may influence the crab's metabolic responses could be controlled. A field experiment was also performed to establish whether the metabolic changes that occurred in the crab had potential for use in monitoring areas at risk from contamination.

Seasonal changes that affect glutathione status and related enzymes within the crab.

The crab is exposed to many stressors in its natural environment, including inter-individual competition, predation, contaminant exposure, climatic changes and seasonality. These influences have potential to affect the crab's behaviour and activity and hence may alter metabolic status. Of these stressors seasonality is known to particularly affect the crabs behaviour and sexual activity (Attrill & Thomas, 1996; Aargaard *et al.* 1995; Hunter & Naylor, 1993; and Crothers, 1968). The

results of the present study showed that seasonal changes also have a significant affect upon the crabs' metabolic responses to contamination, as well as the activities of the enzymes investigated.

During a summer investigation a group of crabs were exposed to a complex mixed effluent. These crabs exhibited reduced gill GST and GR activities compared to control animals. These parameters were also measured in crabs caught later in the year during the winter. In this instance the GST and GR activities of the gill tissues were unaltered. The only reasonable explanation for these contrasting results was that the season that the crabs were caught in was influential upon their metabolic responses to contamination, unless there was additionally the presence of seasonal pollution exposure. Seasonal pollution could occur by increased agricultural run-off during the wet seasons.

Other enzyme activities also varied depending upon the season in which they were measured. Crab gill GST activities were lower in the winter compared to the summer and gill GR activities were higher. The same pattern of seasonal GST activity has also been measured in the freshwater bivalve, *Sphaerium corneum*, (Looise *et al.*, 1996) and the American red crayfish, *Procambarus clarkii* (Nies *et al.*, 1991). However, this fluctuation of activity is not common to all species; GST activities in *Mytilus edulis* were found to be highest during the winter (Power & Sheehan, 1996).

The data obtained from these experiments do not provide insight into the reasons for seasonal variation of enzyme responses. The crab also shows seasonality of other biological parameters which may influence its antioxidant enzyme activities. The extent of xenobiotic accumulation within crab tissues changes according to the crabs' nutritional status (Depledge, 1989 and Scott-Fordsmund & Depledge, 1993) or changes in haemolymph protein composition (Bjerregaard, 1990 and Depledge & Bjerregaard, 1989). The nutritional status of the crab is governed by its foraging activity and this in turn controls the protein composition of haemolymph.

The crabs greatest foraging behavior occurs during the summer when the animals are more active (Attrill & Thomas, 1996, Aargaard *et al.* 1995, Hunter & Naylor, 1993 and Crothers, 1968). In this

set of experiments the crabs had higher haemolymph protein concentrations during the summer than during the winter when foraging activity is reduced. As protein composition affects the accumulation of metals in the haemolymph (Bjerregaard, 1990) this could also influence antioxidant enzyme activities.

Protein levels of other crab tissues were also affected depending upon the nutritional status of the crab. Crab gill and muscle protein concentrations decreased during winter exposure of crabs to high levels of contamination. It was hypothesised that in order to initiate antioxidant mechanisms, during the winter, that the crab had to metabolise protein reserves as nutritional status was low. Muscle and heart tissues are physically similar and share comparable antioxidant enzyme activity. However the muscle tissue protein diminished during toxicity exposure when the crab was of low nutritional status whereas heart tissue protein did not. This demonstrates the selectivity with which tissues are metabolised to meet the amino acid demands for antioxidant processes. The gill and muscle tissues of the crab loose protein content in preference to the vital heart organ.

In order to establish more thoroughly the influences that nutritional status of the crab has upon the protein content of tissues, glutathione status and enzyme activities; a group of crabs were starved prior to mixed effluent exposure. In this study the protein levels of crab haemolymph decreased in those animals that were simultaneously starved and exposed to mixed effluent. Whereas, the haemolymph protein of crabs that were only starved and not exposed to contamination was unaltered.

Haemolymph protein levels have also decreased in other populations of crabs that have been starved and exposed to contamination (Depledge, 1989). When crabs are starved they suppress their metabolism (Marsden *et al.* 1973) and their heart rate and oxygen consumption become depressed and they are less active (Depledge, 1985). These characteristics are also reflected during the seasonal variation of the nutritional state of the crab (Depledge, 1985). Suppressing the metabolism helps to prevent the crab from utilising a significant amount of essential amino acid reserves from

within its tissues. However, if the crab is challenged by toxicity the induction of antioxidant metabolism may be essential for its' survival. Therefore it is advantageous if under these circumstances the crab metabolises proteins from its non-essential tissues in order to release amino-acids for the anabolism of essential anti-oxidant proteins. In order to verify this hypothesis it would be necessary to measure a concomitant increase of antioxidant proteins; this has not been performed. However, decreased haemolymph protein concentrations did coincide with increased total antioxidant scavenging ability in this tissue.

Starvation also affected the enzyme activities of crab tissues. It decreased GST activity of gill tissue, hence lowered nutritional status could account for reduced GST activity during the winter. However, GR activity decreased in this study and was shown to be highest during the winter. Hence nutritional status was not responsible for the seasonal variability of GR activity. The starvation experiment did not mimic crabs experiencing a winter which may be the reason that GR activities did not increase.

It may be necessary for crabs to increase their gill GR activity during the winter to help to maintain glutathione levels during this period of low nutritional status. Starvation resulted in lowered glutathione status in crab gill. This effect of starvation has also been observed in other species (Ogasawara, *et al.*, 1989; Langley & Kelly, 1992; Jung & Henke, 1997; Shimizu & Morita, 1990; Hultberg, 1998; Lautermann *et al.*, 1995; and Vigano *et al.*, 1993). If the crab increases GR activity this may prevent the loss of oxidised glutathione from the cell and reduced glutathione levels can be maintained (Meister, 1989).

This study also revealed another mechanism that may be particularly beneficial to the survival of crabs during the winter. The total antioxidant scavenging ability of haemolymph tissue and the total glutathione content of gill tissue were depressed in starved crabs. However, when these crabs were exposed to mixed effluent their glutathione and total antioxidant scavenging abilities were reinstated. This mechanism has the potential to protect the crab against toxicological damage

during periods of low nutritional status. Other species that have lowered glutathione levels under induced starvation do not produce more glutathione during xenobiotic exposure. The net result is an increase of physiological damage as a result of decreased antioxidant scavenging ability (Schimizu & Morita, 1990; Hultberg, 1998; Lautermann *et al.*, 1995; Hoffman *et al.*, 1987; and Vigano *et al.*, 1993). The crabs therefore are better adapted for toxicological stress responses than other animals that have been investigated. This may also explain why crabs are such a successful species in polluted environments.

During final examination of the seasonality data, the data were analysed using multi-dimensional scaling techniques. This allowed the different experimental groups to be positioned on a two dimensional plot so that those that displayed the most similar results were grouped closest together. The dendrogram groupings, that were based upon the Bray-Curtis similarity matrix, placed the winter and summer exposures into two distinct groups. However, ANOSIM analysis of the data showed that the summer exposed crabs were the only set that exhibited statistically similar results; the exception being crabs exposed to the highest mixed effluent concentration.

A group of animals that are stressed will begin to demonstrate variances of biochemical and/or physiological data that are further removed from the mean result (Forbes & Depledge, 1996). This is explained in greater detail in Section 8.4. During the summer the animals have greater nutritional status, are fitter, and are similar in their ability to tolerate oxidative stress. Therefore, they are likely to display similar biomarker responses even when they are exposed to low amounts of contamination. However, a group of crabs experiencing a winter will be exposed to additional stressors such as reduced nutritional status and climatic changes. This will increase the variability of crabs' ability to tolerate additional stress such as contamination exposure.

The reason that crabs exhibit seasonality in their ability to tolerate stress may be associated with their sexual activity. Crabs are sexually active over the summer months July- September (Crothers, 1967). In order to reproduce successfully they require a high level of fitness and it is important that

they are able to tolerate induced stress levels. The reason for this is that reproduction is taxing upon animals' metabolic resources. For instance, the crab may have to fight with competitors for a mate which increases metabolic requirements and mortality risks; they may need to increase their foraging effort to obtain resources for gamete production or for egg production and spawning. These factors increase metabolic demands on the crab and may also increase their stress levels (metabolic costs of reproduction are reviewed by Sibly and Calow, 1986).

Many animals have an allocation of resources in which to reproduce, if resources are not sufficient then reproduction may not occur (Sibly & Calow, 1986). In some species there is considerable evidence for gamete and embryo resorption by parents that are stressed (Calow, 1973). Invertebrates that inhabit polluted environments have increased costs of maintenance due to increased antioxidant metabolism (Bayne, 1976) and the need for tissue repair and replacement or production of molecules (Sibly & Calow, 1986). Therefore this could reduce the allocation of resources available for reproductive purposes. Crabs that were exposed to the highest mixed effluent concentrations during the summer, displayed biomarker parameters that were more similar to those exhibited during the winter. Therefore, it is possible that they would not be fit enough to reproduce. Further experimental investigations are necessary to evaluate the implications of pollution exposure upon the crabs' reproductive ability.

The role of glutathione with regard to the total antioxidant scavenging ability in the crab.

In many studies, glutathione has been reduced in different species either by nutritional deprivation (Hultberg, 1998, Hoffman *et al.*, 1987 and Lautermann *et al.*, 1995), seasonal changes (Viarengo *et al.* 1991; Power & Sheehan, 1996 and Sheehan & Power, 1999), or exposure to glutathione diminishing compounds (Palmeira *et al.* 1995). The effects of contaminant exposure have been monitored to establish how reduced glutathione status affects the animals' ability to tolerate xenobiotic exposure. The findings have generally shown that decreased glutathione status increases

the animals' sensitivity to contamination (Jones *et al.*, 1995; Cookson & Pentreath, 1996; Kang & Enger, 1988; Kisara *et al.*, 1995; and Shimizu *et al.*, 1997).

In a preliminary experiment a group of *Daphnia magna* were exposed to BSO which reduces cellular glutathione concentrations. *Daphnia magna* that were simultaneously exposed to BSO and cadmium were found to show increased mortality compared to those that were exposed to cadmium alone. There are two well documented possibilities of why reduced glutathione status may have caused this affect; (i) glutathione had a direct role in the detoxification of the metal (Cookson & Pentreath, 1996; Peters *et al.*, 1995 and Kang & Enger, 1988); or (ii) glutathione was involved in the induction of metallothionein which combined with the metal to store it safely in the animals tissues (Chan & Cherian, 1992; Schlenk & Rice, 1998; Brouwer *et al.*, 1993, a & b, and Singhal *et al.*, 1987). The results of this experiment do not allow us to establish which of these mechanisms may have occurred.

A study was also performed using crabs to find how glutathione depletion affected their ability to tolerate pollution exposure. The crabs were injected with a solution of BSO, which depleted cellular glutathione concentrations in some of their tissues (Chapter 6). The total antioxidant scavenging ability of these tissues were determined to establish whether glutathione depletion had affected it. Glutathione-related enzymes were generally unaltered in response to BSO treatment; with the exception of the gill GST activities which were induced.

Reduced glutathione status did not affect the total antioxidant scavenging ability of the crab tissues that were investigated (haemolymph, muscle and gill tissues). This result complies with investigations made by Regoli & Winston (1998), who found that glutathione, ascorbic acid and uric acid only accounted for 35% of the total antioxidant scavenging ability of marine invertebrates. However, other evidence obtained from these experiments show that in certain circumstances glutathione played a greater role towards total antioxidant scavenging ability than could be deduced from measuring total antioxidant scavenging ability alone.

When the crab was subjected to high degrees of physical and toxicological stress, a correlation between glutathione concentrations and total antioxidant scavenging ability were established in several tissues. This shows that during periods of extreme stress glutathione metabolism must play a significant role towards total antioxidant scavenging ability. When glutathione was depleted by BSO the correlation between the two parameters disappeared, however, this did not affect the amount of total antioxidant scavenging ability. Therefore compensatory antioxidant mechanisms must be activated to make up for the loss of glutathione status. Further experimentation would have to be completed to verify which mechanisms these are.

Glutathione status was also correlated with glutathione reductase activity during periods of induced stress. During exposure to contamination the formation of glutathione complexes or the oxidation of glutathione can lead to a reduction in the amount of cellular glutathione. This occurs because enhanced levels of oxidised glutathione may be excreted from the cell more rapidly than they can be reconverted to the reduced form by the action of GR (Meister, 1989). However, if GR activities in the cell are sufficiently efficient, this can prevent cellular loss of glutathione levels (Langley & Kelly, 1992). Therefore, the amount of glutathione that remains in the crab tissues during increased toxicity and stress may be effectively established by the activity of GR. This could result in a correlation forming between GR activity and glutathione levels. The data correlations described here may be useful for establishing when crabs are suffering from induced stress in their natural environment.

Toxicological responses of different crab tissues

The crab tissues that were principally used in these studies were the haemolymph, gill and muscle. It became evident from the results obtained in these experiments that biomarker responses were triggered to different extents in these tissues depending upon the species of chemical used. Some of the toxicological responses were observed as significant changes of the biomarker parameters

measured. In other cases the toxicological response was observed as increased variability from the mean of biomarker responses (Depledge & Lundebye, 1996; Astley *et al.*, 1999; and Forbes and Depledge, 1996).

During the BSO experiment muscle glutathione levels were unaffected by the presence of BSO, however BSO caused increased variability of total antioxidant scavenging ability in this tissue. This response is indicative of increased stress in the muscle tissue. If the muscle specialises in the detoxification of BSO this could account for the chemical having no effect upon its' glutathione concentrations. In addition, correlations between total glutathione content and total antioxidant scavenging ability; and total glutathione content and GR activity were formed in the muscle tissues of BSO treated crabs. These correlations are also associated with a stress response induced by contamination exposure. BSO treatment did not induce these effects from the gill or haemolymph tissues. However, these tissues did display the same characteristics when the crabs were exposed to mixed effluent.

Metal analysis of the tissues showed that only the gill tissue accumulated the metal components of the mixed effluent, cadmium, zinc and copper. None of these metals were shown to accumulate in the muscle tissue. However, the muscle tissue had induced GST activity in response to the mixed effluent exposure. Therefore some other component of the mixed effluent must have caused this effect, (for instance ammonia or components of the engine oil). In contrast, the GST activity of the gill tissues was suppressed in response to mixed effluent exposure; this may have been due to the metal components of the mixed effluent as they accumulated here. Animals that are exposed to xenobiotics frequently have enzymes induced or suppressed in different tissues, the variable accumulation of the toxin within different tissues of the same animal may be responsible for this.

The accumulation of xenobiotics within different animals' tissues is also governed by other factors. These influences include nutritional status, (Scott-Fordsmand & Depledge, 1993; and Depledge, 1989) and seasonality; which has been monitored in several different species (Swaileh, 1996;

Hühnerfuss *et al.* 1995; Smaal & Vonck, 1997 and Weinstein, 1995). Crabs did not always show consistent biomarker responses towards the mixed effluent during different seasons. The biomarkers may have been affected by the seasonality of xenobiotic accumulation, as well as, the fluctuating nutritional status of the crab. Research into the seasonal accumulation of metals and organic pollution within the crabs' tissues must be completed in order to understand how this may affect their enzyme activities.

Xenobiotics accumulate and are detoxified to different extents within different crab tissue types; this results in biomarkers being either induced or suppressed. If biomarkers are applied to a field situation biomarker responses from several different tissues could provide vital information as to the nature of the contaminants that are present in the surrounding water.

Tissues were selected from the crab and the amount that each of them contribute to the crabs total antioxidant scavenging ability was determined experimentally. It was found that the tissues that showed the highest total antioxidant scavenging ability per gram of protein did not necessarily contribute the most towards total antioxidant scavenging ability in the crab. For instance the haemolymph which has the lowest total antioxidant scavenging ability per gram of protein, contributes significantly to the crabs overall antioxidant scavenging ability. The mid gut gland which is believed to be an active site of xenobiotic detoxification contributed less than muscle tissue. The reason that haemolymph and muscle tissues have such strong contributions is in part due to the amount of the crab's body that they occupy. For this reason the amount these tissues contribute towards the animals' total antioxidant scavenging ability can be easily underestimated.

Successful analysis of biomarker data.

The experiments performed in this study have shown that biomarker responses are influenced by many environmental and physiological factors including;

- The type of contaminant
- Season
- The nutritional status of the animal
- Variable accumulation of toxins within tissues (also related to the above parameters)
- Antagonistic effects of combinations of chemicals, (see Chapter 6)
- Sex and age of the animal (not covered in this study, Widdows, 1978 and Kirschen *et al.* 1992)
- Overall health of the animal
- Adaptation of a species to its environment, (Cossu *et al.* 1997 and Regoli & Principato, 1995).

All the above factors play important roles in determining biomarker responses. Therefore it is not realistic to assume that one off biomarker measurements of an area allows us to establish the presence of pollution. There is a necessity for continual monitoring or for monitoring several sites simultaneously.

A multiple biomarker approach was successfully applied in a field study, to identify polluted areas (Section 7). The neutral red retention time was a useful biomarker for distinguishing between contaminated and control sites. However, it was not successful in distinguishing between the extent of contamination at the different polluted sites. The antioxidant enzyme activities were more successful for distinguishing a pollution gradient among the different sites.

Initially the biomarker data were examined using traditional parametric methods of statistical analysis. However, as specific combinations or types of contaminants affect biomarkers of oxidative stress to different extents, a clear distinction of the pollution status from each site was not easily ascertained. The data were therefore analysed using multivariate statistical techniques; this successfully placed the contaminant and control sites into distinct groups. Multivariate analysis

also established differences among the polluted sites based upon different biomarker responses that were measured at them.

Frequently stress induced by factors such as decreased nutritional status and contaminant exposure increases the variation of biomarker responses from the mean in a given population, hence increasing the data distribution at different exposure levels (Lundebye & Depledge, 1996; and Forbes & Depledge, 1996). Some animals are better than others at controlling stress and so their antioxidant systems are induced to different extents. An increased distribution of data is sometimes indicative of a toxicological response of animals and is equally important as observing significant changes of biomarker responses between populations (Lundebye & Depledge, 1996). This is because increases of variation from the mean are often the initial response of a population to contamination before biomarkers are significantly induced (Forbes & Depledge, 1996). Therefore changes of data distributions have a useful application as early warning systems of contamination exposure. For example, metallothionein concentrations were only found to become induced under very high levels of metal exposure, changes of metallothionein distributions may be a better indicator of when metal toxicity is increasing but not yet at critical levels.

Figure 8.1 effectively explains how data distributions change with increased stress. When a group of animals are unchallenged by stress the distribution of biomarker responses may resemble the range shown in Position a; further stress may increase the distribution so it resembles Position b. If ANOVA analysis of the data at Positions a and b is performed there will not be a statistical difference observed between the two populations. If the population at Position b is further stressed the distribution of the data may become smaller. This occurs because the increased stress surpasses the point at which any of the animals are able to tolerate the toxicity, so their biomarker responses become more similar to each other and the distribution of the data decreases (Position c). There is now a statistical difference between Positions a and c using ANOVA analysis. Figures 5.4 and 5.5 demonstrate this type of toxicological response, crabs exposed to mixed effluent exposure had

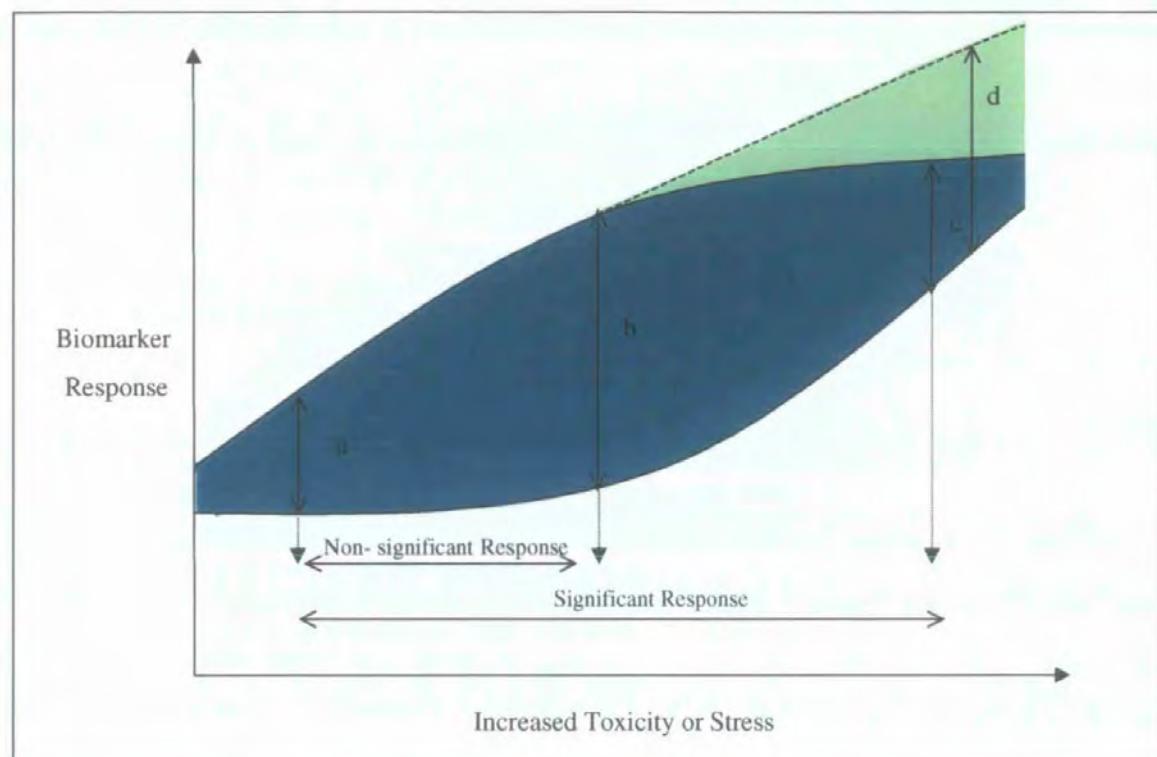


Figure 8.1 Data distributions of biomarker responses from animals responding to stress. The coloured areas show the changes of the distribution of data caused by increased toxicity or stress. The letters signify different positions of the toxicological response.

increased data distributions of their GR and GP activities compared to the controls. When the crabs were further stressed by starvation and also exposed to the mixed effluent the distribution of their activities reduced.

Alternatively, the distribution of the most stressed animals may not reduce as the crabs still have varying abilities of inducing the biomarker response at high exposure levels. However, a statistically significant result is still observed because the data set is significantly removed from the control population (Position d). Figure 5.9 demonstrates this type of toxicological response; the distribution of metallothionein values increased in the fed crabs in response to mixed effluent exposure compared to controls. When the crabs were starved and exposed to contaminant exposure the distribution of the values remained high however, they are significantly removed from the control population.

The ANOVA analysis within the PRIMER programme allows distinctions to be made between exposure groups on the basis of data distributions in addition to significant biomarker responses. This is a particularly effective method for analysing toxicological data as all the factors listed at the beginning of the section are in part responsible for increased variability of biomarker measurements. Therefore, this programme is useful in providing a clear overview of results of biomonitoring toxicity exposures and may be more useful to elicit the early stages of toxicological responses.

Recommendations for future work

The work completed in this study has provided potential for further investigations into antioxidant mechanisms in the crab. Further studies would be particularly beneficial for the future

understanding of how invertebrates tolerate oxidative stress and also how some species are able to be more successful than others in areas that are affected by pollution.

- *Seasonality of biomarker responses*

Research has shown that seasonality affects biomarker responses in the crab. There is also evidence that nutritional status and seasonality influence the accumulation of xenobiotics within different crab tissues. Further research would be useful to establish whether differential xenobiotic accumulation has a direct affect upon the enzyme activities measured in these tissues. This would aid interpretation of biomarker responses during different times of the year. Also, if we can establish which types of compounds accumulate within different crab tissues and the biomarkers that are affected as a result, this would provide clues as to which types of chemicals are causing the effect. For instance increased metallothionein concentrations are indicative of metal accumulation (Pedersen *et al.*, 1997 & Pedersen, 1996).

- *Do crabs metabolise protein reserves to initiate antioxidant mechanisms?*

Crabs were shown to have reduced protein content of some of their tissues when they were of low nutritional status and were exposed to contamination. In addition, reduced haemolymph protein under these conditions was accompanied by a concomitant increase in total antioxidant scavenging ability. These results suggest that protein is metabolised to initiate antioxidant proteins. The measurement of the antioxidant enzyme protein levels would verify this hypothesis and help us to understand mechanisms of toxicological responses in animals of reduced nutritional status.

- *Effects of increased stress upon reproductive capabilities*

Results showed that mixed effluent exposure increased crabs' stress levels during the summer. Does this effect have any influence upon the animals' ability to reproduce? If so, crab populations could be affected by pollution exposure. Crabs are at the bottom of many food chains so their decline would also be influential upon the status of higher species.

- *What are the compensatory mechanisms initiated when glutathione levels are depleted*

Evidence from this investigation has shown that under induced stress glutathione concentration is correlated with total antioxidant scavenging ability. When glutathione is depleted this does not affect the overall antioxidant scavenging ability of these tissues, which suggests that other antioxidant mechanisms may be initiated. The monitoring of alternative antioxidant proteins would be useful to establish which antioxidant mechanisms are compensating for the absence of glutathione.

- *Further use of incorporating data distributions into the interpretation of toxicological data.*

As yet the use of multi-variate analysis of biomarker data is in its infancy (Machala *et al.*, 1997; Fairbrother *et al.*, 1998; and Astley *et al.*, 1999). More research must be undertaken into how the variance of biomarker data increases and diminishes with respect to increasing contamination exposure. Once this phenomenon is better understood then the incorporation of multi-variate statistical analysis techniques into the analysis of biomarker data can be scientifically accepted.

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Multi-variate Analysis of Biomarker Responses in *Mytilus edulis* and *Carcinus maenas* from the Tees Estuary (UK)

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A suite of six biomarkers was measured in shore crabs (*Carcinus maenas*) and mussels (*Mytilus edulis*) deployed at, or native to, the Tees Estuary in north-east England, as well as at several control sites. LC-50 tests on water samples from the field sites were carried out using the marine copepod *Tisbe battagliai* and Microtox assays. Biomarker responses varied among the sites. Results were analysed using novel multivariate statistical procedures. A clearer overall picture was obtained from multi-dimensional scaling plots than by examining the data for different biomarkers individually. It was possible to distinguish among contaminated and control sites, with the contaminated sites being further differentiated to reflect a pollution gradient. It was concluded that multi-dimensional scaling procedures are a valuable additional tool for improving the analysis and interpretation of complex suites of biomarker data. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

The primary aim of most biomarker studies is to detect and quantify the extent of exposure to, and the biological effects of anthropogenic chemical contamination. Most studies performed to date have been undertaken in the laboratory. Several texts describe the diverse techniques available (for example, McCarthy and Shugart, 1990; Peakall, 1992; Phillips and Rainbow, 1993; Calow, 1993, 1994; Forbes and Forbes, 1994; Kramer, 1994; Peakall and Shugart, 1993; Fossi and Leonzio, 1994). Far fewer biomarker studies have been implemented *in situ* (Lange and Lange, 1997; Phillips and Rainbow, 1994; Chapter 9, GEEP workshops, Oslo, Bremmerhaven, Bermuda). In the controlled conditions of the laboratory, it is relatively straightforward to standardise biomarker assays and to regulate the

chemical exposures that organisms receive, so that cause-effect and indeed, exposure-response relationships, can be established. Field studies pose far greater difficulties due to the complex and fluctuating nature of the environment, and interactions among organisms within ecological communities (e.g. predator-prey relationships). However, the latter are arguably more important than laboratory studies since they address the integrated impact of anthropogenic and environmental stressors. Animals *in situ* are exposed to a variety of natural stressors including, salinity stress, hypoxia, thermal stress and predation. These factors, together with phenotypic and ontogenetic differences in susceptibility to stress, result in increased variability in biomarker responses (Depledge and Lundebye, 1996; Forbes and Depledge, 1996). Consequently, data collected in field studies may be much harder to interpret than data from controlled laboratory experiments. To address this problem it may be better to study patterns of biomarker responses as opposed to examining each biomarker singly. An analogy can be drawn with the study of the ecological impacts of pollutants: clearly, it is better to study patterns of change among several species at a site as opposed to changes in just one species (Clarke and Warwick, 1994).

To date few field trials have been undertaken using a multiple biomarker approach. Those that have been performed have usually examined the biomarker responses individually (GEEP workshops, Livingstone *et al.*, 1995; Fossi *et al.*, 1996; van der Oost *et al.*, 1997; Burgeot *et al.*, 1996). A few studies have attempted complex analyses using several biomarkers. These include a risk ranking method designed and tested by Hartwell *et al.* (1997).

In the present study, a suite of biomarker responses was measured in selected marine invertebrates collected from or deployed at, several field sites. Data were analysed using multivariate analysis. Previously, multivariate analysis has been successfully applied to community data by Tapp *et al.* (1993), Olsgard and Gray (1995);

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Hall *et al.* (1996) and Olsgard *et al.* (1997). It has also been employed in an aquatic biomarker study using fish by Machala *et al.* (1997) and in a terrestrial field trial using voles by Fairbrother *et al.* (1998).

Materials and Methods

Experimental protocol

Crabs (*Carcinus maenas*) and mussels (*Mytilus edulis*) were caught from the south-west of Britain and were deployed at selected field sites (see later). In some instances animals indigenous to these sites were also caught. Biomarker responses were measured in both "deployed" and "indigenous" sets of animals and the results compared using multivariate analysis.

Site description

The study was performed near the mouth of the Tees estuary on the north-east coast of England, the Tees was chosen as the study area as it is a highly industrialised area and includes Teesport, the third busiest port in England (Office for National Statistics, 1998). It comprises extensive petrochemical storage and production facilities and a nuclear power station. The number of chemical and petrochemical operations in the Tees estuary is said to be the greatest in Europe (Davies and Tomlinson, 1991). In recent years, water quality has improved, changing it from a chronically polluted estuary supporting no or little life in many reaches, to one that is substantially less polluted (Tapp *et al.*, 1993). To

assess biomarker responses in selected marine invertebrates, control sites, relatively free from contamination were required to give an indication of the sensitivity of the methods. Two control sites were identified in southwest England and one in the north-east of England at which chemical and biological water quality were graded *a* or *b* (very good or good, respectively) by the Environment Agency (1995, 1996).

Crabs and mussels were deployed inter-tidally at sites contaminated to different extents along the Tees estuary (see map – Fig. 1). Sites were selected to represent a presumed pollution gradient. Crabs and mussels were sampled from both sides of the estuary mouth at the Royal National Lifeboat Institute station at South Gare (NZ 555 275) and at the breakwater at the opposite side of the estuary mouth at North Gare (NZ 543 286). Crabs from south-west England were also deployed at the North Gare and South Gare sites. Other crabs and mussels were sampled and deployed in the Seal Sands nature reserve by Greatham Creek (NZ 522 255) and opposite Hartlepool Power Station (NZ 537 263). The final deployment site was opposite Dab Holm Gut on a site owned by Tees Storage Ltd. (NZ 543 243).

Control groups of crabs and mussels from south-west England were deployed at Robin Hoods Bay (NZ 958 049) on the east-Yorkshire coast in the north-east of England, at Bantham (SX 663 441) on the River Avon estuary and Mothercombe (SX 620 477) on the River Erme estuary in Devon. Crabs and mussels were also sampled from these sites, except Robin Hoods Bay

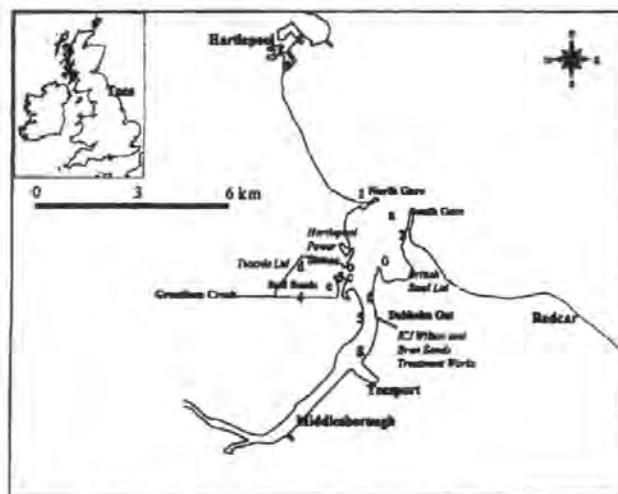


Fig. 1 Detailed map of the Tees Estuary showing the deployment sites (number) and the Environment Agency sampling sites (letter). Major industrial sites are shown in italics and towns in bold. Field sites: 1, North Gare; 2, South Gare; 3, Hartlepool Power Station; 4, Greatham Creek; 5, Tees storage. Environment Agency sampling sites: (a) the Gares; (b) Phillips Approach North; (c) Phillips Approach South; (d) Seaton Snook light North; (e) Tees control site middle; (f) Redcar jetty; (g) Smiths docks.

where crabs were present, but no natural mussel populations were found.

Sampling and deployment

Green, inter-moult male crabs of carapace width 5-7 cm were caught using baited drop nets. Crabs used in the deployment experiment were caught at Bantham on the River Avon in Devon. Prior to deployment they were kept in aerated tanks containing biologically filtered sea water. The test animals were transported in buckets containing small amounts of sea water and tissue paper. The buckets were kept cool by placing them in larger containers fitted with cool-packs. The crabs were fed to satiation with squid prior to deployment. A group of these animals were transported back to the River Avon where they were deployed with animals which had not travelled. This was undertaken to establish whether the transportation of the animals had any effect upon their biomarker responses.

Deployed crabs were held intertidally for two weeks in cages made of stainless steel mesh measuring 80×60 cm, with a perspex lid and divided into eight compartments. Each compartment contained a terracotta flowerpot for the crabs to shelter in. The cages had four extended legs which were wedged into the mud so that they were as stable as possible. The cages were positioned so that they were accessible for approximately 1.5 h either side of high tide time. During the two week deployment the crabs were fed twice with squid.

Mussels in the study were between 4 and 5 cm long and were collected from Sharrow Point, Whitsand Bay in Cornwall. They were maintained in aerated tanks except during transportation when they were held on tissue paper moistened with sea water and placed on ice packs in cool boxes. The mussels were deployed for 10 days in cages made from plastic-coated garden netting measuring 40×10×40 cm, which was attached to the side of the deployed crab cages.

Crab dissection and tissue preparation

Crab haemolymph was removed using a hypodermic syringe inserted at the base of the third walking leg of each animal and snap frozen in liquid nitrogen. Individuals were then killed by destruction of the thoracic ganglion. The gill tissues and midgut gland were removed and snap frozen in liquid nitrogen. Gill tissue was homogenised on ice in (1:2 wet weight of tissue: volume of buffer) 0.01M Tris-HCl pH 7.6 and a 0.03% detergent, NONIDET P-40 (Sigma N3516). The homogenate was sonicated for 15 s at 40% output power and spun for 30 min at 15,000 g at 4°C. The resulting supernatant was removed and frozen at -80°C until required for enzyme analysis. Dissections of the crabs was carried out on site.

Enzyme assays

The enzyme assays were performed on blood and/or gill tissue. The glutathione peroxidase (GP) assay was

adapted from Braven *et al.* (1989). The assay solution was made up to 3 ml in quartz cuvettes containing 20 µl of haemolymph, 50mM Tris-HCl buffer pH 7.6, 4mM GSH (glutathione), 10 µl glutathione reductase and 0.2mM NADPH. The mixture was incubated for 10 min and the reaction was initiated with 75 µl of 15mM t-butyl hydroperoxide. Changes in absorbance of the sample were monitored at 340 nm, for 1 min on a Perkin Elmer lambda 7 uv/vis spectrophotometer. The glutathione reductase (GR) assay was similarly adapted from Braven *et al.* (1989). The assay solution was made up to 2.85 ml with 0.22M sodium phosphate buffer and 2mM EDTA (ethylenediamine tetra-acetate) pH 7.0, 150 µl of 1mM GSSG (oxidised glutathione) and 100 µl of tissue sample in quartz cuvettes. The reaction was initiated using 150 µl of 2mM NADPH. Changes in absorbance were followed at 340 nm for 3 min at 25°C. The glutathione-S-transferase (GST) assay was that of Habig *et al.* (1974). A quartz cuvette containing 1.9 ml of 0.1M potassium phosphate buffer pH 6.5, 1mM GSH and 0.05mM of CDNB (1chloro-2,4-dinitrobenzene) was incubated for 10 min at 25°C. After which the reaction was initiated using 100 µl of sample tissue and the change in absorbance monitored for 3 min at 340 nm.

Metallothionein analysis

Metallothionein concentration (MT) in crab gill was determined by a spectrophotometric method using DTNB (5,5-dithiobis-2-nitrobenzoic acid) (Viarengo *et al.*, 1997). Sample preparation was modified but otherwise the technique was as described originally. To prepare the samples, 1 g wet weight of gill tissue was ground to a fine powder in liquid nitrogen, 3 ml of ice cold 1mM DTT, (dithiothreitol) and 30 µl of PMSF (phenylmethylsulphonylfluoride) solution were added. The mixture was sonicated (3×15 s) at 40% output power and another 30 µl PMSF added. The resulting mixture was ultracentrifuged at 100 000 g and 4°C for 70 min and stored at -80°C until analysis. The absorbance was read at 412 nm and the metallothionein levels were quantified by comparison with a reduced glutathione standard.

Neutral red retention time

Neutral red retention time (RT) was measured on site in crab and mussel haemolymph using the method of Lowe *et al.* (1995). The only modification to the method was the use of an anti-coagulant buffer with crab haemolymph to prevent clotting. The crab physiological saline (0.5M NaCl, 11mM KCl, 12mM CaCl₂.6H₂O, 26mM MgCl₂.6H₂O and 45mM Tris/HCl pH 7.4.) and the crab anti-coagulant solution (0.45M NaCl, 0.1M glucose, 30mM trisodium citrate, 23mM citric acid, and 10mM EDTA, pH 4.6) were based on the protocols of Söderhall and Smith (1983) and Smith and Ratcliffe (1978), respectively. The slides were observed using a portable light microscope every 15 min.

Heart rate

Heart rate was measured for at least 8 h in the laboratory on *M. edulis* using the technique described by Depledge and Andersen (1990). A non-invasive infra red transducer was glued (Loctite 314) directly on to the mid dorsal line of the shell just behind the posterior termination of the hinge. Heart rate was recorded every minute and stored on disk for later analysis. The animals from the different sites were monitored whilst submerged at ambient temperature.

Tisbe battaglii LC₅₀ and Microtox assays

The procedure followed to assess the *Tisbe battaglii* LC₅₀ (lethal concentration to 50% of the animals) was that of Williams *et al.* (1993). The microtox procedure was that laid out in the manual supplied by Microbics Corporation (1992).

Results

Biomarker responses of deployed and indigenous animals as well as toxicity test results are summarised in Tables 1 and 2. There were no significant differences, among the majority of biomarker responses, between different sites for indigenous crab populations ($p > 0.05$). Greatham Creek was the only site where GST activity in the crab haemolymph was decreased, when compared with the crab activities at the other sites. The neutral red retention time was depressed in both indigenous crabs and mussels at the Tees estuary ($p < 0.05$). The indigenous mussels at Hartlepool Power Station and the North and South Gares also had elevated heart rates compared with control sites.

The crabs deployed at Tees Storage had significantly different biomarker responses in most biomarker techniques measured, the exceptions were GR activity in

TABLE 1
Mean and standard deviation of the *Tisbe battaglii* LC-50 assay and the biomarker results on the mussel *Mytilus edulis*.*

Organism Site	<i>T. battaglii</i> LC-50	<i>M. edulis</i>	HR	RT
Avon	106 ± 19	Ind.	22 ± 3	81 ± 24
Erme	87 ± 11	Dep.	23 ± 4	75 ± 32
Whitsand Bay	95 ± 8	Ind.	23 ± 3	88 ± 37
Robin Hoods Bay	81 ± 11	Dep.	26 ± 8	84 ± 28
North Gare	85 ± 10	Ind.	23 ± 3	94 ± 30
South Gare	90 ± 12	Ind.	24 ± 3	103 ± 27
Hartlepool Power Station	56 ± 16	Dep.	31 ± 4	88 ± 34
Greatham Creek	31 ± 19*	Ind.	56 ± 11**	53 ± 20
Tees Storage	37 ± 20*	Dep.	30 ± 3*	54 ± 25
		Dep.	37 ± 7	49 ± 29
		Dep.	27 ± 6*	22 ± 24*

* Ind - indigenous; Dep - deployed; HR - heart rate in beats minute⁻¹. RT - neutral red retention time in minutes. The number of samples (*n*) used in the mussel biomarkers is always 8.

crab gill and also MT concentrations. The neutral red retention time was reduced in both the deployed crabs and mussels ($p < 0.05$). The mussels deployed at Hartlepool Power Station were also found to have significantly increased heart rates.

The Avon control site and the Greatham Creek site were the only sites at which some of the biomarker responses were significantly different between the indigenous and deployed crabs. The biomarker responses of crabs indigenous to the River Avon when compared with those deployed there, revealed significantly elevated MT and GST values, while GP activity was depressed (Tables 1 and 2, $p > 0.05$). At Greatham Creek the deployed crabs blood GST activity was elevated by ca. 3 fold compared to indigenous crabs. The Microtox™ assay did not indicate toxicity in water samples from any of the sites. The *T. battaglii* assay highlighted Greatham Creek and Tees storage as being toxic.

The crabs that had travelled to the Tees estuary and back and were redeployed at the River Avon gave biomarker responses which were not significantly different to crabs that had not travelled.

Multivariate analysis

Multivariate analysis based on Bray-Curtis similarity coefficients and group averaged sorting (Bray and Curtis, 1957) was performed on the data using the PRIMER (Plymouth Routines in Marine Environmental Research) program v4.0, (Plymouth Marine Laboratory). The biomarker data from the indigenous and deployed crabs were analysed separately. Cluster analysis and multi-dimensional scaling (MDS) (Kruskal and Wish, 1978) were performed on the averaged data of each individual biomarker test for every site. If MDS is run on data which is not averaged the resulting stress (a measure of the accuracy of the results) is 0.18, this value is at the limit of reliability for an accurate description (Clarke and Warwick, 1994). For the averaged data the stress improves to a value of 0.00. The dendograms resulting from performing cluster analysis on the averaged biomarker responses for all the indigenous animals are shown in Fig. 2(a) and (c) for all the deployed animals. The MDS plots for the indigenous and deployed animals are shown in Fig. 2(b) and (d), respectively. The biomarkers used in the multivariate analysis for both indigenous and deployed animals were; RT, GST and GP in crab blood; GST, GP, GR, and MT in crab gill. In the multivariate analysis of deployed animals, mussel RT and heart rate data were also included. Statistical analyses involving all the data (at least five replicas from each site) was performed using the ANOSIM program in PRIMER, to ensure that the clusters formed by the dendograms and MDS plots were statistically significant.

The dendrogram of the biomarker results of deployed animals placed the control sites in a discrete group and the Tees sites, with the exception of Tees storage, in another discrete group. Tees Storage site was positioned separately from both of these groups. The groupings

TABLE 2
Mean and standard deviation of all the biomarker results on the crab *Carcinus maenas*.^a

Site	Tissue	No. samples (n)	Blood RT	GST	GP	Gill MT	GST	GP	GR
Avon	Ind.	8	81 ± 38	1.9 ± 0.86	0.327 ± 0.099	37 ± 13	0.368 ± 0.185	0.231 ± 0.064	5.31 ± 1.21
	Dep.	8	86 ± 25	3.85 ± 1.88*	0.499 ± 0.138	103 ± 58**	0.168 ± 0.148	0.106 ± 0.153	3.28 ± 2.25
	Dep.	8	81 ± 42	3.55 ± 1.81	0.557 ± 0.156	76 ± 58	0.184 ± 0.056	0.104 ± 0.029	2.79 ± 1.74
Erme	Ind.	8	62 ± 27	2.73 ± 1.05	0.441 ± 0.099	31 ± 8	0.269 ± 0.105	0.168 ± 0.038	3.92 ± 0.62
	Dep.	6	79 ± 38	3.05 ± 1.31	0.354 ± 0.117	38 ± 16	0.248 ± 0.158	0.205 ± 0.079	4.1 ± 2.14
Robin Hoods Bay	Ind.	6	79 ± 38	3.05 ± 1.31	0.354 ± 0.117	38 ± 16	0.248 ± 0.158	0.205 ± 0.079	4.1 ± 2.14
	Dep.	6	102 ± 16	5.04 ± 1.38	0.419 ± 0.102	30 ± 4	0.295 ± 0.130	0.222 ± 0.16	3.62 ± 2.08
North Gare	Ind.	7	11 ± 11*	2.75 ± 1.01	0.399 ± 0.230	40 ± 15	0.296 ± 0.244	0.141 ± 0.027*	5.24 ± 1.74
South Gare	Ind.	8	15 ± 16*	3.46 ± 1.74	0.311 ± 0.153	35 ± 16	0.199 ± 0.057	0.142 ± 0.034*	3.65 ± 0.8
	Dep.	7	45 ± 34	3.63 ± 1.55	0.415 ± 0.19	24 ± 4	0.175 ± 0.086	0.148 ± 0.039	4.35 ± 1.91
Hartlepool Power Station	Dep.	8	26 ± 13*	4.84 ± 1.54	0.302 ± 0.057	31 ± 7	0.274 ± 0.126	0.166 ± 0.047	3.22 ± 1.14
Greatham Creek	Ind.	8	4 ± 7*	1.11 ± 0.73*	0.496 ± 0.112	27 ± 5	0.201 ± 0.034	0.168 ± 0.035	6.24 ± 2.99
	Dep.	8	24 ± 14*	3.25 ± 1.62	0.38 ± 0.109	30 ± 7	0.222 ± 0.052	0.163 ± 0.054	2.97 ± 1.2
Tees Storage	Dep.	8	15 ± 14*	7.62 ± 4.63*	0.708 ± 0.31**	42 ± 12	0.083 ± 0.026**	0.092 ± 0.032*	4.91 ± 2.99

^a Ind - indigenous; Dep - deployed; Dep* - Crabs re-deployed at the Avon having travelled up to the Tees estuary and back; RT - neutral red retention time in minutes; GST - glutathione-S-transferase activity in $\mu\text{M mg}^{-1}$ protein min $^{-1}$ for gill tissue and in nM mg^{-1} protein min $^{-1}$ for blood; GP - glutathione peroxidase activity in $\mu\text{M mg}^{-1}$ protein min $^{-1}$; MT - Metallothionein concentration in $\mu\text{g g}^{-1}$ wet weight $^{-1}$; GR - glutathione reductase activity in $\text{nM mg protein}^{-1}$ min $^{-1}$; * - statistically and significantly different from the majority of control sites, ** - statistically and significantly different from the majority of other sites.

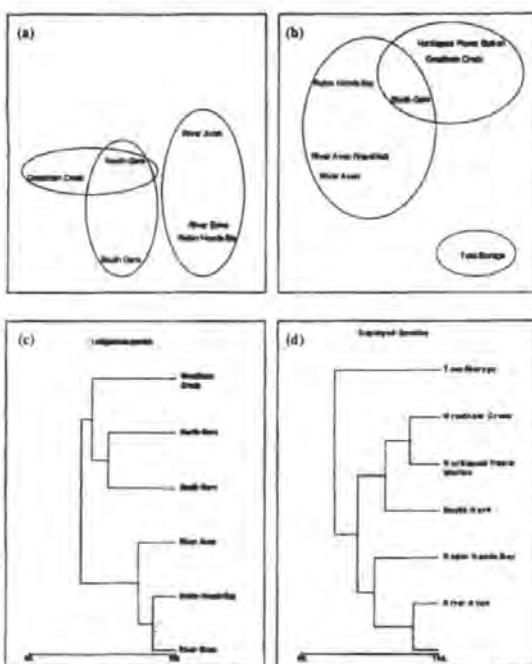


Fig. 2 (a)-(d) MDS plots and dendograms for the indigenous and deployed biomarker responses in crabs and mussels.

were confirmed by the MDS plot. Statistical analyses showed that all the biomarker results from the control sites were significantly different from those from con-

taminated sites ($p < 0.001$). The South Gare site was an exception. Tees storage group was statistically significantly different from the other two groups ($p < 0.01$).

The dendrogram of the indigenous animal biomarker results clustered all the control sites and all of the contaminated sites together, as did the MDS plots (see Fig. 2(a) and (b)). Statistical analysis confirmed this; the indigenous animals grouped at the control sites were significantly different from the indigenous animals from the Tees sites ($p < 0.01$). No statistical differences between any of the control sites were observed; however on the Tees estuary, Greatham Creek was statistically significantly different from the South Gare site ($p < 0.05$).

Environmental data

The chemical data available from the Environment agency water quality analyses is shown in Table 4 and results from the literature are shown in Table 3. The chemical data confirmed the divisions observed using multi-variate analyses of biomarker data. The Tees storage site has the highest concentration of chemicals present, especially organics, followed by Greatham Creek and Hartlepool Power Station sites, and finally the Gare sites.

Discussion

Laboratory exposures of invertebrates to different chemical pollutants produce biomarker responses which fluctuate depending upon the species of animal used and also the nature of the chemical. GST activity is increased in response to copper and cadmium in the crab *Oziotelphusa senex senex*. In contrast GST activity is reduced in the same animal in response to pesticides, (Reddy *et al.*, 1996). However GST activity has been shown to decrease in response to pesticides in the fresh water mussel, *Sphaerium corneum* (Boryslawskyj *et al.*, 1988). The fluctuation of different biomarkers in response to different toxicants provides a pattern of results which can give clues as to the type of pollutant that is causing the observed effect.

Multivariate analysis techniques are a useful tool for interpreting biomarker data as they produce a two-dimensional pattern of the degree of similarity between different groups of data. MDS is particularly useful for highlighting sites at which more detailed analysis of chemical contamination would be useful. The clustering of the Tees sites formed from using multivariate techniques corresponded well with the available chemical data. The highly polluted nature of the Tees storage site which was singled out by the biomarker responses was confirmed by the Environment Agency data and information found in the literature. The results also indicated a pollution gradient, suggesting that animals from the Tees sites were more stressed than the control animals and that there was a pollution stress gradient down the estuary. The presence of a pollution gradient was further supported by the overall responses of indigenous crabs at the Gares which were similar to one another and closer to the results of indigenous crabs at control sites

than the crabs at Greatham Creek. The South Gare and Greatham Creek sites were significantly different to each other. Similarly, the crabs deployed at South Gare were not significantly different to the control crabs suggesting that the combined biomarker responses were following a pollution gradient.

The results were also consistent with multivariate analyses of ecological community structures at the same sites which have been performed by other authors (Shilabeer and Tapp, 1989; Tapp *et al.*, 1993; Hall *et al.*, 1996). Tapp *et al.* (1993) found evidence of increased community stress at the end of Dabholm Gut which is opposite the Tees storage site, which was the most contaminated site in this study.

The significant differences observed in biomarker responses between crabs which were indigenous to the River Avon and caught prior to those that were deployed there, could be explained by a period of heavy rainfall during the first three days of the deployment. This results in high nutrient and phosphate loading from agricultural run off or a high concentration of phenols from Dartmoor. Phenols are a recognised natural contaminant source in the area (Environment Agency, 1998). The differences in biomarker responses due to the incident did not influence the division of the sites into control and contaminated groups after multivariate analysis. This emphasises the advantages of using more complex statistical techniques. The differences observed did however highlight the need for several control sites.

If biomarkers and multivariate analysis are to become established as recognised monitoring tools it is important to find a suite of biomarkers that most compliment each other. This can be achieved by looking at the amount each biomarker contributes towards the Bray-Curtis similarity matrix. Using the primer programme, an analysis known as SIMPER analysis can be carried out which lists in decreasing order the influence of each biomarker towards the dissimilarity matrix. Following SIMPER analysis Tables 5 and 6 were produced ranking the contribution of each biomarker towards dissimilarity between sites. The tables show that the biomarker that distinguished the most among sites was the neutral red retention time assay and the least, were the mussel heart rate and metallothionein assays. The method can be refined by replacing the least discriminatory biomarkers with others that are more selective. Established techniques could also be included such as *Tisbe battagliai* LC-50, which indicated toxicity at three of the sites in this study.

The analyses of biomarker data using multi-variate statistical techniques has the potential of being an effective ecotoxicological tool. In this particular instance the method proved to be more sensitive than using conventional toxicity tests, *Tisbe battagliai* and Microtox™. The method could be useful for use in Direct Toxicity Assessment, if used in conjunction with the toxicity tests already in use.

TABLE 3
The water quality information available from the literature about the Tees estuary field sites.*

Information source	Contaminant	Monitoring site: Tees estuary or Offshore Tees (*)	South Gare	Phillips approach	Redcar Jetty	Smiths Docks
Blackburn and Waldock, 1995 (not detected at 'clean sites')	Nonylphenol				3.1 µg l ⁻¹ (D) 5.2 µg l ⁻¹ (TE)	1.3 µg l ⁻¹ (D) 1.42 µg l ⁻¹ (TE)
Dawes and Waldock, 1994 (all sampled September 1992 volatile organic compounds below detection limits at most other 'clean sites')	Octylphenol Chloroform 1,1,1-trichloroethane	286 ng l ⁻¹ 29.8 ng l ⁻¹			13 µg l ⁻¹ (TE) 1490 ng l ⁻¹ 94.2 ng l ⁻¹	11500 ng l ⁻¹ 602 ng l ⁻¹
Laslett, 1995	1,2-dichloroethane bromodichloroethane dibromochloroethane Cadmium Copper Lead	1210 ng l ⁻¹ < 10 ng l ⁻¹ 0.02-0.042 µg l ⁻¹ (1991) 0.015-0.097 µg l ⁻¹ (1992) 1.3-1 µg l ⁻¹ (1991) 0.449-4 µg l ⁻¹ (1992) 0.096-0.920 µg l ⁻¹ (1991) 0.054-0.820 µg l ⁻¹ (1992)	10 ng l ⁻¹		1410 ng l ⁻¹ 151 ng l ⁻¹ 67 ng l ⁻¹	4020 ng l ⁻¹ 1160 ng l ⁻¹ 172 ng l ⁻¹
Law, 1997 (samples taken 15/6/ 94) unless otherwise indicated	total PAH's	*61 ng l ⁻¹ (U,HW) 21 ng l ⁻¹ (U,LW)	2193 ng l ⁻¹ (U,HW) 4220 ng l ⁻¹ (UR,LW) 2403 ng l ⁻¹ (D,HW)	1120 ng l ⁻¹ (U,HW) 2537 ng l ⁻¹ (U,LW) 1730 ng l ⁻¹ (U,LW) 3408 ng l ⁻¹ (D,HW) 9404 ng l ⁻¹ (U, 7/7/95) 3888 ng l ⁻¹ (D,7/7/95)	2689 ng l ⁻¹ (U,HW) 2364 ng l ⁻¹ (U,LW) 3312 ng l ⁻¹ (D,HW) 713 ng l ⁻¹ (D,LW) 816 ng l ⁻¹ (D,7/7/95) 548 ng l ⁻¹ (D,7/7/95)	

* D - dissolved; TE - total extractable; U - water sample unfiltered; HW - high water; LW - low water; (Date) - date water sample taken.

TABLE 4
Environment Agency water quality samples taken from the Tees estuary between May and October 1998.*

Sampling Site	Pb	Cd	Cu	Zn	Hg	NH ₃ -N (mg l ⁻¹)	TBT	C ₂ Cl ₄	CHCl ₃	1,2-DCE	DO%
Gates											
Tees at the gates	<2.5-3.61	<0.25-0.508	<0.5-2.02	5.72-28.6	<0.02-0.02	<0.02-0.07	<0.005	<0.1	<0.1-0.18	<0.1	49-82
Greatham Creek											
Phillips Approach North	<2.5-2.58	<0.25-0.121	2.02-3.02	11.6-15.8	<0.02	na	na	na	na	na	35
Phillips Approach South	2.5-53.6	0.147-<0.25	0.647-5.33	7.9-39.3	0.02	<0.02-0.085	0.008	0.14	0.17-0.43	0.24	43-113
Seaton Snook light north	<2.5-2.39	<0.25-0.164	1.17-2.43	9.17-26.3	<0.02	na	na	na	na	na	48-62
Tees control site middle	2-5.07	0.116-<0.25	1.22-3.19	7.84-23.7	0.06	<0.02-0.13	na	na	<0.1-0.99	na	21-78
u/s dabholm gut											
Redcar jetty	2.57-6.85	0.094-0.311	1.42-7.41	10.1-163	<0.02-0.02	0.05-2.48	<0.005-0.012	<0.1-0.68	<0.1-2.1	<0.1	25-110
d/x dabholm gut											
Smiths docks	3.3-21.6	0.105-0.503	2.36-7.41	23.7-64.8	<0.02-0.07	0.23-13.4	<0.005-0.013	<0.1-0.19	0.1-1.6	<0.01-0.77	68-109

*2-4 samples were taken at each site during this period. The concentrations shown are the maximum and minimum detected during the sampling period in µg l⁻¹ unless otherwise stated. na - no analysis done.

TABLE 5

Showing the results of Simper analysis on all the indigenous and deployed animal biomarkers used for the multivariate analysis.*

Position	1	2	3	4	5	6	7
Crab RT	12		2	1			
GST Blood		3	2	4	5	1	
GP Blood		7	6	1	1		
GST Gill		2	2	4	5	1	1
GP Gill				2	2	7	4
GR Gill		3	3	3	1	2	
MT Gill					1	4	10

* The table lists the number of times each biomarker was the main cause (position 1) to the least cause (position 7 or 9) in identifying dissimilarity between sites.

TABLE 6

Showing the results of Simper analysis on all the deployed animal biomarkers used for the multivariate analysis.*

Position	1	2	3	4	5	6	7	8	9
Crab RT	9	5							1
GST Blood				1	4	3	5	2	
GP Blood		2	2	1	6	4			
GST Gill	5	2	4	3		1			
GP Gill		3	3	2	2	4	1		
GR Gill		5	4	2	2		1	1	
T Gill	1	2	1			1		4	6
Mussel RT	5	3	2	1	3	1			
Mussel HB						1	6	8	

* Same as in Table 5.

We thank Rod at the RNLI on South Gare, Mike Leakey at English Nature and Tees Storage Ltd. for permission to catch and deploy animals; Sara McMahon for technical assistance and the Environment Agency for supplying chemical data as well as advice prior to deployment.

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