The sublethal effects of sulphide on *Arctica islandica* and *Mytilus edulis*

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Candidates Declaration

I declare that the work reported in this thesis, unless otherwise acknowledged, is of my own composition. No part of this work has been submitted for any other degree.

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Table of Contents

Summary

The aims of this thesis were to examine the effects of sulphide on two species of bivalve, *Arctica islandica* and *Mytilus edulis*. All the experiments were designed to maintain aerobic conditions throughout, in an attempt to separate the effects of sulphide from those of hypoxia.

Initially the LT_{50} values for both *A. islandica* and *M. edulis* were established (Chapter 2). It was apparent from the results of the LT_{50} experiments that the *A. islandica* from a sulphide-rich habitat had longer survival times than those from a low-sulphide habitat. There was also a significant difference in the average shell height of the *A. islandica* from both areas, with the mean height of the *A. islandica* from the low-sulphide area being almost double the size of those from the sulphide-rich area.

A. islandica were very resistant to sulphide, with an LT_{50} of 6 – 8 weeks, depending on the concentration of the sulphide, whereas *M. edulis* had an LT_{50} of only 1 – 3 weeks. However, both *A. islandica* and *M. edulis* were found to undergo an increase in body condition upon exposure to sublethal concentrations of sulphide for extended periods (Chapter 2).

Further analysis of the LT_{50} survivors showed that the condition index (shell meat volume to inner shell volume ratio) increased consistently with sulphide exposure in both *A. islandica* and *M. edulis*. These results may indicate that the bivalves are able to utilise the reducing potential of sulphide as an energy source.

Experiments were carried out to examine the effect of sulphide on the ventilation rate of *M. edulis* (Chapter 3). During sulphide exposure, *M. edulis* remained open and continued to ventilate at all the sulphide concentrations tested. This appeared to indicate that this bivalve was unable to detect sulphide at concentrations between 0 µM and 1200 µM. Furthermore, the results suggested that *M. edulis* was maintaining aerobic respiration in the presence of sulphide.

Experiments were designed to examine the physiological response of isolated hearts from *M. edulis* to sulphide exposure (Chapter 4). The results indicated that it is the H_2S ion species of sulphide that elicits a response in the isolated hearts. It was possible to acclimatise *M. edulis* to sulphide, and the acclimatisation appears to occur at an intracellular level, suggesting that a response to sulphide exposure is induced.

The concentrations of succinate in selected tissues of intact *M. edulis* and *A. islandica* showed no significant changes upon exposure to increasing concentrations of sulphide. Thus there was no evidence of anaerobiosis. It appears, therefore, that the intact *M. edulis* and *A. islandica* did not switch to anaerobiosis during exposure to sublethal concentrations of sulphide under normoxic conditions. Furthermore, succinate was demonstrated not to be a reliable indicator of anaerobiosis in the presence of sulphide, due to the effect of the redox potential of sulphide on succinate dehydrogenase and fumarate reductase (Chapter 6). The Krebs cycle was shown to continue in an aerobic direction in the presence of sulphide, with the net oxidation of succinate to fumarate, due to the higher redox potential of oxygen compared with that of sulphide.

The oxygen consumption of isolated hearts from *M. edulis* increased significantly as the sulphide exposure concentration was increased. Hence, isolated hearts from *M.*

edulis may maintain aerobic respiration, and an aerobic electron flow through the Krebs cycle, in the presence of sublethal concentrations of sulphide. Since succinate does not accumulate in intact *A. islandica*, and they display an even higher resistance to sulphide under aerobic conditions than *M. edulis* (Chapter 2), it is likely that this is also true of *A. islandica*.

The *A. islandica* with the highest sulphide tolerance, collected during the course of this study, were from Laholm Bay in the Kattegat on the west coast of Sweden (Chapter 2). Laholm Bay is a sulphide-rich hypoxic habitat characterised by fine mud. Thiol production in these *A. islandica* was compared to that in *A. islandica* from Kockholmen. Kockholmen is situated north of Laholm Bay in the Skagerrak, and is a sulphide-free normoxic habitat characterised by coarse sand. The concentrations of cysteine in the blood of the *A. islandica* from Kockholmen were significantly higher than those in the *A. islandica* from Laholm Bay. Furthermore, upon exposure to sulphide, the cysteine concentrations in the blood of the *A. islandica* from Kockholmen displayed a significant linear increase, unlike those in the blood of the *A. islandica* from Laholm Bay, which remained constant (Chapter 7).

The concentrations of cysteine were also determined in *M. edulis* that had been acclimatised to sulphide, and in *M. edulis* not acclimatised to sulphide, and compared to the concentrations of cysteine in *A. islandica* collected from the Clyde Estuary. Cysteine concentrations were higher in *M. edulis* that had not been acclimatised to sulphide, when compared to *A. islandica* and sulphide acclimatised *M. edulis* (Chapter 5). These findings support those reported in Chapter 7 and add credibility to the theory that cysteine is involved in the re-activation of succinate dehydrogenase after its inhibition by sulphide. This process is known to occur *in vitro* but has not yet been demonstrated *in vivo*.

Glutathione concentrations were significantly higher in the tissues of *M.* edulis that had not been acclimatised to sulphide, when compared to *A. islandica* and sulphide acclimatised *M. edulis* (Chapter 5). It is possible that the differences in glutathione concentration were due to glutathione activity in sulphide detoxification in *M. edulis*.

Subsequent experiments demonstrated the occurrence of sulphide-stimulated glutathione (GSSG and GSH) production in *A. islandica* (Chapter 7). Sufficient GSSG was produced in *A. islandica* upon exposure to sulphide to oxidise the sulphide in the tissues (Chapter 7). GSSG promotes sulphide oxidation (Chapter 8), and it therefore appears that *A. islandica* uses GSSG as a "sulphide oxidase" to detoxify sulphide at a cellular level.

The reduction of GSSG to GSH by sulphide has the dual advantage of not only detoxifying the sulphide, but the reduction of the glutathione also occurs without the energy expenditure usually needed to maintain the glutathione redox cycle. It is possible that this energy saving contributes to the increase in body condition observed in the *A. islandica* during the LT_{50} experiments (Chapter 2). There is an additional benefit. The reduction of GSSG to GSH is the rate-limiting step in the glutathione redox cycle. Sulphide-stimulated GSSG reduction increases the turnover rate of the cycle and thus makes more GSH available to deactivate reactive oxygen species (ROS).

Thiosulphate accumulated in the tissue samples from *M. edulis* and *A. islandica* in the presence of sulphide (Chapter 5 and 7), confirming that thiosulphate is the main product of sulphide detoxification in these two species of bivalve. The concentrations of sulphite were significantly lower in *A. islandica* than in *M. edulis*. Sulphite is produced as a transient in the two step oxidation of sulphide to thiosulphate. Because sulphide oxidation to thiosulphate is more efficient in invertebrates from

sulphidic habitats, lower concentrations of sulphite are expected in the more sulphide tolerant *A. islandica* than in *M. edulis*.

Differences in the concentrations of thiosulphate between the blood and tissues of the *A. islandica* from Laholm Bay and Kockholmen indicated that the sites of sulphide detoxification between the two areas were different. The first mechanism is active in the blood of the *A. islandica* from Laholm Bay, and the second is active in the tissues of the *A. islandica* from Kockholmen. A possible explanation is that the detoxification mechanism in the blood is induced by long-term sulphide exposure. The second mechanism is a short-term detoxification mechanism active in the tissues of the *A. islandica* not acclimatised to sulphide.

Chapter 1

Hydrogen sulphide toxicity

Hydrogen sulphide is highly toxic to aerobic organisms (National Research Council, 1979). This is true for both aquatic and terrestrial animals. For example, in cats the minimal LD_{50} is 25 $\mu mole.kg^{-1}$, which is of the same order of toxicity as hydrogen cyanide (Evans, 1967). Hydrogen sulphide is used as an intermediate in the preparation of reduced sulphur compounds, and is a by-product of sewage treatment, leather tanning and desulphurisation processes in the oil and gas industries (Amman, 1986). An early survey showed that there are approximately 23 occupations in which sulphide poisoning is a hazard to man (Milby, 1962). In the United Kingdom between 1960 and 1964 there were 8 fatalities from hydrogen sulphide as compared to only 4 from hydrogen cyanide, and in one factory alone, in the United States, there were 174 cases of sulphide poisoning over 15 years (Poda, 1966). The toxicity of hydrogen sulphide combined with its common usage has led to hydrogen sulphide being the leading cause of sudden death in the workplace (National Institute for Occupational Safety and Health, 1977). The toxic effects of hydrogen sulphide have been recognised and studied for some time (Bernard, 1857), and it has even been used as a chemical agent during World War 1 (Evans, 1967).

It is not only humans and terrestrial animals that need to cope with the toxic effects of sulphide. Sulphide is toxic to many aquatic animals (Kang and Matsuda, 1994; Gopakumar and Kuttyamma, 1996; Vismann, 1996a; Sandberg *et al.*, 1999), but some animals appear to cope with high concentrations (Theede *et al.*, 1969; Vetter *et*

al., 1987; Johns *et al.*, 1997). In 1977 the hydrothermal vents at the East Pacific Rise-Galápagos Rift were discovered (for a review see Childress and Fisher, 1992; Juniper and Martineu, 1995). These habitats were subsequently shown to support whole communities of animals that are specially adapted to cope with concentrations of sulphide up to 20 mM (Powell and Somero, 1985). Generally, the inhabitants of the hydrothermal vent systems utilise chemolithoautotrophic symbioses with sulphur bacteria, which fix carbon using the reducing power of sulphide as an energy source (Wilmot and Vetter, 1992). However, sulphide is swiftly oxidised by contact with oxygen, haem compounds, metal ions and some organic compounds (Millero, 1986; Powell and Arp, 1989). Thus, animals that utilise sulphide as an energy source need to be close enough to the hydrothermal vents to be exposed to sulphide, but access to an oxidant (O_2 or NO_3^-) is also needed. These preconditions create a unique problem where the hydrothermal vent animals need to prevent the oxidation of sulphide within the tissues so that it can be utilised by the sulphur bacteria.

The haemolymph of the vestimentiferan tube worm, *Riftia pachyptila,* contains a sulphide binding protein that concentrates the sulphide, protecting the sulphide sensitive tissues of the tube worm, whilst transporting the sulphide to the tissues containing the symbiotic bacteria (Arp and Childress, 1983). Thiotaurine (a combination of taurine and sulphide) has been shown to transport sulphide from the haemolymph, to the symbiotic bacteria in the tissues (Albéric and Boulegue, 1990; Pruski *et al.*, 2001). Once the symbiotic bacteria receive the sulphide, they utilise its reducing power to generate ATP and NADPH, reduce nitrate, and fix CO_2 via the Calvin-Benson cycle (Felbeck and Somero, 1982; Anderson *et al.*, 1987). The hydrothermal vent animals can only occupy very narrow niches around the vent systems, where temperature and sulphide concentrations are not too high, and oxygen is still available. The optimum conditions are temperatures below 50 °C, with sulphide concentrations below 400 µM (Spiess *et al.*, 1980). Subsequent studies on

the hydrothermal vent inhabitants revealed that not all of these animals utilise symbiotic bacteria to reduce sulphide. The hydrothermal vent crab, *Bythograea thermydron*, although not containing symbiotic bacteria, is able to maintain stable heart and ventilation rates upon exposure to up to 1.4 mM sulphide (Vetter *et al.*, 1987).

Sulphide is commonly produced during eutrophication processes where it is formed as a by-product during the reduction of sulphates by sulphur-reducing bacteria (Theede *et al.*, 1969). However, in the presence of oxygen sulphide is quickly oxidised to less toxic substances. Thus sulphide accumulation occurs only under hypoxic conditions, primarily in the sediment (Hagerman *et al.*, 1996). The sulphate-reducing bacteria generally belong to the genera *Desulfovibrio*, *Desulphobacter* and *Desulphobulbus*, and are widely distributed in the terrestrial, limnic and marine environments (Jørgensen, 1982b). These bacteria commonly inhabit the anoxic areas of sediments, where they oxidise almost half of all organic matter settling onto the sediment to CO_2 at the expense of sulphate, with sulphide as a by-product (Jørgensen, 1982a). Hence, sulphate-reducing bacteria occupy the terminal step of the anaerobic microbial food chain.

At the oxic/anoxic interface there are several genera of bacteria, including *Thiobacillus*, *Beggiatoa*, *Thioploca* and *Thiovulum*, which oxidise sulphide back to sulphate (Jørgensen, 1982b). Both the sulphate-reducing and the sulphide-oxidising bacteria of the sulphur cycle are commonly found in the sediments of salt marshes, mangrove swamps, fjords, shallow marine basins and stratified lakes or seas, such as the Baltic Sea and the Black Sea (Meadows *et al.*, 1981; Jørgensen, 1982b; Hagerman, 1998).

Sulphide can accumulate to very high concentrations, with 1.1 mM having been recorded in intertidal mud flats of the Wadden Sea (Thiermann *et al.*, 1996). Concentrations of up to 20 mM have also been reported in interstitial water (Grieshaber *et al.*, 1992). Unfortunately, the authors did not elaborate on the conditions under which such high concentrations of sulphide occurred. These high concentrations of sulphide would normally be toxic to aerobic organisms (National Research Council, 1979). However, there are numerous cases of polychaetes (Völkel and Grieshaber, 1992; Gamenick *et al.*, 1998), priapulids (Oeschger and Vetter, 1992), crustaceans (Johns *et al.*, 1997; Bourgeois and Felder, 2001), bivalves (Theede *et al.*, 1969; Oeschger, 1990; Oeschger and Storey, 1993) and some fish (Bagarinao and Vetter, 1990, 1993) that encounter sulphide in their habitats and seem to have some resistance to its toxic effects. The ribbed mussel *Guekensia demissa*, a salt marsh inhabitant of North America, tolerates sulphide concentrations of approximately 1 mM and even up to 8 mM (Lee *et al.*, 1996). The ostracod, *Cyprideis torosa*, commonly found in the eulittoral zone of the Baltic Sea, can withstand hypoxia combined with sulphide concentrations of up to 1.8 mM for several weeks (Jahn *et al.*, 1996). *C. torosa* accumulates succinate, which gives a higher ATP yield per mole of glucose than lactate (Chapter 6). But most importantly, *C. torosa* has a high capacity for long term anaerobiosis while still retaining mobility (Jahn *et al.*, 1996).

Thus there are sulphide tolerant organisms that are able to inhabit sulphide-rich sediments. Hence, there is an interaction between the bacteria of the sulphur cycle in the sediments, and the sulphide tolerant organisms that inhabit these sediments which is an important facet of the marine environment, giving rise to the *sulphide biome* concept on a global scale (Fenchel and Reidl, 1970). The mechanisms employed by sulphide tolerant species to survive sulphide exposure have attracted substantial interest from researchers (for reviews see Beauchamp *et al.*, 1984;

Vismann, 1991b; Grieshaber *et al.*, 1992; Grieshaber and Völkel, 1998). But to explore the mechanisms behind sulphide tolerance, an understanding of the nature of sulphide and the mechanisms of sulphide toxicity is needed.

Total sulphide is the sum of three ion species, H_2S, HS^- and S^{2-}. Usually, unless otherwise stipulated, researchers use the term 'sulphide' or 'hydrogen sulphide' as defining total sulphide. But Vismann (1996a) showed that the HS^- ion is non-toxic to the brown shrimp, *Crangon crangon* (which is very sensitive to the H_2S ion). Hence the ratios of the three ion species have important implications for sulphide toxicity. The ratio of the three species present at any time is pH dependent. The pK_a for dissociation of the first proton is 7.04, and 11.96 for the second (Beauchamp *et al.*, 1984). Thus at pH 8 the ratio of the three species is as follows: $H_2S < 10$ %, $HS^- < 90$ % and $S^{2-} < 0.01$ %. Due to the very small amounts of the S^{2-} ion species present, and because it is highly charged, the S^{2-} ion species will be disregarded in this review.

Interstitial water can have a pH as low as 6 - 6.5 (Vismann, 1996a). Such a low pH shifts the proportion of the H_2S ion species to more than 70 %. However, Vopel *et al.* (1998) found that pH had no effect on the survival rates of the copepod, *Cletocamptus confluens*. It is tempting to speculate, however, that such a finding is due to *C. confluens* having very similar permeability coefficients for both the H_2S and HS^- ion species due to it small size. But for larger animals, the pH of the water dictates the toxicity of total sulphide.

Both the H_2S and HS^- ion species play a role in how much total sulphide enters an animal. The H_2S ion species, because it has no charge, is able to diffuse freely into aquatic animals. But because of its charge, the HS^- species is less freely diffusible.

Julian and Arp (1992) calculated the permeability coefficients for the H_2S and HS^- ion species as 0.17 $cm.h^{-1}$ and 0.063 $cm.h^{-1}$, respectively in the echiuran worm, *Urechis caupo*. These values are not absolute, and permeability coefficients for the H_2S ion species have been estimated from between 4 x $10^{-5}cm^2.s^{-1}$ and 8 x $10^{-7}cm^2.s^{-1}$ (0.38 $cm.h^{-1}$ and 0.053 $cm.h^{-1}$) in the peanut worm, *Sipunculus nudus* and the ostracod, *Cyprideis torosa*, respectively (Völkel and Grieshaber, 1992; Jahn *et al.*, 1996). Thus the H_2S ion species diffuses into animals approximately 2 times faster than the HS^- ion species. However, unless stated otherwise, throughout this thesis, sulphide or hydrogen sulphide refers to the sum of the three ion species (total sulphide).

As mentioned above, hydrogen sulphide is highly toxic to aerobic organisms. The site of action of hydrogen sulphide once inside the cell is cytochrome c oxidase, the last of the three proton pumping assemblies of the respiratory chain (Beauchamp *et al.*, 1984). Here, H_2S binds reversibly to the haem on the cytochrome aa_3 sub-unit of the cytochrome c oxidase, which inhibits aerobic respiration (National Research Council, 1979). In addition, hydrogen sulphide also reduces disulphide bridges and reacts with haem components in the blood (Patel and Spencer, 1963; Smith and Gosselin, 1964, 1966; Bagarinao and Vetter, 1989; Oeschger and Vetter, 1992). H_2S has also been shown to have an inhibitory effect on vertebrate axonal conduction, synaptic transmission, neurotransmitter content and neuronal growth (Julian *et al.*, 1998), all of which are thought to be independent of metabolic rate. Hence, although the most immediately apparent effect of sulphide exposure is the inhibition of aerobic respiration, there are a range of other effects that can potentially complicate an attempt to examine one effect independently of the others.

Exposure to sulphide can elicit a number of responses in aerobic organisms. Highly mobile animals may be able to migrate to sulphide free areas. Sessile animals such as bivalves can close their shell valves and hence exclude sulphide as a short-term

solution. Animals which are unable to migrate or exclude sulphide can either attempt to maintain aerobic respiration during sulphide exposure, or switch to anaerobic respiration which is not inhibited by sulphide exposure (Hagerman and Vismann, 1993; Vismann and Hagerman, 1996). Interestingly, animals with a high tolerance of hypoxia also seem to demonstrate a tolerance of sulphide exposure (Theede *et al.*, 1969; Gorodesky and Childress, 1994; Jahn *et al.*, 1996; Vopel *et al.*, 1998). Oeschger and Vetter (1992) suggest that animals use anaerobiosis "to passively outlast sulphide" but only as a last resort. The strategies that have drawn the most interest are those where aerobic respiration appears to continue during exposure to sulphide. Researchers have proposed a number of mechanisms that may be involved in sustaining aerobic respiration during sulphide exposure, most of which involve the detoxification of sulphide.

Childress and Fisher (1992) are of the opinion that sulphide detoxification is pH driven; whereas Bagarinao and Vetter (1989) reported that the oxidised form of the tri-peptide glutathione has some detoxifying ability, becoming reduced in the process. Although whether this is an important factor in detoxification, or just another result of sulphide poisoning, is unknown. Powell and Somero (1986b) demonstrated sulphide-stimulated oxygen consumption in the symbiont-containing clam, *Solemya reidi.* Subsequently, it was shown that sulphide is oxidised via sulphite to thiosulphate in the mitochondria of this bivalve (O'Brien and Vetter, 1990).

Since the oxidation of sulphide to sulphate releases 8 electrons (Doeller *et al.*, 1999a), oxidation of sulphide in the mitochondria, has the potential to provide energy to the cell. Hence researchers looked for a link between sulphide oxidation and the entry of electrons into the respiratory chain. These studies revealed that there is some evidence that an alternative terminal oxidase exists in the respiratory chain of the lugworm, *Arenicola marina* (Völkel and Grieshaber, 1994), which can either

oxidise sulphide or transfer electrons to oxygen during sulphide oxidation. This led to the theory of sulphide detoxification in *A. marina*. It was proposed that, during periods of low sulphide exposure, a sulphide oxidase in the mitochondria of the body-wall musculature, transfers electrons from sulphide to oxygen via cytochrome c oxidase. This process is thought to be coupled to oxidative phosphorylation. However, during episodes of high sulphide exposure, electrons from sulphide are transferred to oxygen through the sulphide oxidase via an alternative oxidase, which is independent of oxidative phosphorylation (Völkel and Grieshaber, 1997). Thus it is possible that both a sulphide oxidase, that is able to oxidise sulphide in the mitochondria, and an alternative terminal oxidase, that replaces cytochrome c oxidase during periods of high sulphide exposure, are active in some sulphide resistant aerobic organisms (Völkel and Grieshaber, 1996). Thus animals with these enzymes may be able not only to detoxify, but also to gain an energy benefit from the detoxification process, upon exposure to sulphide.

The main product of sulphide detoxification in marine animals is thiosulphate ($S_2O_3^{2-}$) which is formed from the oxidation of hydrogen sulphide (Gorodezky and Childress, 1994; Johns *et al.*, 1997). Small amounts of sulphite and sulphate are also formed (Johns *et al.*, 1997), but the formation of thiosulphate from sulphide has the lowest oxygen cost to the animal (Vismann, 1991a), as each thiosulphate molecule represents the detoxification of 2 sulphide molecules (Jahn *et al.*, 1996). A visible sign of sulphide detoxification activity is the blackening of the tissues involved (Oeschger and Vetter, 1992; Völkel and Grieshaber, 1994; Johns *et al.*, 1997). The explanation given for such a phenomenon is the presence of iron. Some marine animals are known to concentrate iron in the hepatopancreas (Vismann, 1991b). Oeschger and Vetter (1992) found that the priapulid, *Halicryptus spinulosus* turned black in the presence of sulphide, suggesting that the mucus coating the animal contains iron and is used as a first defence. Once inside the animal, haem-containing

molecules have the ability to bind sulphide and may play an important role in the detoxification process (Bagarinao and Vetter, 1989; Oeschger and Vetter, 1992). The effect of hydrogen sulphide on the respiratory pigment haemocyanin has also been the subject of a number of studies (Gorodesky and Childress, 1994; Hagerman and Vismann, 1995).

There is some evidence that haem containing molecules in the haemolymph play a role in sulphide detoxification (Oeschger and Vetter, 1992). Lactate, a product of anaerobic metabolism in many Crustacea, is a modulator of the oxygen affinity of the haemocyanin, but only if present in high concentrations (Truchot, 1980; Hagerman and Vismann, 1995). This is significant since the only time that concentrations of lactate are sufficient to increase the oxygen affinity of the haemocyanin, is during exposure to sulphide or severe hypoxia. These studies were carried out using *Crangon crangon* (Hagerman and Vismann, 1995). Similarly, during sulphide exposure, thiosulphate accumulates in the haemolymph of *B. thermydron* and has been shown to increase the oxygen affinity of the haemocyanin which in turn allows the crabs to maintain higher oxygen consumption rates at lower Po_2 levels (Gorodesky and Childress, 1994).

Thus, there are a number of mechanisms that are employed by different aquatic animals to overcome the toxic effects of sulphide. Initially, there may be some attempt to oxidise sulphide prior to entry into the animal (Oeschger and Vetter, 1992). Subsequently, the blood is involved in sulphide transport (Arp *et al.*, 1987; Frenkiel *et al.*, 1996; Zal *et al.*, 1997, 1998; Cerda-Colon *et al.*, 1998; Silfa *et al.*, 1998; Cheeseman *et al.*, 2002; Rosando-Ruiz *et al.*, 2002) and possibly in detoxification. Once inside the tissues, thiotaurine is involved in the transport of sulphide in some symbiont-containing species (Albéric and Boulegue, 1990; Pruski *et al.*, 2001), and part of the sulphide is detoxified via the reduction of iron containing molecules (Völkel

and Grieshaber, 1994; Johns *et al.*, 1997). Finally, it is possible that an alternative terminal oxidase and sulphide oxidase exist in the mitochondria of some sulphide-tolerant species which oxidise sulphide whilst using the reducing power of the sulphide molecule as an energy source. It seems likely, therefore, that an individual species may utilise more than one of these mechanisms upon exposure to sulphide.

A number of questions remain unanswered about the different aspects of sulphide toxicity and the mechanisms of sulphide detoxification used to protect aquatic animals during sulphide exposure. Firstly, the effects of sulphide on the physiology and sulphide biochemistry of aquatic invertebrates must be separated from that of hypoxia. The effects of both render the respiratory chain either non functional, or only partially functional. Hence the investigation of sulphide on the physiology and sulphide biochemistry of invertebrates carried out under normoxic conditions would overcome any conflict between these environmental parameters. Subsequently, a comprehensive study of sulphide detoxification mechanisms would be in part reliant on choosing species that strongly express the different aspects of sulphide detoxification, which would aid characterisation.

The aims of this thesis were to examine the effects of sulphide on the physiology and sulphide biochemistry of selected species of bivalves, firstly in intact animals, and then in isolated tissues. Two species, the ocean quahog, *Arctica islandica*, and the blue mussel *Mytilus edulis* were selected, both of which are resistant to hypoxia, but only one known to be resistant to sulphide (Theede *et al.*, 1969; Chapter 2). Subsequently, the effects of sulphide on these species could be contrasted to highlight differences in the responses of a sulphide tolerant species and a non-sulphide tolerant species, to sulphide.

Chapter 2

Sulphide tolerance in *Arctica islandica* and *Mytlus edulis*

Introduction

To examine the sub-lethal effects of sulphide on invertebrates, species selection is important, primarily as the individual characteristics of a species dictate the direction in which subsequent experiments will be aimed. For example very small species such as the polychaete *Capitella capitata* have some resistance to sulphide (Gamenick *et al.*, 1998). However, due to the small size of the organs, accurate quantification of biochemical indicators, or changes in physiological responses, such as heart rate in the presence of sulphide, is difficult to achieve. For this reason two species of bivalve were selected that were not only expected to display resistance to sulphide, but also of a sufficient size, and available in sufficient quantities, to allow subsequent in-depth studies to be performed.

The blue mussel, *Mytilus edulis* L. is economically the most important of the mussel species found in the North Atlantic, and is commonly cultured in North West Europe (Hayward *et al.*, 1996). *M. edulis* has also been extensively used as a model species to examine physiological processes in bivalves (Bayne, 1971; Hildreth, 1976; Famme *et al.*, 1986; Jones *et al.*, 1992; Clausen and Riisgård, 1996). Consequently, the effects of fluctuating parameters such as starvation (Bayne, 1973), oxygen content (Helm and Trueman, 1967; Bayne, 1971; Taylor and Brand, 1975), temperature

(Pickens, 1965) and even parasitic infestations such as the polychaete *Polydora ciliata* on *M. edulis* (Ambariyanto and Seed, 1991) are well documented. Both aerobic and anaerobic metabolism have also been exhaustively studied in *M. edulis* (Glaister and Kerly, 1936; de Zwaan and Van Marrewijk, 1973; Holwerda and De Zwaan, 1979; Klutmans *et al.*, 1981; Shick *et al.*, 1983) leading to an extensive knowledge of both the biochemical and physiological processes in this bivalve. However the effects of sulphide on *M. edulis* have not been examined in any depth, possibly as it is a toxin not usually associated with the type of habitats where *M. edulis* predominantly occurs. But *M. edulis* is resistant to hypoxia (Bayne, 1971; Coleman, 1974; Holwerda and De Zwaan, 1979), and there is some evidence that invertebrates that are resistant to hypoxia also display some resistance to sulphide (Childress and Fisher, 1992; Grieshaber *et al.*, 1994).

Arctica islandica, commonly known as the Icelandic cyprine or the ocean quahog, is a bivalve that is found in the Baltic Sea, the Western and boreal North Atlantic Ocean (Oeschger and Storey, 1993). It also occurs in the White and Barents Seas, off the East Coast of North America, from Newfoundland to Cape Hatteras, and as far south as the Bay of Cadiz in Spain. *A. islandica* is a sublittoral species that is commonly found in soft sediments at depths of 4 m to 256 m (Merrill and Ropes, 1969).

A. islandica is an edible bivalve that has been harvested on a small scale since 1943 in the Rhode Island area as part of the World War 2 food production programme (Kennish and Lutz, 1995). Intensive fishing started in the 1970s when the surf clam *Spisula solidissima* (Dillwyn) stocks crashed due to over fishing, and it is now a multimillion dollar fishery (Steimle *et al.*, 1986). Unfortunately, due to slow adult growth rates, low adult mortality, long time to maturity, and the absence of recruitment the fishery is very vulnerable to commercial exploitation (Kennish and Lutz, 1995). Investigations in Icelandic waters have revealed that stocks of *A.*

islandica represent a major resource that could support a major fishery (Thórarinsdóttir and Jóhannesson, 1996).

The age of bivalves can be accurately measured by taking acetate peels of umbo growth lines (Richardson *et al.*, 1981; Ramon and Richardson, 1992; Richardson *et al.*, 1993; Ramon *et al.*, 1995; Richardson *et al.*, 1999). *A. islandica* is a long lived bivalve species with specimens up to 200 years old living on the U.S. Atlantic coast (Witbaard *et al.*, 1999) and specimens of over 100 years old commonly found in the North Atlantic (Swaileh and Adelung, 1994). The longevity of *A. islandica* exceeds the maximum age for any bivalve, previously quoted as 70 – 80 years for *Margaritana margaritifera* (Forster, 1981).

Initially, for the first 15 – 20 years *A. islandica* grows quite rapidly, possibly because predation is size dependant. For example, cod cannot predate individuals with valves larger than 4 cm in height, and the lobster *Homarus americanus* is only able to open valves smaller than 7 cm high (Witbaard and Klein, 1994). After the first 15 – 20 years, growth rates decline with the average increment of valves over 100 mm being approximately 0.1 mm per year (Forster, 1981). The longevity of *A. islandica* has been utilised by researchers for a range of studies. Witbaard (1996), by quantifying the periodic growth increments on *A. islandica* valves, was able to correlate changes in local conditions with changes in the valves. Further studies of *A. islandica* from different grounds highlighted a 33 year cycle in growth trends that reflected long term changes in water masses known as the Russell cycle (Witbaard *et al.*, 1997). Utilisation of the periodic growth increments has also been used to examine long term changes in beam trawl fisheries (Witbaard and Klein, 1994), where the tickler chains used in the beam trawl leave scars on *A. islandica* valves which can be examined chronologically.

Chemical contamination is a growing concern in fishery management, and *A. islandica* have been used to quantify organic and trace metals in the North West Atlantic (Steimle *et al.*, 1986). Smaller individuals accumulate Cu and Zn (essential metals), whilst larger individuals accumulate Cd and Pb (non-essential metals) (Swaileh and Adelung, 1994). Unfortunately, the authors did not discuss any possible explanations for this difference in trace metal accumulation. However, non-essential metal accumulation is higher in winter and is thought to be due to annual changes in the soft tissue weight (Swaileh, 1996). Changes in growth rate have been correlated to temperature (Witbaard *et al.*, 1997) and food availability, and inversely correlated to depth and silt content (Witbaard *et al.*, 1999).

Early experiments showed that *A. islandica* is very resistant to both hypoxia and hydrogen sulphide, with LD_{50} survival times of 800 -1000 hours when exposed to 200 µM sulphide and 0.15 ml $O_2.l^{-1}$ (Theede *et al.*, 1969). Calorimetric measurements have shown that after prolonged anoxia, energy release in *A. islandica* is less than 1% of the aerobic rates (Oeschger, 1990). Such high resistance to both sulphide and hypoxia has been attributed to a variety of mechanisms, both behavioural and physiological. These include detoxification of sulphide to thiosulphate, avoidance of H_2S by valve closure and a reliance on very efficient anaerobic mechanisms when aerobic respiration is no longer possible due either to low concentrations of O_2 or inhibition of the respiratory chain by high concentrations of H_2S (Oeschger and Storey, 1993).

The two selected species, *M.* edulis and *A. islandica* are both resistant to hypoxia as discussed above, but only *A. islandica* commonly encounters sulphide in its natural habitat. However, *M. edulis* does appear to have some resistance to sulphide (Theede *et al.*, 1969). It might be expected, therefore, that there could be some discrepancy in the resistance of these two species to sulphide.

There is, however, another consideration to take into account which may be relevant to this series of experiments. There is some evidence of sulphide stimulated ATP production in the ribbed mussel *Guekensia demissa* (Parrino *et al.*, 2000). If sulphide exposure stimulates ATP production, it is possible that the affected species will be receiving a net energy gain by exposure to sulphide. By monitoring changes in the condition indices of these two species after sulphide exposure, changes in the relative wet meat volume to inner shell volume would indicate whether sulphide may be providing extra energy which is of practical benefit to the bivalves.

There are a range of methods used to estimate the condition of bivalves. These methods, collectively named condition indices, give an estimation of the physiological condition of bivalves (Etim *et al.*, 1997). Most of the research into condition in bivalves has been on commercially important species, especially in aquaculture, in an attempt to quantify the effects of environmental parameters on growth (Rodhouse, 1977; Nagasawa and Nagata, 1992; Esquivel and Voltolina, 1996). The first attempts to quantify condition in bivalves occurred almost a century ago in oysters (Grave, 1912). The basic method utilised by Grave has not been modified much over the years. All the methods are reliant on the ratio of meat volume to shell volume or the difference between total volume of the bivalve and shell volume as shown in the formula below:

$$\text{Condition index} = \frac{\text{Meat volume}}{\text{Shell volume}} \times 100$$

Davenport and Chen (1987) compared seven variations on this basic theme using *M. edulis*. The variations of the method rely on using wet meat weight or wet meat volume, dry meat weight and cooked meat weight. Additionally, the shell weight or volume as described above was used. The authors found that all the methods gave comparable results unless the meat was frozen prior to measurement. A point to note

is that the ratios given by dry meat weight will be somewhat lower than those using wet meat weights simply due to differences in water content. However, there is a direct correlation between wet meat weight and dry meat weight (Baird, 1957) in *M. edulis*. The relationship (y = 0.36x +1.55) between wet meat weight and dry meat weight was calculated from the data presented by Baird (1987). Where y is the wet meat weight and x is the dry meat weight.

Even if the method used for calculating condition index is accurate there are a range of other variables that make interpretation of the results difficult. A study of the condition index of *M. edulis* from two distinct areas in the Baltic Sea and two areas in the North Sea highlighted the impact of locality on the condition of *M. edulis* (Gilek *et al.*, 1992). The mean condition indices of the mussels in the Baltic Sea (61 – 54) were approximately half those in the North Sea (126 – 84) for unpolluted and polluted sites, respectively. Changes in other environmental parameters can also affect condition in bivalves. Salinity, temperature, pH and dissolved oxygen were all shown to have an impact on the condition of the eastern oyster *Crassostrea virginica* (Fisher *et al.*, 1996). Parasitic infestations also affect condition. For example, there is a negative correlation between the numbers of parasitic copepods *Pectenophilus ornatus* and the condition index of the Japanese scallop *Patinopecten yessoensis* (Nagasawa and Nagata, 1992). Another parameter to consider is the effect of season on condition indices. The seasonal cycle of gonad development and spawning has been shown to affect the condition indices of *Mytilus galloprovincialis* (Soto *et al.*, 1995), and even laboratory handling has a detrimental effect on mussel condition indices. A measurement of condition index is therefore a sensitive method of examining changes in condition in bivalves from which changes in physiological condition can be estimated.

By measuring the condition of *A. islandica* and *M. edulis*, and comparing this condition to that in sulphide exposed *A. islandica* and *M. edulis*, the effect of long term exposure to sulphide on both these species can be assessed. The primary aim of this experiment was to determine the LT_{50} of both *A. islandica* and *M. edulis* exposed to similar concentrations of sulphide. The secondary aim was to evaluate the condition of the surviving *A. islandica* and *M. edulis* compared to a control group not exposed to sulphide.

Materials and Methods

M. edulis were collected from the banks at the top of Loch Fyne (Strachur, Scotland) (56° 10` N; 5° 05` W) where the salinity ranged between 5 - 25°/$_{oo}$. *A. islandica* were dredged from Laholm bay, in the Kattegat near the Swedish coast (56° 34` N; 12° 43` E; Ca 17m), a sulphide-rich hypoxic site with very fine muddy substratum (Chapter 7), and from Irvine Bay, Firth of Clyde (55° 37` N; 4° 44` W), from a depth of about 6m using a hydraulic dredge (Chapter 5), where the substratum is a mud/sand mixture.

All animals were subsequently transported to the University Glasgow, Scotland in seawater where they were maintained in a re-circulating sea water aquarium at 10°C ±1°C (salinity = 35 ± 1°/$_{oo}$) under a 12:12 hour light: dark regime. Individuals were not fed whilst under observation but some particulate matter was present in the circulating seawater and may have provided a source of food. All experiments were begun within a week of capture.

The sulphide stock solution (100mM) was prepared from $Na_2S.\ 9H_2O$ (Sigma, S-4766 Assay 100%) every day prior to use and maintained under a nitrogen atmosphere to

prevent oxidation. The concentration of the stock solution was determined using iodometric back titration (Parsons *et al.*, 1984), where 10 ml of the stock sulphide solution was added to 250 ml oxygen free (degassed) distilled water. Subsequently, 20 ml of 0.1 N iodine and 25 ml of 0.1 N HCl were added. The excess iodine was titrated with 0.1 N thiosulphate using starch as an indicator. The pH of the sulphide stock solution was firstly adjusted to pH8 using 6 N HCl, and then maintained at pH8 by the buffer qualities of the seawater. Colorimetric determination of sulphide modified from Parsons *et al.* (1984) was used to monitor sulphide concentration in the exposure tank, and during experimentation. To fix the sulphide, 500 µl of 0.12 M zinc acetate and 125 µl of 0.6% NaOH were added to a 50 µl sample. The samples were subsequently stored at -20°C. For colorimetric determination 125 µl N,N-dimethyl-p-phenylenediamine (0.3%) and 125 µl of 11.5 mM $FeCl_3$ was added. After 20 minutes the absorbance was measured at 670 nm (range = 0 – 0.8).

Each species was placed separately in an exposure tank (n = 20) and a lid fitted. The lid was larger than the exposure tank with an overhanging lip so that seawater pumped into the tank could overflow, displacing any air below the lid and preventing any interaction between the air and the seawater in the exposure tank as illustrated in Fig. 2.1.

Aerated seawater was pumped continuously through the exposure tank at a slow rate (74 – 240 $ml.min^{-1}$) using a peristaltic pump. A second peristaltic pump was used to pump a stock sulphide solution into the mixing chamber located upstream of the exposure tank in which the animals were kept. By adjusting the relative flow rates of the two pumps it was possible to obtain the required sulphide concentration within the exposure tank whilst still maintaining normoxic conditions. The Po_2 of the water was initially monitored in the exposure tank, using an microcathode oxygen electrode (Strathkelvin Instruments, Oxygen Meter 781) that had been calibrated prior to use

against aerated sea water (at 10°C) and against a solution having a Po_2 of zero (sodium sulphite in 0.01 M sodium tetraborate). The actual sulphide concentration in the exposure tank water was measured using the colorimetric method described by Parsons *et al.* (1984) above.

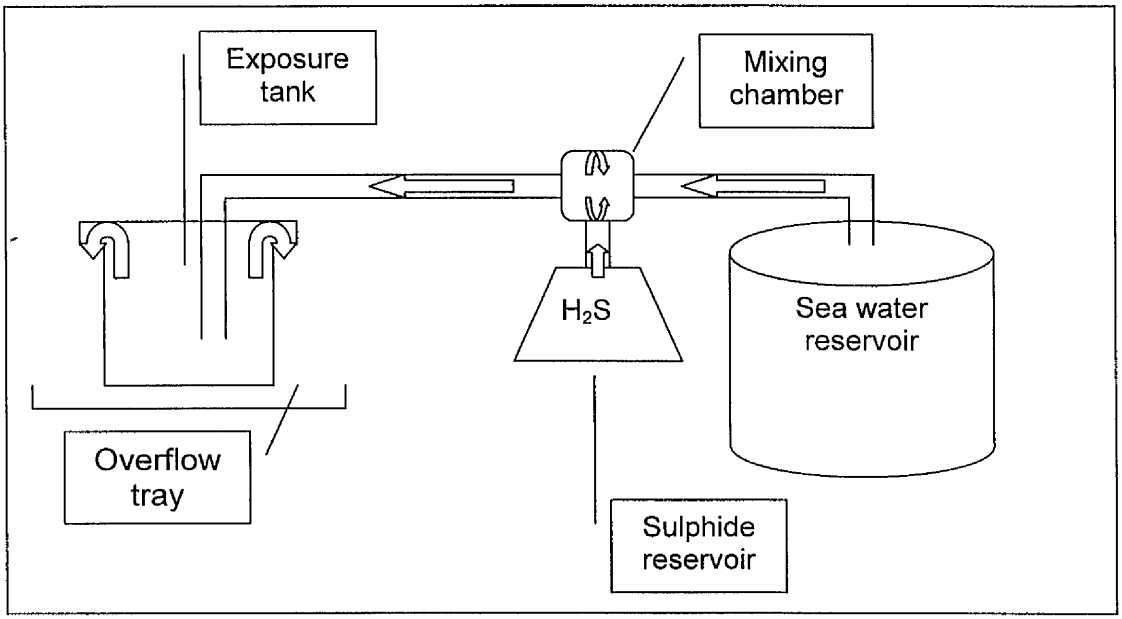

Figure 2.1: Diagrammatic representation of the closed system used to expose Mytilus edulis *and* Arctica islandica *to a constant concentration of sulphide whilst minimising sulphide oxidation caused by exposure to air.*

The bivalves were allowed to settle overnight before a known concentration of sulphide was introduced to the tank. A steady sulphide concentration was maintained until 50% mortality was achieved. Mortality was assumed when shell gape was observed, and the bivalves no longer responded to mechanical stimulation. The first exposures were to 150 µM sulphide. After 50% mortality, the condition index of the survivors was assessed as described below. A new batch of bivalves was subsequently exposed to 500 µM sulphide, and the experiment was repeated.

To examine the condition of the surviving bivalves the following formula, shown to be accurate in assessment of condition indices (Davenport and Chen, 1987), was employed:

$$\text{Condition} = \frac{\text{Wet meat volume}}{\text{Total volume} - \text{Shell volume}} \times 100$$

As soon as LT_{50} was reached the survivors (n = 10), and bivalves not exposed to sulphide (n = 10), were used to calculate condition. The wet meat volume, total volume and shell volume were measured by the displacement of water in a graduated cylinder upon the addition of the intact bivalve, total soft tissue or empty shell valves. The condition indices of the bivalves exposed to sulphide were compared to those of a similar number of bivalves from the same area maintained under sulphide-free conditions.

Statistical analysis

All data were analysed using Minitab 11 statistical package. The data were normally distributed ($0.960 < \text{normal scores} < 0.982$), there was no significant heterogeneity of variance (Bartlett's value = 6.059; $p = 0.049$), and a one way ANOVA was used to check for significance ($p < 0.05$). Statistical significance are all quoted at a 95% Confidence interval ($p < 0.05$) unless otherwise stated.

Results

During the LT_{50} experiments, progress was regularly monitored, and some behaviour was observed. However, there was no discernable difference in behaviour between the sulphide-exposed bivalves and those not exposed to sulphide.

There was a marked difference in the mean sizes of the *A. islandica* from the 2 areas sampled, and so the mean height of the valves of all the invertebrates examined is shown in Table 2.1. The mean height of the *A. islandica* from the Clyde Sea was approximately double that of those found in Laholm Bay.

Table 2.1: Table of the mean size range of Arctica islandica *and* Mytilus edulis, *the largest of which were selected from each area for experimentation (n = 20). The diameter from umbo to opposing shell margin was measured. The O_2 and H_2S concentrations displayed were measured in the bottom water where possible.*

Species	**Mean ± Std dev. (mm)**	**O_2 (ml.l^{-1})**	**H_2S (µM)**	**Capture location**
A. islandica	48.01 ± 6.75	0	33,8 ± 2	Laholm Bay, Sweden
A. islandica	95.44 ± 9.39	6.45	0	Little Cumbrae, Scotland
M. edulis	61.52 ± 4.33		emersed	Loch Fyne, Scotland

The results of the LT_{50} experiments are detailed in Table 2.2 below. *A. islandica* had the longest survival time, 80% longer than *M. edulis*. The *A. islandica* from Laholm Bay had a 25% longer survival time than those from the Clyde Sea, whilst *A. islandica* from Laholm Bay exposed to 150 µM sulphide had a 21% higher survival rate than those from the same location exposed to 500 µM sulphide. *M. edulis* exposed to 150 µM sulphide survived 51% longer than those exposed to 500 µM sulphide.

Table 2.2: LT_{50} values for Arctica islandica *and* Mytilus edulis *exposed to 500 µM sulphide under normoxic conditions in a through flow system (n = 20). Two groups of* Mytilus edulis, *and two groups of* Arctica islandica *from Laholm Bay, Sweden were used, the first exposed to 150 µM sulphide and the second to 500 µM sulphide.*

Species	**Capture location**	**150 µM H_2S**	**500 µM H_2S**
A. islandica	Laholm Bay, Sweden	1436 hours	1145 hours
A. islandica	Little Cumbrae, Scotland		867 hours
M. edulis	Loch Fyne, Scotland	484 hours	238 hours

Upon completion of the LT_{50} experiments, the condition indices of the surviving bivalves were calculated to examine the effect of long term sulphide exposure on bivalve condition. The condition indices of the *A. islandica* are summarised graphically in Fig. 2.2.

Although there was a 3% mean increase in the condition index of the *A. islandica* exposed to 150 µM sulphide, and a 10% mean increase in the condition index of the *A. islandica* exposed to 500 µM sulphide, compared to the control animals, the increases in condition index were not significant.

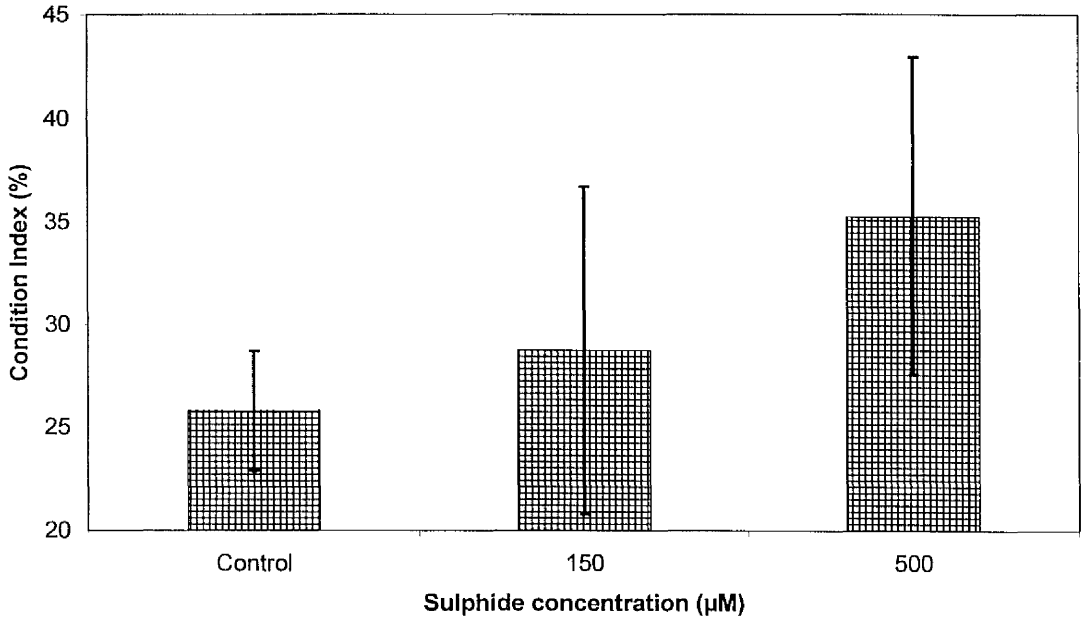

Figure 2.2: The condition indices of survivors of the LT_{50} experiments in which Arctica islandica *were exposed to 150 µM sulphide and 500 µM sulphide. Values are means ± 95% CI (n = 10).*

The condition indices of the *M. edulis* that were exposed to 150 µM sulphide showed a 3% mean increase in condition index compared to the control *M. edulis* (Fig. 2.3). However these increases are not statistically significant.

By use of a general linear model (GLM) the change in condition indices with increasing sulphide concentration in *A. islandica* can be compared with those of *M. edulis*. There is no significant difference ($F = 0.1$; $p = 0.749$) in the change in condition indices with sulphide exposure (slope) between *A. islandica* and *M. edulis*.

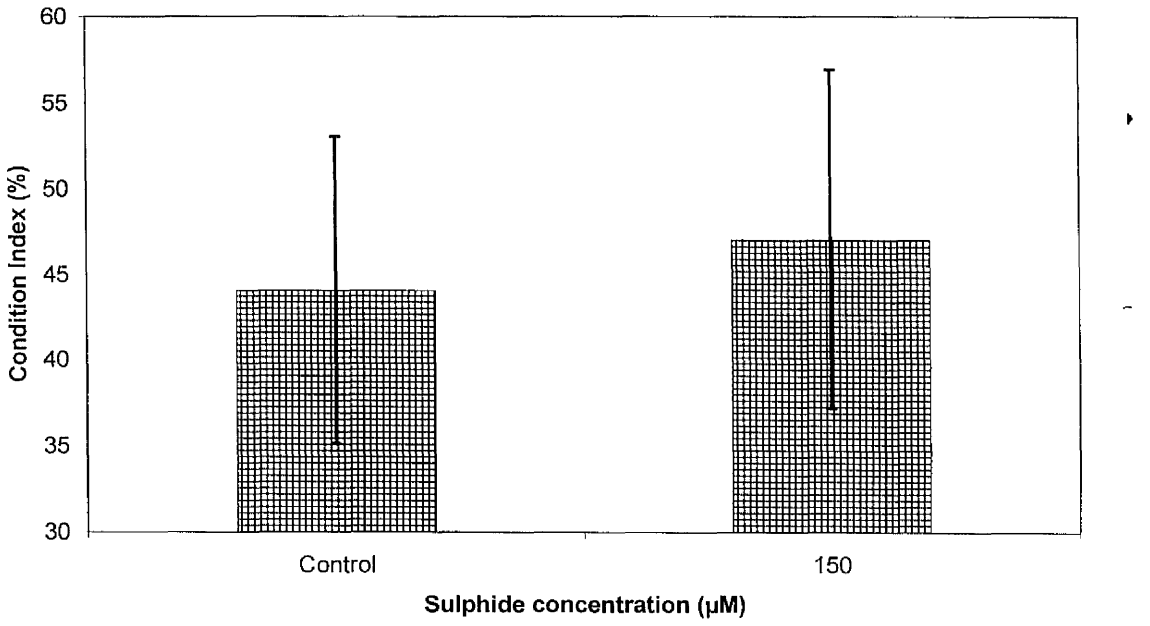

Figure 2.3: The condition indices of survivors of the LT_{50} experiments in which Mytilus edulis *were exposed to 150 µM sulphide. Values are means ± 95% CI (n = 10).*

Discussion

There are some difficulties associated with examining species from a "sulphide rich" area for sulphide tolerance since sulphide is very prone to oxidation, and as such sulphide formation in the sediments is patchy and irregular as well as seasonally dependant (Thiermann *et al.*, 1996). The irregular nature of sulphide distribution can lead to sulphide tolerant species being found in close association with non-tolerant species (Hagerman, 1998). Furthermore, seasonal mass mortalities can occur due to a combination of H_2S and oxygen deficiencies, which is a seasonal occurrence in some areas caused by stratification and eutrophication (Theede *et al.*, 1969; Baden *et al.*, 1990). Hence the presence of a species in a "sulphide rich" habitat does not necessarily mean that that species is sulphide tolerant. The peanut worm, *Sipunculus nudus* and the lugworm, *Arenicola marina* are both inhabitants of mud flats. But only up to 13 µM sulphide has been found in associated pore water of *S. nudus* burrows,

whilst 340 µM sulphide has been found in the *A. marina* burrows (Völkel and Grieshaber, 1992). Higher levels (up to 1mM) have been found in very fine mud sediments (Thiermann *et al.*, 1996), and concentrations of 3.4 mM have been recorded in the sediments of salt marshes (Bagarinao and Vetter, 1989). These concentrations are very much higher than the concentration (33.8 µM) recorded at the site from which the *A. islandica* were collected. But interestingly the bottom water oxygen level was 0 ml. l^{-1} and the *A. islandica* were the only living macrobenthic organism found in Laholm Bay during the sampling period (Chapter 7) which suggests that they have some resistance to sulphide and hypoxia.

Early work examining the resistance of various Baltic Sea and North Sea invertebrate species found LT_{50} values of 800 – 1000 hours in seawater with 0.15 $ml.l^{-1}$ oxygen and 200 µM sulphide, for *A. islandica* and 600 hours for *M. edulis* (Theede *et al.*, 1969). Theede *et al.* did not make use of a flow through system and hence there is always the possibility of a reduction in sulphide concentration with time, due to detoxification mechanisms that have been shown to be present in some species (Powell and Somero, 1986b; Johns *et al.*, 1997). Even so, their results are similar to those described above, with *A. islandica* having by far the greatest resistance to sulphide, but with *M. edulis* also showing some resistance to sulphide.

Upon completion of the LT_{50} experiments, the condition indices of the surviving *M. edulis* and *A. islandica* were calculated using one of the formulae described by Davenport and Chen (1987). The condition indices recorded prior to and after sulphide exposure (44 – 47%) are somewhat lower than those recorded by Gilek *et al.* (1992) in *M. edulis*. These authors recorded condition indices approximately 3 times higher (126) in *M. edulis* from unpolluted areas of the North Sea, and 2 times higher in *M. edulis* from polluted areas. Such a massive discrepancy is more likely to be due to differing local conditions in Loch Fyne where the mussels for this study

were collected. As described in the method section, the salinity ranged between 5 - 25°/$_{oo}$ at the collection site caused by the close proximity of a fresh water stream which may affect the condition indices (Fisher *et al.*, 1996). The close proximity of the village Strachur may also add to local pollution in the area. However, the reason for the differences remains unclear, and could be due to any number or combination of factors, such as salinity, temperature, pH, dissolved oxygen and even season, as discussed in the introduction.

Although the condition indices of the *A. islandica* increased with increasing sulphide exposure concentration, this increase was not significant. There appears to be no corresponding data in the literature with which the condition indices of the *A. islandica* can be compared. Thórarinsdóttir and Jóhannesson (1996) compared the wet meat weight/shell length ratios between *A. islandica* from three distinct sampling areas in Icelandic waters, using the equation $W = c L^p$, where w = tissue weight (g), L = length (mm) and p = slope. The authors described finding the same condition factor in all three areas. However, the slopes differed significantly between areas ($W = 0.637^{-3}L^{(2.981<p<3.053)}$). Using the same equation the relationship of the shell length and wet meat weight for the *A. islandica* from Laholm Bay (Sweden) was calculated ($W = 0.24^{-2}L^{2.028}$).

Both the condition and the slope of the length : weight relationship of the *A. islandica* from Laholm Bay were lower than those quoted by Thórarinsdóttir and Jóhannesson (1996). Such a distinct difference between sampling areas, is possibly a testament to the pristine condition of Icelandic waters where fluctuation due to local pollution is negligible. Gilek *et al.* (1992) when sampling *M. edulis* reported that the condition indices of *M. edulis* also differ between sampling areas. Hence in both species it may be unwise to use condition index as a measure of difference between local habitats, however, using the same species from the same habitat in a closely controlled

laboratory based experiment, will highlight differences caused by manipulation of experimental parameters.

Although the habitat and general behaviour of *M. edulis* and *A. islandica* are very different, both species were maintained under identical conditions during the experimental period. Hence the only variable was the addition of sulphide. It was therefore possible to compare the rate of change in condition index with increasing sulphide exposure between these two species. Both *M. edulis* and *A. islandica* displayed similar increasing rates of change in condition index with increasing sulphide exposure. Although the increase in condition index with sulphide exposure is not significant, the increasing trend is similar in both species. The similarity in the trends may indicate that both *M. edulis* and *A. islandica* gain in condition as a direct result of sulphide exposure. An increase in condition index means that there is an increase in the wet meat volume/weight suggesting that more energy is available for soft tissue growth in the presence of sulphide. Sulphide-stimulated ATP production has been described in the ribbed mussel, *Guekensia demissa* (Parrino *et al.*, 2000) and in callianassid ghost shrimp (Bourgeois and Felder, 2001). However, the ecological benefits of sulphide-stimulated ATP production have remained vague. But if the gain in condition is a direct result of sulphide stimulated ATP production, the benefit of this process to the bivalves is tangible.

In summary, *A. islandica* that encounter sulphide in their natural habitat have the highest survival times, followed by *A. islandica* from a sulphide free area, with *M. edulis* having the shortest survival times. However, both species appear to gain some benefit in condition as a direct result of sulphide exposure, even though exposure to these concentrations of sulphide eventually leads to mortality. These results suggest that both *M. edulis* and *A. islandica* have mechanisms in place that not only allow

prolonged survival upon exposure to sub-lethal concentrations of sulphide, but also a mechanism that can exploit sulphide to create an energy benefit.

Chapter 3

Ventilation rates of *Mytilus edulis* in the presence of sulphide

Introduction

In *M. edulis,* the terminal electron acceptor in the respiratory chain, cytochrome c, is maximally inhibited by exposure to 200 μM sulphide (Lee *et al.*, 1996). However, the results of Chapter 2 indicate that *M. edulis* is able to survive 2 – 3 weeks in the presence of concentrations of sulphide that should result in maximum inhibition of the respiratory chain. Hence *M. edulis* appears to possess mechanisms to deal with a sulphide challenge. Because sulphide inhibits aerobic respiration it presents the animals with an interesting physiological problem. Increasing ventilation would bring more oxygen into contact with the gills in an attempt to restore aerobic respiration, but it would also increase exposure of the gills to sulphide, further inhibiting the respiratory chain. Reducing the ventilation rate would decrease sulphide exposure to the gills, but would also limit oxygen uptake and hence reduce the level of aerobic respiration and sulphide oxidation. Closing the valves and excluding sulphide completely is another alternative, but even though *M. edulis* has a high anaerobic capacity, it cannot survive indefinitely without oxygen (Theede *et al.*, 1969; Bayne, 1971). Although *M. edulis* does appear to have some resistance to sulphide (Chapter 2), it is not known how it modifies its physiological responses when exposed to

sulphide. However, it is possible that the strategies utilised by *M. edulis* to survive respiratory stress caused by sulphide exposure may be present in other invertebrates.

The effects of respiratory stress in invertebrates, whether exposed to low ambient oxygen concentrations, or to toxins that inhibit respiratory pathways, have been well documented (Theede *et al.*, 1969; Vetter *et al.*, 1987; Bagarinao and Vetter, 1989). Generally, an initial increase in respiratory stress is characterised by an increase in heart rate and in gill ventilation (Wheatley and Taylor, 1981; Morris and Taylor, 1985). Such a strategy is only effective up to a point, beyond which invertebrates are forced into a switch to anaerobic metabolic pathways. To decrease energy expenditure during anaerobiosis, the metabolism of the affected animal is reduced to a minimum and this is manifested as a decrease in heart rate, gill ventilation rates and in general activity (Helm and Trueman, 1967; De Fur and Magnum, 1979).

Unlike crustaceans, most bivalves are fairly sessile and unable to avoid environmental toxins such as sulphide. It has been shown that the marine animals most resistant to sulphide are those that have the most well developed anaerobic metabolism (Gorodesky and Childress, 1994; Jahn *et al.*, 1996; Vopel *et al.*, 1998). Since many sessile or burrowing invertebrates are particularly well adapted to hypoxic conditions, it is these animals that have been the subject of most research (for reviews see Childress and Fisher, 1992; Grieshaber *et al.*, 1992; Grieshaber *et al.*, 1994). *M. edulis* is resistant to hypoxia (Coleman, 1974), and appears to have some resistance to sulphide even though it is an animal that is not usually associated with this environmental toxin (Chapter 2).

The changes in the ventilation rates of *M. edulis* as a consequence of exposure to declining oxygen tension (Po_2) have been examined previously (Bayne, 1971; Taylor and Brand, 1975). At slightly reduced Po_2, *M. edulis* increased the ventilation rates to compensate for lower oxygen availability (Bayne, 1971). However, if Po_2 continued to fall, ventilation rates declined steadily (Taylor and Brand, 1975).

Methods by which ventilation rates may be measured in bivalves suffer from the difficulty of trying to quantify the flow from a flexible exhalent siphon that, in many species, cannot easily be separated from the inhalent siphon without considerable interference to the bivalve. Researchers have developed a wide range of methods either to separate the water flow between the inhalent and exhalent siphons, or to bypass this problem altogether. These methods can be broadly separated into two categories. Simply, those that rely on the quantification of particulate matter removed by the ctenidia (gills) (indirect methods) and methods that rely on a range of novel approaches to quantify directly the flow rate from the exhalent siphon (direct methods). Throughout this chapter the term ventilation rate will be used to describe the volume of water that is expelled from the exhalent siphon per unit time and is therefore equivalent to the term 'ventilation volume' used by some authors in studies of crustaceans and fish (Clark and Potts, 1998; Parsons and Carlson, 1998; McKim *et al.*, 1999; Rose *et al.*, 2001).

The methods that quantify the removal of particulate matter from the water column are all variations of the same technique, commonly known as the clearance method (Fox *et al.*, 1937; Jørgensen, 1943). If a known concentration of particulate matter (micro-algae are often used) is introduced into a tank containing a suspension feeding

bivalve, the decrease in the concentration of algae as a function of time is directly related to the volume of water passed over the gills. The clearance method has been shown to be a reliable method (Jørgensen, 1949; 1955), but it has some immediately apparent constraints. The most severe is that the concentration of algae decreases with time. The obvious solution is to maintain the concentration of algae in the tank constant (Loosanoff and Engle, 1947; Jørgensen *et al.*, 1988). Part of the inhalent and exhalent water can also be siphoned off and the difference in algal concentrations calculated (Famme *et al.*, 1986). There are a number of other variations of the same basic clearance method (for a review see Riisgård, 2001a), for example, the steady state method where the concentration of algae is kept stable by the constant addition of algae by means of a dosing pump. The algal concentration is measured at fixed intervals, during the experimental period, and hence a mean ventilation rate may be calculated (Winter, 1973; Clausen and Riisgård, 1996). A further modification of the clearance method is where a constant flow of algae is passed through an experimental chamber and the difference in the concentration of algae in the inflow and outflow water is measured by means of either an electronic particle counter (Thompson and Bayne, 1972; 1974; Vahl, 1972) or a haemocytometer (Taylor and Brand, 1975). Other methods utilised by researchers include the use of a photoaquarium, to quantify the algal concentrations (Winter, 1973).

The direct methods can be separated into three categories, although some consist of a combination of methods. The dye method consists of replacing the inhalent seawater with gravity fed dyed seawater from a reservoir of dyed water and hence the rate of flow can be measured. A capillary tube is placed close to the inhalent siphon,

and a steadily increasing flow of dyed seawater passes into the inhalent siphon until the inhalent seawater is totally replaced with dyed seawater (Coughlan and Ansell, 1964). There have been modifications to this method, such as the elaborate system employed by Hamwi and Haskin (1969) to enable oxygen concentrations to be measured simultaneously in the exhalent dyed seawater, but the principle remains the same.

A commonly utilised method to measure ventilation activity of bivalves is the thermistor method (Heusner and Enright, 1966; Deaton and Mangum, 1976). This technique enables accurate measurements of ventilation activity to be made without disturbance. Two thermistors with identical current versus resistance characteristics are utilised. One is placed close to the exhalent siphon, and the other shielded from the flow but in the same water. Both thermistors are slightly heated using a small current, and the thermistor in the flow of the siphon cooled by the water flow. The difference in the temperature of the 2 thermistors can be compared using a Wheatstone bridge circuit (for schematic see McCammon, 1965). The thermistors can provide accurate recordings of the speed of water movement once they have been calibrated using known flow rates. To calculate the actual ventilation volume, however, it is necessary to measure the area of the exhalent siphon and then multiply by the speed of water movement (Lowe and Trueman, 1972; Meyhöfer, 1985).

There are a number of methods that rely on physically separating the flow from the exhalent siphon from that of the inhalent. One of the earliest was developed by Galtsoff (1926) who relied on forcing the valves apart, inserting a rubber tube into the mantle cavity before packing any leaks with cotton wool, and collecting the flow from

the gills in a measuring cylinder. The obvious problem with this method is that there is considerable disturbance to the bivalve, which could affect the accuracy of the data obtained. Other researchers have attached a tube or apron to the valves which mechanically separates the siphons and, in the case of attachment of a tube, allows the exhalent flow to be quantified directly (Loosanoff and Engle, 1947; Drinnan, 1964). The attachment of a tube to the valves is not a new idea (Moore, 1908), and has been utilised with a great deal of success over the years (Hildreth, 1976; Davenport and Woolmington, 1982; Famme *et al.*, 1986). All these methods rely on an overflow system to quantify the flow from the exhalent siphon, and therefore have the drawback that the flow needs to be measured manually using a measuring cylinder. A further development was the invention of an impeller that was driven by the water flow, and allowed constant recordings to be made (Jones *et al.*, 1992)

All the methods described so far have some drawbacks, in particular, those methods that quantify the removal of particulate matter. The obvious drawback is that the assumption made is that a fixed percentage of particulate matter is retained by the gills (Coughlan and Ansell, 1964; Ali, 1970; Hildreth, 1976). Research has shown that different sizes of particulate matter have different retention percentages; retention of the micro-alga *Chlorella* sp. varies from 0 – 92% in *Ostrea virginica* (Loosanoff and Engle, 1947). Furthermore, the same oysters were shown to retain less than 50% of the particulate matter that passed over the gills (Loosanoff and Engle, 1947). Vahl (1972), who examined the effect of size on particle retention in *Chlamys opercularis* supported these findings. In addition, there is now evidence that the concentration of algae in the water may affect the rate of ventilation in *M. edulis* (Winter, 1973, Taylor and Brand, 1975).

The dye and thermistor methods are accurate methods that do not interfere with the bivalves. Both methods are reliant on the correct placement of the capillary tube or thermistor, in the flow of the siphon, and work best with bivalves that maintain a fairly constant position in the sediment, such as *Mya arenaria* and *Mercenaria mercenaria*. Of the two methods, the thermistor method is more attractive as a continuous recording can be made, but suffers from the need to accurately determine siphonal aperture area to enable the actual volume of water pumped to be determined.

The attachment of an apron or tube to the valves in order to separate the inhalent and exhalent flow does not seem to impede normal pumping activity Riisgård (2001a), but nevertheless has 2 distinct disadvantages. Firstly, the bivalve is attached to an overflow tube, and hence cannot move freely, and secondly there is the danger that back pressure from the overflow system could seriously reduce pumping efficiency and therefore ventilation rate (Hildreth, 1976).

For the purposes of the series of experiments described in this chapter, the ventilation rate needed to be recorded continuously. The method had to be sufficiently sensitive to show small changes in ventilation rate with changing sulphide exposure concentration, over short periods of time. With these criteria only two methods were deemed appropriate, the thermistor method, or the attachment of a tube to the valves in order to separate the siphons. By accurately quantifying the flow of seawater over the gills of *M. edulis* it is possible to study the changes in ventilation rate upon exposure to differing concentrations of sulphide. As the gills are the main point of entry for both oxygen and sulphide into *M. edulis*, it would be interesting to discover the changes in ventilation rate (volume) that might result from exposure to sulphide.

The aim of this experiment was to measure directly the effect of sulphide on the ventilation rate of *M. edulis* under normoxic conditions.

Materials and Methods

M. edulis were collected from the banks at the top of Loch Fyne (Strachur, Scotland) (56° 10` N; 5° 05` W) where the salinity ranged between 5 - 25°/$_{oo}$. They were transported to the University Glasgow and maintained in re-circulating seawater aquaria (salinity = 32°/$_{oo}$; temperature = 10°C), under a 12:12 hour light: dark regime. Individuals were not fed whilst under observation but some particulate matter was present in the circulating seawater and may have provided a source of food. A large number of mussels covering a wide range of sizes were collected, however only a selected size range, (mean length, from umbo to opposing shell margin, of 61.5 mm ± 4.3 mm), were used for the experiments described in this chapter.

The sulphide stock solution (50mM) was prepared from Na_2S. $9H_2O$ (Sigma, S-4766 Assay 100%) every day prior to use and maintained under a nitrogen atmosphere to prevent oxidation. The concentration of the stock solution was determined using iodometric back titration (Parsons *et al.*, 1984), and colorimetric determination of sulphide modified from Parsons *et al.* (1984) was used to monitor sulphide concentration in the exposure tank, and during experimentation (see Chapter 2).

Two direct methods of quantifying ventilation rate were tested to find the most accurate for use with *M. edulis*. The first method used was the thermistor method. Thermistors with identical current versus resistance characteristics (2.2 kΩhms) were

utilised. One was placed close to the exhalent siphon, and the other shielded from the exhalent flow, but in the same water. Both thermistors were slightly heated using a small current (8 mA), and the thermistor in the flow of the siphon was cooled by the water flow. The difference in the temperature of the 2 thermistors was compared using a Wheatstone bridge circuit (for schematic see McCammon, 1965) and recorded on a pen-recorder. The thermistors were calibrated using the flow from a peristaltic pump with a tube of known diameter at a number of known flow rates. Subsequently, the areas of the siphons were estimated by carefully measuring the length and width of the siphon using callipers enabling the calculation of the ventilation rate ($ml.min^{-1}$) (Lowe and Trueman, 1972; Meyhöfer, 1985).

The second method used relied on the attachment of a tube to the valves to separate the siphons. A soft latex tube (finger from a latex surgical glove), was fitted over the exhalent siphon and sealed in place against the valves using cyanoacrylate adhesive to create a water tight join between the latex tube and the shell valves. Care was taken not to impede the natural valve movements. When the valves re-opened the individual *M. edulis* were examined to ensure that the siphons were successfully separated. The latex tube was subsequently connected via a cannulating probe (SP7507), to a blood flow meter (Spectramed Blood Flow meter SP2202). The blood flow meter was calibrated *in vitro* using the Spectramed Calibrator (SP7010). The blood flow meter monitors the change in pulsatile EMF (voltage) caused by changes in flow through a cannulating probe (electromagnet), which allows a continuous recording of the actual ventilation volume ($ml.min^{-1}$) to be made without impeding movement or mobility in any way. The mussels were placed in an exposure tank (volume = 355 ml), and an airtight lid fitted. Nitrogen was bubbled into the seawater in

the exposure tank at a slow rate, which displaced any oxygen in the gap between the water and the lid of the exposure tank to minimise the oxidation of sulphide as a result of contact with the air. The rate of flow of the nitrogen was adjusted to create a positive nitrogen atmospheric pressure in the exposure tank. But care was taken to ensure that the nitrogen did not lower the Po_2 of the exposure tank. Po_2 was monitored as described below. The exposure tank was held in a constant temperature room at 10 °C for the duration of the experiments.

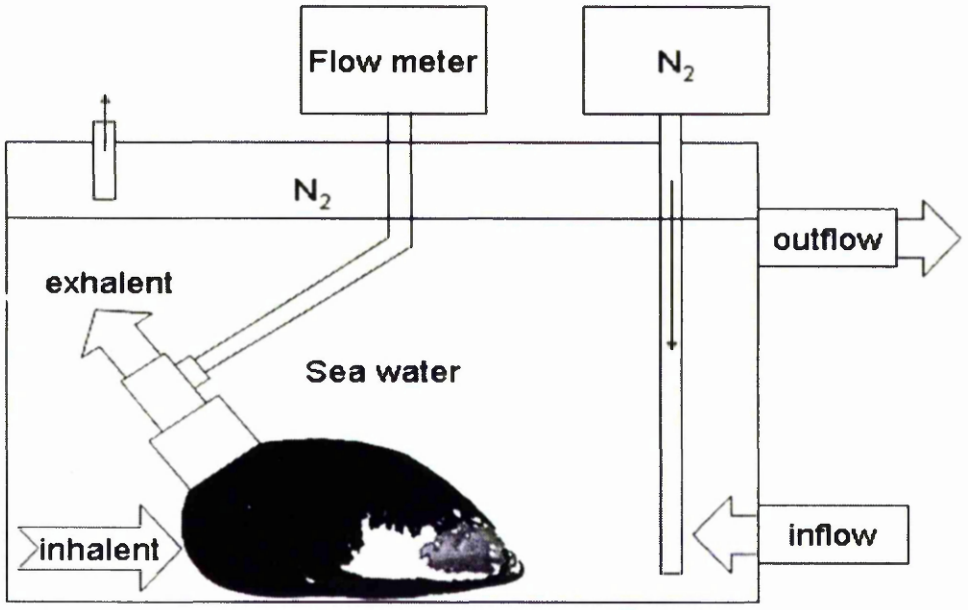

Figure 3.1: A stylised diagram of the apparatus constructed to measure ventilation rates in Mytilus edulis *using a blood flow meter. The inflow is a mixture of H_2S and normoxic seawater, as described in the method section above.*

Aerated seawater was pumped continuously through the exposure tank at a slow rate (74 – 240 $ml.min^{-1}$) using a peristaltic pump. A second peristaltic pump was used to

pump the stock sulphide solution into a mixing chamber located upstream of the experimental exposure tank. By adjusting the relative flow rates of the two pumps it was possible to obtain the required sulphide concentrations within the exposure tank whilst still maintaining normoxic conditions.

The Po_2 of the water was monitored continuously in the outflow water using a microcathode oxygen electrode (Strathkelvin Instruments, Oxygen Meter 781). The electrode was calibrated prior to use against aerated sea water (at 10°C) and against a solution having a Po_2 of zero (sodium sulphite in 0.01 M sodium tetraborate). Due to the rapid oxidation of sulphide in the presence of oxygen, the actual sulphide concentration in the exposure tank water was measured using the colorimetric method described by Parsons *et al.* (1984), and the relative flow rates of the 2 peristaltic pumps re-adjusted to compensate for sulphide oxidation, until the required sulphide concentration was achieved.

Initially, changes to the gape of the valves were measured using a strain gauge (Strathkelvin Instruments), connected to a Washington pen recorder via a FC116 strain gauge coupler (Strathkelvin Instruments). The individual mussels had one valve fixed to the bottom of the exposure tank using wax, and the strain gauge was connected to the opposite valve via wax and thread. This enabled continuous recordings to be made of the shell gape.

The mussels were allowed to settle overnight, with the latex tube in place, before the experiment commenced. Individual *M. edulis* were exposed to sulphide at concentrations 200 µM, 500 µM, 800 µM and 1200 µM for 60 minutes and allowed to

recover (each mussel was exposed to only one concentration). The fluctuations in ventilation rate recorded by the blood flow meter were monitored using a Maclab interface and Macintosh computer.

Statistical analysis

All data were analysed using Minitab 11 statistical package. The data were normally distributed (Normal scores > 0.917), there was no significant heterogeneity of variance (Bartlett's value = 6.318; $p = 0.958$), and a one way ANOVA was used to check for significance ($p < 0.05$).

Results

Methodology evaluation

Initially, pilot studies were carried out using the thermistors as described in the introduction. Good recordings of the speed of the water flow from the exhalent siphon were achieved. However, it was observed that *M. edulis* are able to vary the size of the siphonal apertures, by partially closing the siphon, or closing one end, and hence the exhalent current often bypassed the thermistor. Furthermore, it proved difficult to accurately calculate the area of the siphonal aperture. The mussels were shown to change the direction of flow from the exhalent siphon which, due to the operating nature of the thermistor, was recorded as a positive flow direction, all of which compounded the problem of obtaining accurate recordings from this species. The *M. edulis* were also very prone to disturbance. Any vibration, even from the adjoining room, would cause the mussels to cease ventilation temporarily, although the valves would remain open. The mussels displayed the ability to vary the flow rate through the exhalent siphon, as well as to reverse the direction of flow as mentioned above, even

in the absence of external stimuli. Although the method enabled recordings of pumping activity to be obtained it was very difficult to obtain quantitative data for the ventilation volume of *M. edulis*. As a result, this method was subsequently abandoned.

Observations of the behaviour of *M. edulis* showed that when the shell margin is fouled with byssal threads from other mussels, an individual will still extend its siphons, and press them against the byssal threads to maintain as large an inhalent and exhalent area as possible (personal observation). If a soft latex tube is fixed in place over the exhalent siphon, the mussel will push the mantle edge against the latex hence sealing the gap between the siphons.

A typical trace of the ventilation rate of *M. edulis* recorded prior to a challenge with sulphide is displayed in Fig. 3.2. Utilising the blood flow meter allowed continuous recordings to be made. The sensitivity of this methodology enabled very accurate readings of ventilation rate to be made over varying time scales from seconds to hours.

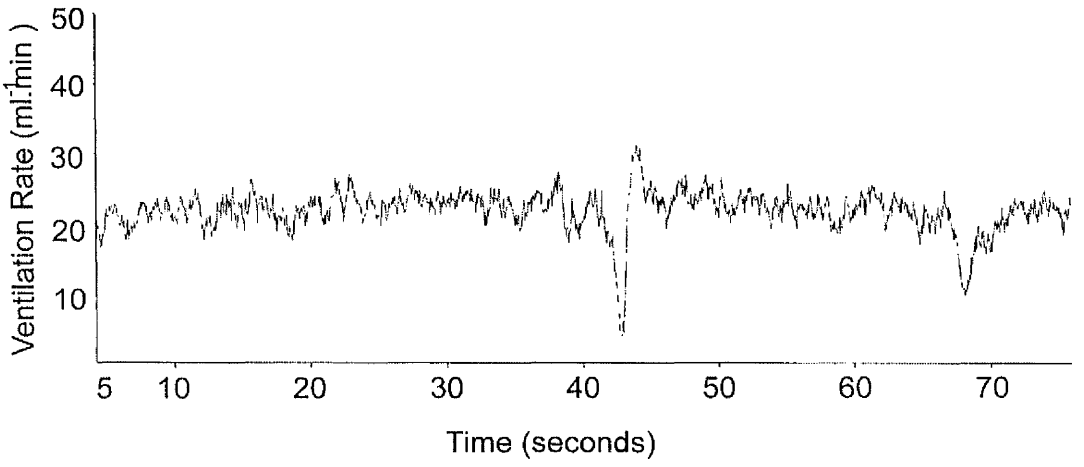

Figure 3.2: Trace of the ventilation rate of Mytilus edulis *prior to sulphide exposure (control rate) at 10 °C. Recordings were carried out using a Spectromed flow meter and Maclab 3.4 software. For further details see text.*

Any mechanical disturbance to the *M. edulis* resulted in the valves either partially or fully closing. However, the recordings of valve closure made with the strain gauge showed that sulphide had no effect on valve closure at any of the concentrations used although there was a marked effect on the ventilation rate.

The mean ventilation rates (20 – 40 $ml.min^{-1}$) have been converted to a percentage of the resting ventilation rate (100%) measured prior to the addition of sulphide. The ventilation rates were converted to percentages of the resting (control) rate, as it is the change in ventilation activity that is of interest in this series of experiments and this conversion enabled comparisons to be made between animals showing different initial ventilation rates. The reduction in flow rate after 43 seconds (Fig. 3.2) is due to the slight vibration caused by hitting a table in the room adjoining the experimental temperature controlled room, in an attempt to examine the sensitivity of *M. edulis* to disturbance. This demonstrated that *M. edulis* are very sensitive to any such

disturbance and that this may result in a sudden closure of the shell and a reduction in ventilation rate.

Effects of sulphide on the ventilation rate

The mean ventilation rates of *M. edulis* exposed to varying concentrations of sulphide for 60 minutes were recorded (Fig. 3.3). Following the sulphide exposure period the ventilation rate during the post exposure, or recovery period, was recorded for an additional 60 minutes.

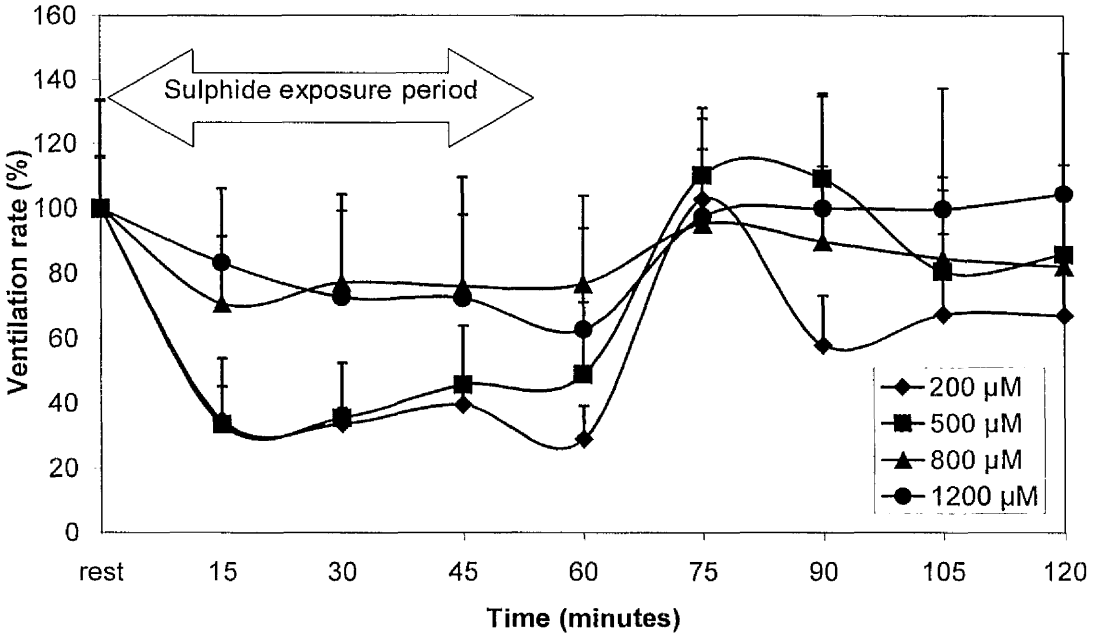

Figure 3.3: Mean ventilation rates of Mytilus edulis *exposed to sulphide (µM) for 60 minutes and then allowed to recover for 60 minutes (pH = 8; temperature = 10°C). Values are expressed as a percentage of the initial ventilation rate for each animal. Values are means + standard error (n = 8).*

Upon the addition of sulphide the mean ventilation rates decreased by between 60 % and 80 % for the mussels exposed to 200 µM and 500 µM sulphide, but only by between 20 % and 40 % for those mussels exposed to 800 µM and 1200 µM sulphide. Within the first 15 minutes of the recovery period the mean ventilation rate increased in all cases. The increase of the ventilation rate during the first 15 minutes of the recovery period is particularly pronounced for the mussels exposed to the lower concentrations of sulphide (200 µM and 500 µM), and overshoots the resting ventilation rate before stabilising.

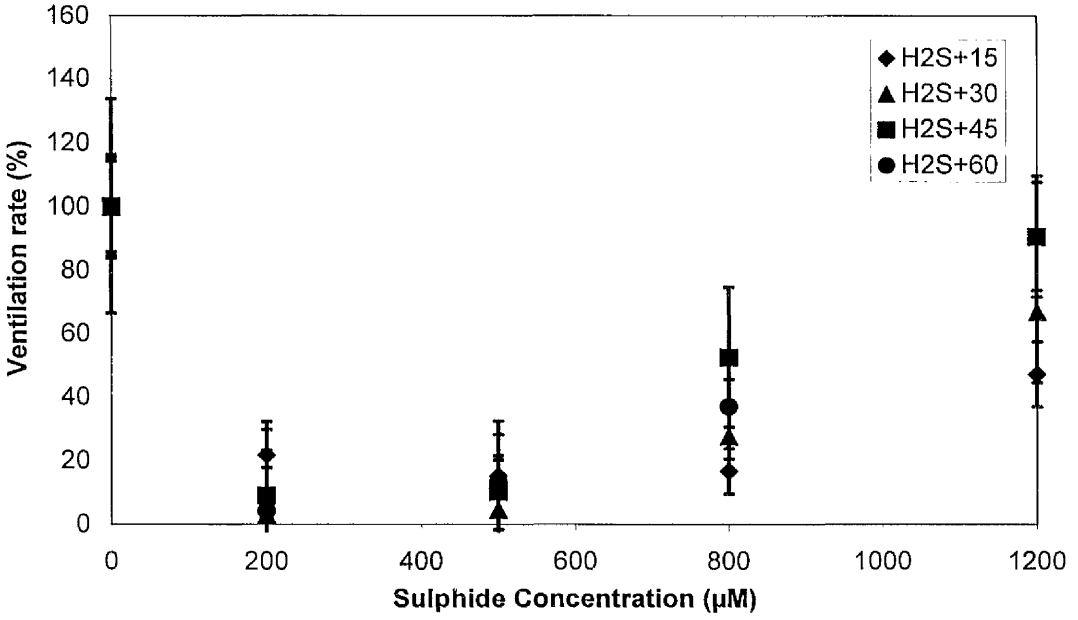

Figure 3.4: Mean ventilation rates of Mytilus edulis *after 15 minutes, 30 minutes, 45 minutes and 60 minutes exposure to varying concentrations of sulphide (pH = 8; temperature = 10°C). Values are expressed as a percentage of the initial ventilation rate for each animal. Values are means ± standard error (n = 8).*

The ventilation rates for animals exposed to differing concentrations of sulphide for 15, 30, 45 and 60 minutes are summarised in Fig. 3.4 again with the resting

ventilation rates shown as 100 %. Although the trend is consistent at each of the 15 minute time increments, there was no significant difference between the ventilation rates at each concentration. The largest decrease in ventilation rate from the resting rate (100%) was shown by those mussels exposed to 200 µM sulphide. The ventilation rates subsequently increased approximately linearly with increasing sulphide concentrations to the maximum sulphide concentration used (1200 µM).

Discussion

Methodology evaluation

M. edulis seem to suffer no ill effects from the attachment of a soft latex tube to the valves in the area of the exhalent siphon, and still fully extended the siphon after a short recovery period (15 - 30 minutes). Careful consideration would be advised before using this method on other bivalves as success in this case could simply be due to the gregarious nature of *M. edulis* which frequently encounters similar obstructions around the siphons in its natural habitat.

If the siphons can be successfully separated without disturbance to the natural behaviour of the bivalve, as in this case, the use of a blood flow meter to measure bivalve ventilation rates provides a sensitive and accurate method of direct measurement that is especially useful at low ventilation rates (below 10 $ml.min^{-1}$). The ventilation rates recorded varied between 20 – 40 $ml.min^{-1}$ in the pre-exposure or control *M. edulis*. Previously, researchers have quantified the ventilation rates of *M. edulis* using an indirect algal clearance method described in the introduction. Bayne (1971) recorded ventilation rates of 11 – 19 $ml.min^{-1}$, whilst Thompson and Bayne

(1972) recorded slightly higher rates of 16 and 30 ml.min^{-1} in *M. edulis* weighing 0.31 g dry wt and 1.20 g dry wt, respectively. A slightly modified algal clearance method was utilised by Clausen and Riisgård (1996), who measured similar flow rates in this species of 11 – 33 ml.min^{-1} in individuals ranging from 25 – 30 mm in length. However, Davenport and Woolmington (1982) recorded much higher ventilation rates using a direct method in the presence of algal cells. These authors found that in the presence of a food source *M. edulis* increased the ventilation rates to a maximum of 80 ml.min^{-1}, whereas in the absence of a food source ventilation rates were reduced to 20 – 30 ml.min^{-1}. It is therefore probable that any attempt to measure changes in ventilation rate due to respiratory stress could be frustrated if an algal clearance method is used to quantify ventilation rate, due to the resulting feeding response of *M. edulis* overriding respiratory considerations.

Effects of sulphide on the ventilation rate

Upon the addition of sulphide to the water in the tank containing *M. edulis*, there was a marked effect on the ventilation rate. It is perhaps surprising that the mussels did not close the valves when exposed to sulphide, especially at the higher exposure concentrations, in an attempt to limit uptake. An explanation for this observation may be that since *M. edulis* does not usually encounter sulphide in its natural habitat, it may be unable to detect sulphide in the surrounding water. The two pallial sense organs in mussels are the osphradium which is assumed to be chemosensory (Thompson and Bayne, 1972) and the abdominal organ (White, 1937). The ability of this species to detect sulphide does not appear to have been the subject of any previous study. The lack of any response by *M. edilus* even to the high concentrations of sulphide used during the present study may indicate either that *M. edulis* only has a

limited ability to detect sulphide, or is totally unable to detect sulphide in water (at least at these concentrations).

Rather unexpectedly, mussels exposed to the lower concentrations of sulphide (200 µM and 500 µM) showed the greatest reduction in ventilation rate. The ventilation rate of the *M. edulis* when exposed to both these concentrations decreased to approximately 30 % of the resting rate. This may be indicative of an attempt by *M. edulis* to limit the amount of sulphide that is pumped through the valves. Similar results were obtained by Doeller *et al.* (1999a) where excised gills from the sulphide tolerant salt marsh species *Geukensia demissa* and from *M. edulis* showed a decrease in ciliary beat frequency with increasing sulphide exposure concentration. Unfortunately, the authors did not use sulphide exposure concentrations greater than 500 µM making further comparison impossible. The same authors showed that oxygen consumption was stimulated upon exposure to 200 µM – 500 µM sulphide in excised *G. demissa* (Lee *et al.*, 1996). This conflict between increasing oxygen consumption rates and decreasing gill ciliary beat frequencies may not be due to changes in metabolic pathways or oxygen transport. Investigations into the neuromuscular sensitivity of the echiuran worm *Urechis caupo* to sulphide have shown that sulphide directly inhibits muscular contraction by an unknown mechanism that was thought to be independent of changes in metabolic pathways or oxygen transport (Julian *et al.*, 1998). The authors argue that the most likely cause was due to the increasing inability of the muscle tissue to contract in the presence of sulphide due to the HS^- ion binding, possibly via a disulphide bridge, to an unknown target in the muscle and reversibly inhibiting contraction.

As soon as the period of sulphide exposure ended, the ventilation rates of all the mussels increased to a higher rate than those recorded during the pre-exposure (control) rates. This might have resulted from an attempt either to flush any remaining sulphide from the gills, or to repay an oxygen debt incurred during the sulphide exposure period. Previous work on the recovery of invertebrates from sustained hypoxia has shown that there is an elevated period of oxygen consumption at the beginning of the recovery period (Helm and Trueman, 1967; Bayne, 1971; Vismann and Hagerman, 1996; Bourgeois and Felder, 2001). This is accompanied by increased ventilatory rates (Bayne, 1971; Taylor, 1977; Vetter *et al.*, 1987), possibly in an attempt to pass more oxygen over the gills to repay the oxygen debt incurred during the hypoxic event. Unfortunately, it is not possible to separate an attempt by *M. edulis* to flush the sulphide from the gills from an attempt to increase oxygen availability to repay any oxygen debt.

Following sulphide exposure, the excised gills from *M. edulis* and *G. demissa* showed no increase in the ciliary beat rate above the resting rates (i.e. no 'overshoot'), as displayed by the intact bivalves, instead it returned quickly to the resting rate (Doeller *et al.*, 1999a). The discrepancy between the reactions of the intact *M. edulis* and those of excised gills could possibly be due to direct physiological control in the intact mussel reacting to fluctuations in the sulphide exposure concentration, which is absent in excised gills.

The ability of the mussels actively to control ventilation rate as a response to changes in environmental parameters is the focus of some debate. Either ventilation rate and bivalve feeding are highly automated and unregulated processes, with a limited set of

responses to environmental conditions (Clausen and Riisgård, 1996; Riisgård, 2001a,b,c), or there is direct physiological control of ventilation rate by the mussel (Cranford, 2001; Widdows, 2001). The significance of direct physiological control is that there can be a wide range of responses by a mussel to changing environmental conditions such as an increased supply of food, or the presence of an environmental toxin. If ventilation rates were highly automated, there would be no difference in the response of excised gills and that of the gills in an intact mussel upon exposure to sulphide. From the results discussed above this is not the case, suggesting that there is direct physiological control of ventilation in *M. edulis*.

Interestingly, the mussels exposed to 800 µM and 1200 µM sulphide showed a different response to those exposed to 200 µM and 500 µM sulphide. The ventilation rates of those mussels exposed to the higher concentrations of sulphide only decreased to approximately 70 % of the resting rate upon sulphide exposure. A possible explanation for the marked difference in response to sulphide exposure between the mussels exposed to 200 µM/500 µM and to the 800 µM/1200 µM concentrations is that at the higher sulphide exposure concentrations either *M. edulis* was unable to detect sulphide, or the direct control of ventilation, as described above, is inhibited. The former phenomenon is known to occur in terrestrial animals exposed to sulphide (National Research Council, 1979), but has not previously been observed in bivalves. Sulphide is a neuromodulator in mammals (Abe and Kimura, 1996); subsequent research showed that sulphide is also a smooth muscle relaxant (Hosoki *et al.*, 1997). Hence it is possible that exposure to high concentrations of sulphide affects the efficiency of the control of ventilation rate in *M. edulis*. There is very little 'overshoot' in the ventilation rates of the mussels exposed to 800 µM/1200 µM at the

beginning of the recovery period when compared to the mussels exposed to 200 µM/500 µM sulphide which adds evidence to the latter theory. However the reason for the discrepancy remains unknown.

After 15, 30, 45 and 60 minutes of sulphide exposure, the mussels exposed to 200 µM sulphide showed the greatest reduction in ventilation rate. Subsequently, there was a steady increase in ventilation rate with increasing sulphide concentration. However, sulphide inhibits aerobic respiration by binding to the terminal oxidase in the respiratory chain (National Research Council, 1979). Later research revealed that the maximum amounts of the inhibited or reduced form, of cytochrome c in *M. edulis* gills (70% inhibition) occurs upon exposure to 200 µM sulphide (Lee *et al.*, 1996). It seems probable, therefore, that at higher sulphide exposure concentrations *M. edulis* must be utilising non oxygen-dependent, sulphide-insensitive pathways for energy production. However, utilisation of such pathways is characterised by a reduction in ventilation rates (Helm and Trueman, 1967; De Fur and Magnum, 1979), not an increase as displayed above. As ventilation rates continued to increase upon exposure to sulphide concentrations above those needed to maximally inhibit cytochrome c, it seems probable that *M. edulis* does not resort to anaerobic metabolism in the presence of sulphide. Instead, *M. edulis* may still be utilising aerobic pathways even when exposed to 1200 µM sulphide. As mentioned above, the cytochrome c of *M. edulis* is sensitive to sulphide. If aerobic respiration does continue in the presence of sulphide there must be a mechanism in *M. edulis* that allows aerobic respiration to occur even when exposed to sulphide concentrations sufficient to inhibit the cytochrome c, and hence the respiratory chain.

In summary, the use of the blood flow meter provides a sensitive method of continuously recording ventilation rate with minimum disturbance to the mussel. Intact *M. edulis* appear to display direct physiological control of ventilation, which is not apparent in isolated *M. edulis* gills. The maximal inhibition of ventilation occurred on exposure to the lowest sulphide concentration. Higher ventilation rates were recorded at higher sulphide exposure concentrations suggesting that aerobic respiration may continue in the presence of sulphide.

Further research using a specific tissue from *M. edulis* as a model of an aerobically active tissue would be useful to examine the effect of sulphide exposure on respiration and metabolism in *M. edulis*. The isolation of an aerobically active tissue would possibly avoid the effects that different behavioural activities, such as feeding, would have on any attempt to quantify respiration and metabolism upon exposure to sulphide.

Chapter 4

The effect of hydrogen sulphide on isolated heart preparations from the bivalve mollusc, *Mytilus edulis*

Introduction

In Chapter 3 the effect of sulphide on ventilation rate of *Mytilus edulis* was examined and although it was shown that there was a profound effect of sulphide on ventilation rate, the causes remain to be established. The fact that hydrogen sulphide has also been shown to have an inhibitory effect on vertebrate axonal conduction, synaptic transmission, neurotransmitter content and neuronal growth (Julian *et al.*, 1998), raises the possibility that sulphide may have a similar effect on invertebrates. As there is more than one possible effect of sulphide on ventilation rate, a number of questions arise. Is the observed effect due to the inhibitory effects of sulphide on respiratory processes in the gills, a physiological reaction by the mussel to control the toxic effects of sulphide, the effects of sulphide on the nervous system, or due to the inhibition of the ciliary mechanism by sulphide?

In order to clarify some of these points another tissue needed to be selected to study the effects of sulphide on metabolism. The criteria for selection were first to select a tissue that can be used as a model of aerobically active tissue, with very little or no neurogenic control. And then to isolate the tissue so that changes in activity caused by modifications in behaviour, due to nutritional and respiratory requirements, can be minimised. The most likely candidate in *Mytilus edulis* is the ventricle of the heart. The ventricle of bivalves is highly conserved and not dissimilar to that found in gastropods.

It is therefore possible to examine the function of the heart in other bivalve species and in gastropods, to gain a better insight into the function of the heart in *M. edulis*.

The vascular system of bivalves is characterised by a dorsal heart that consists of a central ventricle and paired lateral auricles. These three chambers are enclosed in a pericardium; however, there is no open flow of haemolymph between the heart and the pericardium (White, 1937). The ventricle usually surrounds the rectum of the animal, but the morphology can vary from species to species. The auricles are fine structures and are easily damaged during dissection (Hill and Welsh, 1966). The ventricle is a more muscular structure that has considerable contractile power (Krijgsman and Divaris, 1953). The variations in pressure between the ventricle and pericardium were investigated by Jones (1971). He showed that in the gastropod, *Helix pomatia* L., the ventricle generated by far the highest pressure (24cm of water), followed by the auricles (5cm) and then the pericardium. Although his work was on a gastropod mollusc, it shows that the ventricle provides the main driving force for blood flow. Later work by Brand and Morris (1984) showed that this system is relatively inefficient in the dog cockle, *Glycymeris glycymeris* (L.). The authors speculated that in bivalves all tissues in contact with seawater are involved in oxygen uptake. The ventricle of bivalves is composed of muscle fibres of the smooth unstriated type that are interwoven to form the inner wall. The outer wall consists of an epicardium made of a single layer of epithelial cells interspersed with mucous cells (Motley, 1933). Furthermore, Motley showed that the epicardium forms a barrier restricting the free passage of substances between the blood and the pericardial fluid. Early histological work gave no evidence of a neurogenic pacemaker mechanism (Krijgsman and Divaris, 1953) which had been the focus of some debate for a number of years (Carlson, 1906). Further research by Ebara (1967), who experimented on half-ventricles connected only by muscle fibres, demonstrated that the pacemaker

mechanism in bivalves, as with most molluscs, is myogenic, and as such is a result of the integration of the rhythm of the individual cells of the ventricle.

Speculation as to the role of the heart in molluscs in general has led to research on a number of aspects of the cardiac physiology of these animals. The heart rate was shown to vary with activity and with the breathing cycle in *H. pomatia* L. (Sommerville, 1973), even in the absence of the visceral nerve connection. The heart rate in the freshwater mussel *Anodonta anatina* varies as the animal changes activities (Brand, 1976). For example, digging, gaping, and feeding all produce different heart beat responses. Others have examined the effect of respiratory stress on the heart. Earll (1975) commented that although there was some evidence that the amplitude of the heart varied with respiratory stress in bivalves, it was somewhat inconclusive. Further research by Brand (1976) showed that the systolic pressure of the heart decreases with sustained respiratory stress lending credence to the possibility of the effect of respiratory stress on the amplitude of the heart stroke.

Most researchers, however, have concentrated on the beat frequency response of the heart to different stimuli including respiratory stress. Bayne (1971), using *M. edulis*, looked at the heart rate response in some depth and reported on some very interesting findings. Better fed mussels were able to maintain higher rates of oxygen consumption when exposed to declining oxygen tension; consequently these animals were called regulators, and those that could not, conformers (Bayne, 1971, 1973). Coleman (1974) was able to show that the regulators achieved higher rates of oxygen consumption by an increase in heart rate. From these findings a direct link, previously only suspected (Hill and Welsh, 1966), can be made between the metabolic rate of bivalves and their heart beat frequency. Such a phenomenon is not limited to bivalves, but has been found in vertebrates, including the bottle-nosed dolphin, *Tursiops truncatus*, and to a lesser extent in the smallest mammal, the etruscan

shrew *Suncus estruscus*. In these mammals, a direct correlation between not only heart rate and metabolic rate, but also oxygen consumption has been established (Williams *et al.*, 1993; Jurgens *et al.*, 1998). Oxygen consumption is used as an indicator of metabolic activity, and researchers have for many years been refining methods of quantifying this relationship (Nomura, 1950). Although heart rate is indicative of a change in metabolic rate (Coleman, 1974), the relationship is by no means a simple one since heart rate can also be affected by a range of other stimuli perceived by the animal (MacKay and Gelperin, 1972; Earll, 1975).

Sessile bivalves need to be able to deal with periods of low oxygen availability by modification of their respiratory rate. This is achieved not only by a reduction in activity, to reduce metabolic rate, but also by switching from aerobic to anaerobic respiration when oxygen becomes severely limited. Such a response is not limited to bivalves, but is common among facultative anaerobes (Theede *et al.*, 1969; Vetter *et al.*, 1987; Bagarinao and Vetter, 1989). Generally, an initial increase in respiratory stress is characterised by an increase in gill ventilation (Baldwin and Lee, 1979), however heart beat rate remains stable (Wheatley and Taylor, 1981; Morris and Taylor, 1985). Such a strategy is only effective up to a point beyond which the energy demand needed to maintain aerobic respiration is no longer sustainable. Invertebrates are subsequently forced to switch to anaerobic respiration. Anaerobic respiration does not produce as much energy as aerobic respiration, hence to minimise the energy deficit, the metabolism of the affected animal is reduced to a minimum and this is manifested as a decrease in heart rate, gill ventilation rates and in general activity (Helm and Trueman, 1967; De Fur and Magnum, 1979). However, it is not possible to demonstrate that anaerobic metabolism is occurring unless the biochemical indicators are quantified. Indicators of anaerobic metabolism in molluscs are the accumulation of octopine, lactate, succinate and alanine in the affected tissues (Grieshaber *et al.*, 1992; 1994).

The decrease in heart rate during periods of anaerobiosis can lead to the almost complete cessation of the heart beat (severe bradycardia) during periods of prolonged exposure. Bradycardia occurs commonly in intertidal bivalve molluscs such as *M. edulis* (Helm and Trueman, 1967), especially during emersion; although it is noticeably absent in the high littoral species *Geukensia demissa* Dillwyn and *Polymesoda caroliniana* Bosc (Deaton, 1991). Periods of bradycardia during emersion in bivalve molluscs have been compared to the diving response in air breathing vertebrates (Feinstein *et al.*, 1977). As the molluscan heart rate can be used as an indicator of metabolic rate, researchers have recorded heart rate both *in vivo* and *in vitro* in an attempt to examine changes in metabolic rate.

Throughout this chapter, heart rate refers to the myogenic ventricle contractile or pulsatile rhythm. To record the heart rate of bivalve molluscs *in vivo* either an invasive or a non-invasive method can be used. The invasive method makes use of an impedance technique, by which a small oscillating current is passed between two fine silver electrodes inserted through small holes drilled in the shell valves adjacent to the heart. Any changes in impedance between the electrodes, resulting from the contraction of the ventricle, are converted to a voltage signal that can be recorded on a pen-recorder (Helm and Trueman, 1967; Trueman *et al.*, 1973). The use of the impedance technique means that the animal can be some distance away from the recorder, and this has enabled accurate recordings to be made in the field for some months at a time (Trueman *et al.*, 1973). By a combination of impedance techniques and electrocardiogram (ECG) recordings, changes in heart rate as well as ventricular and pericardial cavity pressure can be recorded in mussels (Brand, 1976). This author suggested that pericardial cavity pressure is directly related to the stroke volume of the ventricle. If so, this technique would provide a very accurate method of measuring changes in heart action in bivalve molluscs. The drawback of an impedance technique is that it is difficult to use on very small animals.

A commonly used non-invasive method is the photo-electric method (Kokocinski and Kadziela, 1961) which makes use of the phenomenon by which the current intensity in a photo-electric cell is proportional to the intensity of the light reaching the photo-electric cell. In practice, this means that by placing a light source on one side of the mollusc's shell, and a photo-electric cell on the other, with the heart in-between, the heart rate can be monitored. The heart rate is monitored as a function of the fluctuating light absorption caused by the heart changing shape as it beats. This method was shown to be most effective on animals from 4 – 40 mm (Kokocinski and Kadziela, 1961; Cobb *et al.*, 1997). The method has subsequently been use with success in decapod crustaceans (Depledge, 1985; Aagaard *et al.*, 1995; Depledge and Lundebye, 1996; Styrishave and Depledge, 1996; Lundebye and Depledge, 1998; Curtis *et al.*, 2000).

The molluscan heart has been studied since about the end of the 19th century (Baker, 1898). However, it was only some years later that an attempt was made to examine the molluscan heart *in vitro.* Carlson in 1905 was already wrestling with the problems of removing bivalve molluscs from their valves without damaging the pericardium, and one or both of the auricles. The auricles were subsequently removed due to their frailty, and apparent reliance on the ventricle for mechanical stimulation. The ventricle was then suspended by a silk thread attached to the hindgut to obtain a record of the rate of beating of the ventricle (Carlson, 1906). There have been few modifications to this basic method over the years, but the only real development has been to pierce the wall of the ventricle with a cannula which can be used to perfuse the heart (Civil and Thompson, 1972; Wilkens, 1972; Sommerville, 1973). This method of introducing solutions directly into the ventricle is especially helpful when looking at the effect of substances that are normally excluded from the ventricle by the cuticle which surrounds it (McKay and Gelperin, 1972).

When analysing the heart rate of molluscs, both *in vitro* and *in vivo*, it is important to bear in mind that the rate will vary with size, and that larger animals will have a slower heart beat rate than smaller animals. Pickens (1965) showed that this relationship is linear in *M. edulis* on a double log scale. As has been demonstrated for many other ectothermic animals, the ambient temperature also has a profound effect on heart rate, with the heart beat rate increasing with increasing temperature in *M. edulis* (Pickens, 1965; Jones, 1972). The relationship between temperature and heart beat rate is linear, but varies as the tolerance temperatures for each species is approached (Jones, 1972).

In isolating the heart from *M. edulis*, direct physiological control of the tissue (if any) allowing the heart rate to adapt to changing behaviour such as feeding response is effectively limited, and any change in heart rate may be directly correlated to changes in metabolic rate. It may therefore be possible to examine the direct effect of sulphide on metabolic rate by recording the heart rate of isolated hearts perfused with sulphide. Thus the aim of the present study was to examine the effect of hydrogen sulphide on isolated heart preparations from *M. edulis*.

Materials and methods

M. edulis were collected from the littoral zone in Loch Fyne, (Strachur, Scotland) (56° 10` N; 5° 05` W). The salinity was measured using a refractometer and was found to vary between 5 - 25°/$_{oo}$. Further investigation revealed that the variation was due to the close proximity of a fresh water stream. The animals were then transported alive but on ice, to the Institut für Zoophysiologie, Heinrich-Heine Universität, Düsseldorf, Germany. Here the animals were maintained in re-circulating artificial seawater aquaria (salinity = 20°/$_{oo}$; temperature = 15 ± 1°C). The *M. edulis* were divided into two groups. The first group was kept in a highly aerated sulphide-free environment,

and the second group was placed in anoxic sulphidic mud periodically seeded with organic matter to maintain a sulphide concentration of approximately 200 µM as described by Doeller *et al.* (1999a). The sulphide concentration of the holding tank was monitored using the colorimetric method (see Chapter 2). The aeration of this tank was manipulated to be as high as possible without mixing the sediment into the water column. Mussels were left for 1 month prior to experimentation to allow them to acclimatise to the sulphide concentrations in the tank. Throughout this chapter, these mussels will be referred to as sulphide acclimatised. Since there is a correlation between the heart rate of *M. edulis* and size (Pickens, 1965), the mussels were selected to be of a similar size (Mean = 62 ± 9 mm), to limit variations in heart rate due to animal size. The mussels were not fed during the experimental period but there was a flow-through system in the sulphide-free tank which could provide a supply of food.

The method used to isolate and suspend the ventricle followed that of Carlson (1906). The mussels were removed from the shell valves before the ventricle was dissected out. Auricles were removed due to their frailty and lack of strong contractile properties. A silk thread was carefully attached to the hindgut to suspend the ventricle. Following this procedure, the ventricles were perfused with seawater (salinity = 20°/$_{oo}$; temperature = 15 °C), using a modified hypodermic needle (Civil and Thompson, 1972; Wilkens, 1972; Sommerville, 1973). The needle also provided the base of attachment for the suspended heart. The end of the hypodermic needle was bent using a pair of long nose pliers. The point of the needle was subsequently inserted into the base of the ventricle so that the outer wall of the ventricle was pierced. Care was taken to prevent the fragile tissue of the ventricle tearing when the heart was suspended. It was also necessary to ensure that the needle did not pierce the intestine that runs through the centre of the ventricle. The heart was then perfused with seawater at a fixed rate using a gravity fed, temperature controlled

burette. A second burette with a similar flow rate, containing sulphide at the appropriate concentration was connected to the same system. Hence the flow of seawater into the ventricle could be changed to a sulphide solution and *vice versa* by adjusting a system of valves, without any physical disturbance to the ventricle Fig. 4.1.

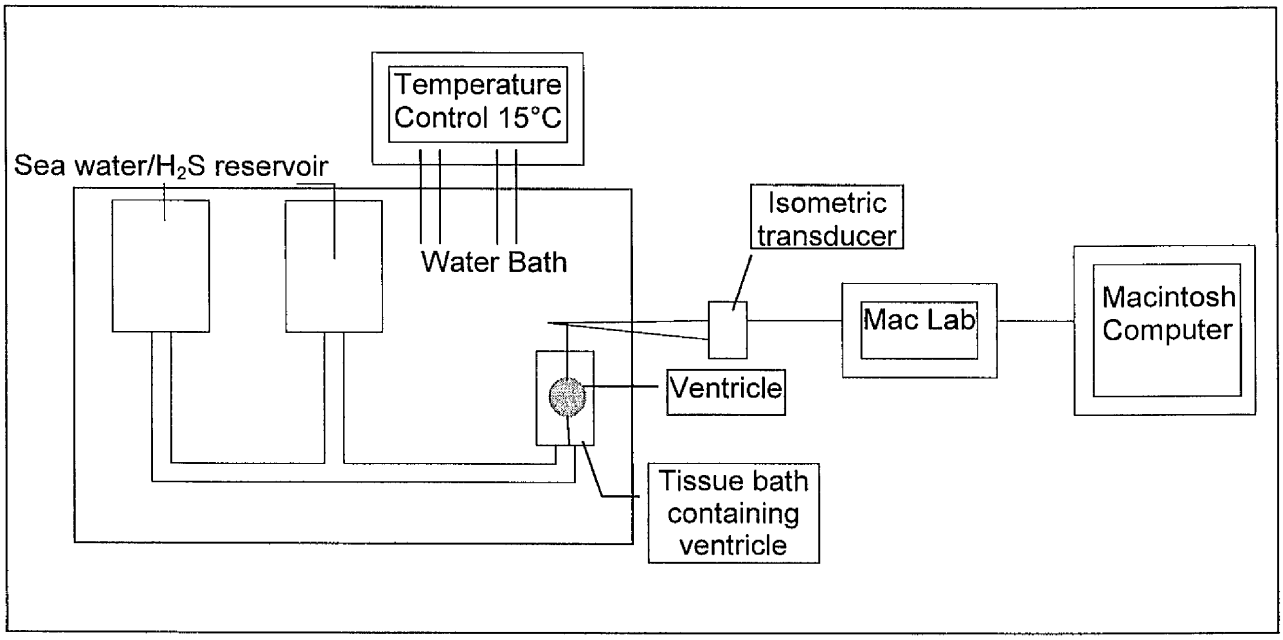

Figure 4.1: A stylised diagram of the system used to monitor and record the heart rate of isolated Mytilus edulis *hearts exposed to varying concentrations of hydrogen sulphide.*

The pulsations of the ventricle were monitored using an isometric force transducer (CB Sciences Inc. FT-102 Force Transducer), and recorded on an Apple Macintosh Computer by means of an 8 channel Maclab interface, and Maclab 3.4 software. The ventricles from *M. edulis* that had not been "acclimatised" to sulphide were exposed to 200 µM, 500 µM, 1000 µM and 1200 µM sulphide. In addition, isolated ventricles from the sulphide acclimatised animals (n = 10) were exposed to 1200 µM sulphide. Each ventricle was exposed to only one concentration of sulphide. Immediately upon the addition of sulphide, the heart rate was recorded to show the initial reaction of the ventricles. All isolated ventricles were exposed to the appropriate sulphide

concentration for 30 minutes and the heart rate recorded at 15 minute intervals for 150 minutes.

The sulphide stock solution (50mM) was prepared from $Na_2S.\ 9H_2O$ (Sigma, S-4766 Assay 100%) every day prior to use. Due to the highly volatile nature of sulphide in the presence of oxygen, the stock solution was maintained under a nitrogen atmosphere to prevent spontaneous oxidation. The concentration of the stock solution was determined using iodometric back titration (Parsons *et al.*, 1984) however colorimetric determination of sulphide modified from Parsons *et al.* (1984) was used to monitor sulphide concentration during the experiments. Both methods of determining sulphide concentration are described in detail in Chapter 2. The pH of the sulphide solution was initially adjusted to pH 8 using 6N HCl, and then maintained at pH 8 by the buffering properties of the seawater.

Statistical Analysis

The data were analysed using Minitab 10.1 statistical software. The data were normally distributed ($0.872 < \text{Normal scores} < 0.999$). There was no significant heterogeneity of variance (Bartlett's value = 11.34; $p < 0.05$). Analysis of Variance was used to determine if there was a significant difference in percentage heart rate between the different sulphide concentrations. Tukey's test (CI = 95%) was used to quantify the differences ($p < 0.05$).

Results

Initially, the heart rate of the isolated hearts was monitored for 48 h under control conditions (no sulphide present). There was a slow decrease in heart rate with time, but the decrease in heart rate was not significant over the 180 minutes of the experimental period (30 minutes sulphide exposure and 150 minutes recovery). The

heart rate (mean ± standard deviation) under control conditions was 23 ± 3 BPM (n = 100). The hearts were perfused with sulphide shortly after the heart rate had stabilised following the isolation and suspension process. It took approximately 30 minutes for the heart rate to stabilise. Fluctuations in stroke amplitude were not quantified; however visual inspection indicated that there were no detectable changes between the control heart amplitude, the heart amplitude upon the addition of sulphide, and heart amplitude during the recovery period.

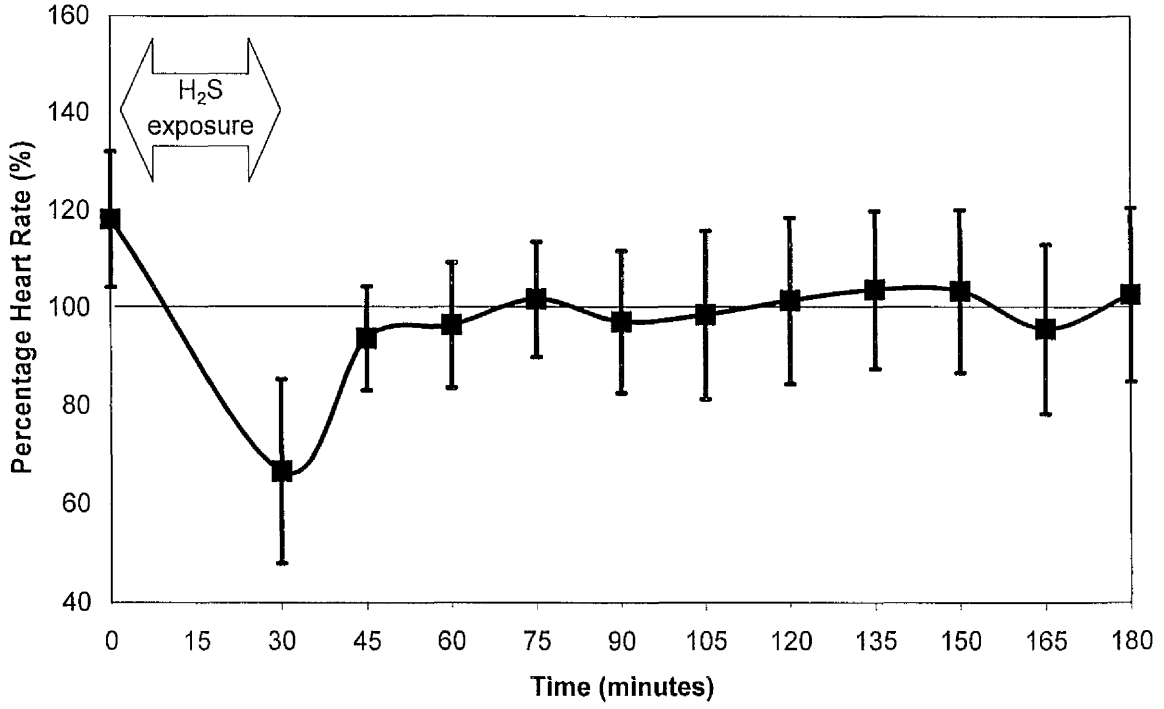

Figure 4.2: The ventricle beat rate, as a percentage change from the resting rate (100%). The isolated ventricles were obtained from Mytilus edulis *maintained in a highly aerated aquarium, and then perfused with 1200 µM hydrogen sulphide for 30 minutes. Values are means ± standard deviation (n = 10).*

The axes of the graphs in Fig. 4.2 and Fig. 4.3 have been adjusted to show the resting percentage heart rate (control rate) as 100 percent. The resting heart rate was recorded after the ventricle had been allowed to settle in the tissue bath and the rate

of contraction was stable. Fig. 4.2 illustrates the same trend that is shown by the isolated ventricles in response to exposure to 200 µM 500 µM and 1000 µM sulphide. The perfusion of the ventricles with sulphide produced an immediate increase in the rate of contraction of the isolated hearts, the degree of which differed with sulphide concentration. After 30 minutes of sulphide exposure, however, the ventricular beat rate had fallen below that of initial rate (control rate). It was observed that the recovery of the heart rate to normal levels, as recorded prior to sulphide exposure, occurred within 15 minutes of the end of the sulphide exposure period. The heart rate, in all cases, proceeded to decrease slowly with time during the recovery period in which the ventricles were perfused with aerated seawater without sulphide.

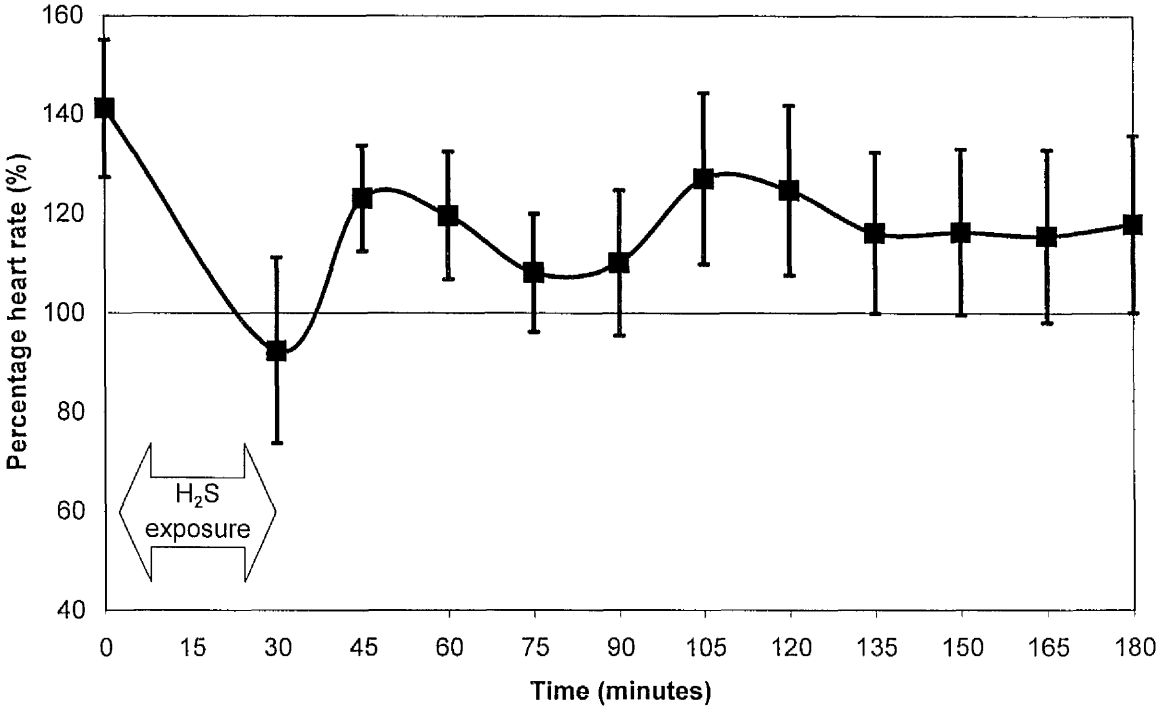

Figure 4.3: The ventricle beat rate, as a percentage variation from the resting rate (100%), of the isolated ventricles from the sulphide acclimatised Mytilus edulis, *and then perfused with hydrogen sulphide for 30 minutes at a concentration of 1200 µM. Values are means ± standard deviation (n = 10).*

The ventricles from the sulphide acclimatised mussels (Fig. 4.3) showed the same overall trends upon exposure to 1200 µM sulphide as the ventricles from the mussels that had not been previously acclimatised to sulphide (Fig. 4.2). But the initial increase in percentage heart rate upon the addition of 1200 µM sulphide to the ventricles from the sulphide acclimatised mussels (Fig. 4.3) is approximately double that of the mussels that had not been previously acclimatised to sulphide (Fig. 4.2). After 30 minutes of exposure to 1200 µM sulphide, the change in percentage heart rate from the sulphide acclimatised mussels (Fig. 4.3), was less than half that shown by the ventricles from the mussels that had not been previously acclimatised to sulphide, exposed to 1200 µM (Fig. 4.2).

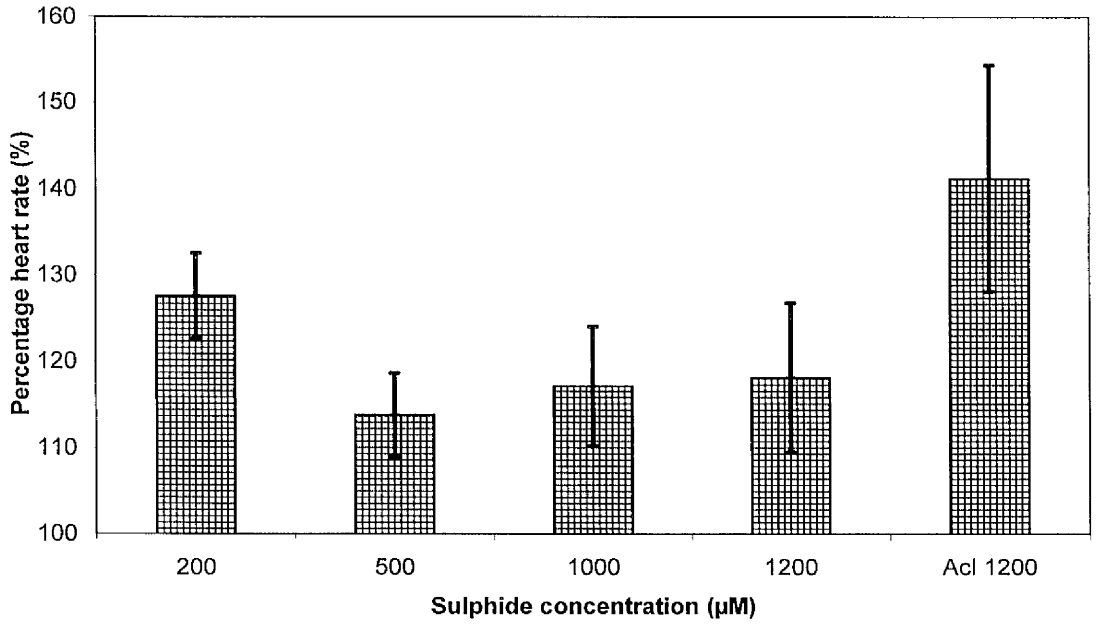

Figure 4.4: The mean ventricle beat rate, as a percentage variation from the resting rate, of the isolated ventricles immediately upon being perfused with hydrogen sulphide (pH = 8; temperature = 15°C). Acl 1200 refers to the sulphide acclimatised Mytilus edulis *exposed to 1200 µM. Values are means ± standard deviation (n = 10).*

The percentage change in heart rate is greater upon the addition of 200 µM sulphide than during exposure to 500 µM, 1000 µM and 1200 µM sulphide. The percentage heart rate upon the addition of 500 µM, 1000 µM and 1200 µM sulphide is similar in all three cases (Fig. 4.4). The sulphide acclimatised mussels exposed to 1200 µM showed approximately double the percentage change in heart rate compared with those mussels that had not been previously been acclimatised to sulphide, when exposed to 500 µM, 1000 µM and 1200 µM sulphide.

An ANOVA of the change in percentage heart rate immediately upon the addition of sulphide (Fig. 4.4) showed there are significant differences ($F_{6,31} = 4.39$; $p = 0.003$) in the effect of the different sulphide concentrations on the percentage change in heart rate. Further analyses showed that the addition of 500 µM, 1000 µM and 1200 µM elicited a significantly lower response ($p < 0.05$) than the addition of 200 µM sulphide.

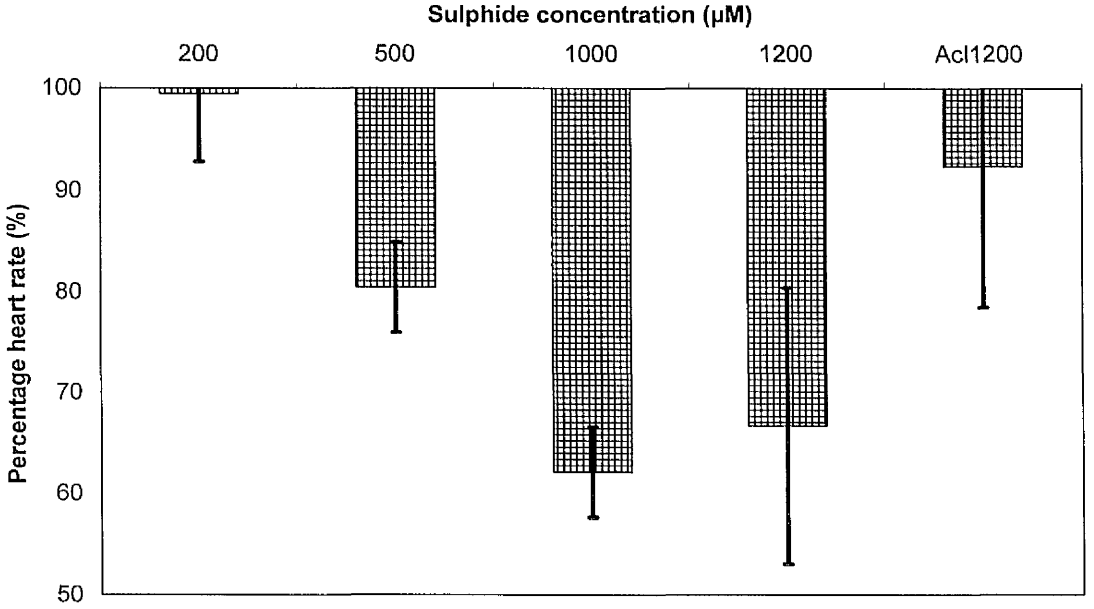

Figure 4.5: The mean ventricle beat rate, as a percentage variation from the resting rate, of the isolated ventricles after 30 minutes of hydrogen sulphide exposure (pH = 8; temperature = 15°C). Acl 1200 refers to the sulphide acclimatised Mytilus edulis *exposed to 1200 µM. Values are means ± standard deviation (n = 10).*

The addition of 1200 μM sulphide to the isolated ventricles from the sulphide acclimatised mussels initially elicited a significantly higher change ($p < 0.05$) in percentage heart rate than was observed for the ventricles from the mussels not acclimatised to sulphide and exposed to 1200 μM and 1000 μM.

After 30 minutes of exposure to 200 μM sulphide (Fig. 4.5), the percentage heart rate was similar to that of the control mussels. The percentage heart rate of the 500 μM, 1000 μM and 1200 μM sulphide exposed ventricles decreased with increasing sulphide concentration. An ANOVA of the change in percentage heart rate showed, however, that there were no significant differences ($F_{6,43} = 1.41$; $p = 0.232$) in the percentage change in heart rates after 30 minutes of sulphide exposure. But the percentage heart rates of the isolated ventricles from the sulphide acclimatised mussels remained similar to the pre-exposure (control) rate, and somewhat higher than was recorded in the ventricles from the mussels not acclimatised to sulphide and exposed to 500 μM, 1200 μM and 1000 μM displaying the same trend as Fig. 4.4.

Discussion

The mean rate of contraction of the isolated hearts was approximately 23 ± 3 BPM (n = 100) at 15 °C. These heart rates are similar to those described by Helm and Trueman (1967) who recorded 24 – 26 BPM at 17 °C. Coleman (1973) recorded mean heart rates of 20 - 22 BPM in intact *M. edulis* at 5 °C. Thus the rates of contraction of the isolated hearts recorded in this series of experiments were similar to those found in intact *M. edulis*.

In all cases, the effect of the exposure of the ventricles to sulphide is immediate. As soon as the period of sulphide exposure ceases, there is a very fast recovery of the heart rate to the rate recorded prior to sulphide exposure (control rate). Such rapid

changes in heart rate suggest that the substance affecting the ventricle is able to enter and leave the cells very rapidly. There are three ion species of sulphide, but because only the H_2S ion species is freely diffusible through membranes it is probable that it is the H_2S species that is eliciting the response. However, both the H_2S and HS^- species play a role in how much total sulphide enters an animal (Chapter 1), and H_2S diffuses approximately 2 times faster than the HS^- ion. Previous studies that have manipulated the pH of the sulphide exposure solution have shown that sulphide toxicity in the brown shrimp, *Crangon crangon* is dependent on the concentration of the H_2S ion species (Vismann, 1996a). It is possible that this is also the case in *M. edulis*. At pH 8, the experimental pH, there is approximately 16 µM, 32 µM, 80 µM and 101 µM of H_2S species present in 200 µM, 500 µM, 1000 µM and 1200 µM total sulphide, respectively.

Previous work has used the ciliary beat frequency of isolated gills from the ribbed mussel, *Geukensia demissa* as a model of aerobically active tissue to examine the metabolic reaction to sulphide (Doeller *et al.*, 1999a). But this appears to be the first time that isolated hearts have been used as a model system for the study of sulphide exposure on the metabolic rate. However, these results simply reflect changes in metabolic rate at an intracellular level in the smooth muscle tissue that makes up the isolated ventricle. It is possible that these results do not reflect the overall changes in metabolic rate in intact *M. edulis* exposed to sulphide. Increases in the heart rate of the isolated ventricles are indicative of an increase in metabolic rate, as more energy is consumed at higher heart rates. Similarly, less energy is consumed at lower heart rates, indicating a decrease in metabolic rate.

It is known that sulphide inhibits the terminal oxidase of the respiratory chain (National Research Council, 1979), hence inhibiting aerobic respiration. Generally, although an initial increase in respiratory stress is characterised by an increase in gill

ventilation in invertebrates, heart beat rate remains constant (Baldwin and Lee, 1979; Wheatley and Taylor, 1981; Morris and Taylor, 1985). But if respiratory stress continues to increase, eventually it is no longer energetically effective to maintain aerobic respiration. Anaerobic respiration replaces aerobic respiration, which is characterised by a reduction in both heart rates and gill ventilation rates, as discussed in the introduction (Helm and Trueman, 1967; De Fur and Magnum, 1979). Subsequent research demonstrated a similar trend in decapod crustaceans, where the most sulphide resistant (the hydrothermal vent crab, *Bythograea thermydron*) maintained a constant heart rate in the presence of sulphide (Vetter *et al.*, 1987). The same study showed that decapods that are less resistant to sulphide display a rapid decrease in heart rate as the maximum aerobic tolerance to sulphide of the species is reached and anaerobic respiration replaces aerobic respiration. However, because the metabolic end products of anaerobic respiration such as succinate, octopine, or alanine were not recorded in this series of experiments, it is not possible to show directly that the hearts become anaerobic at any stage (Baldwin and Lee, 1979).

It is possible to speculate that an increase in the rate of contraction of the isolated hearts, which is accompanied by an increase in metabolic rate, is also accompanied by an increase in aerobic respiration. This is due to higher energy demands, caused by higher metabolic rates, needing higher aerobic respiration rates to compensate for the energy deficit. But equally, an immediate increase in heart rate may be due to an initial energy benefit gained by the presence of sulphide by an unknown mechanism (Chapter 2), hence metabolic rate would be elevated, but the effect on aerobic respiration would be unknown.

If the reductions in heart rate are examined, again there are a number of possible explanations. It may be that a decrease in heart rate is accompanied by a reduction in aerobic respiration, due to the lower energy demand of reduced metabolic rates. It is

also possible, however, that a decrease in heart rate is due to the inhibition of muscle action by sulphide (Chapter 3). Investigations into the neuromuscular sensitivity of the echiuran worm, *Urechis caupo,* to sulphide have shown that sulphide directly inhibits muscular contraction by an unknown mechanism (Julian *et al.*, 1998).

The myogenicity of the *M. edulis* ventricle has been demonstrated to depend on changes in the ionic conductance's for Ca^{2+} (and possibly Na^{+}) and K^{+} (Devlin, 1993; Curtis *et al.*, 1999), in a manner similar to that occurring in vertebrate smooth muscle and cardiac muscle.

A number of recent findings have shown that H_2S can act as a muscle relaxant in mammalian smooth muscle (Hosoki *et al.*, 1997), possibly due to a direct effect on ion-channel function (Zhao *et al.*, 2001). The smooth muscle in invertebrates are similarly affected, as demonstrated by Julian *et al.* (1998) using the Echiuran worm, *Urechis caupo.* The possibility should therefore be considered that the effects of sulphide on the *M. edulis* ventricle contraction frequency may be in part due to a direct effect of sulphide on the ionic channels controlling myogenicity.

The initial reaction of the ventricles to sulphide was a sudden increase in heart rate. Such an increase is indicative of elevated metabolic rates. Even though the initial reaction of the ventricles to the sulphide solution was recorded, the optimum level of heart rate or metabolism that was elicited by each concentration of sulphide was approached almost immediately, an eloquent reminder of the speed with which metabolic rate can be modified to cope with environmental stress.

The increase in heart rate recorded upon the addition of 200 μM sulphide, was significantly greater than upon the addition of 500 μM, 1000 μM and 1200 μM concentrations and may be indicative of a significantly higher metabolic rate upon

exposure to 200 µM sulphide compared with the addition of 500 µM, 1000 µM and 1200 µM sulphide. Hence there is a significant decrease in metabolic rate with increasing sulphide exposure. The mussels exposed to the higher concentrations were already displaying the switch over from increasing metabolic rate to decreasing metabolic rate as indicated by the lower percentage heart rates of the 500 µM, 1000 µM and 1200 µM exposure concentrations. The optimum rate of metabolism in the presence of sulphide must therefore be reached upon exposure to concentrations of less than 500 µM. But it is impossible to tell whether it is the stimulation of metabolism by sulphide that is being recorded upon exposure to 200 µM sulphide, the optimum rate of sulphide stimulated metabolism, or, the subsequent decrease of metabolism that is characterised by the reduction in percentage heart rate shown during exposure to 500 µM, 1000 µM and 1200 µM sulphide. However, on the basis of the results from the *M. edulis* ventilation experiments, the evidence suggests that maximum metabolism was reached upon exposure to approximately 200 µM sulphide (Chapter 3).

Theoretically, 30 minutes of exposure to sulphide should result in a reduction in heart rate due to respiratory chain inhibition leading to reduced aerobic respiration and hence a lower metabolic rate. The rate of contraction of the ventricles decreased upon exposure to higher sulphide exposure concentrations as expected, but not significantly. The reason for this is surmised to be that as maximum inhibition of heart rate by sulphide is approached; increasing sulphide concentrations would be less able to inhibit heart rate, since the site of inhibition would be approaching saturation.

The ventricles from the sulphide acclimatised mussels exposed to 1200 µM sulphide showed a significantly higher initial increase in heart rate due to sulphide exposure, than the ventricles from the *M. edulis* not acclimatised to sulphide and exposed to 1000 µM and 1200 µM sulphide. This may be indicative of a higher metabolic rate in

the isolated hearts from the sulphide acclimatised *M. edulis*. The greater increase in heart rate upon exposure to sulphide, shown by the ventricles from the sulphide acclimatised mussels, is very interesting as it indicates that the metabolic rate was not being inhibited by sulphide to the same extent as the metabolic rate in the ventricles from the mussels not acclimatised to sulphide. In addition, after 30 minutes of sulphide exposure, the ventricles from the acclimatised mussels still have a much higher percentage heart rate than the ventricles from the mussels not acclimatised to sulphide and exposed to 500 µM, 1000 µM and 1200 µM sulphide, again showing a higher level of metabolism being maintained by the hearts from the acclimatised *M. edulis*.

Throughout this chapter the term sulphide acclimatised has been used to describe the *M. edulis* that were exposed to 200 µM sulphide for 1 month prior to experimentation in an attempt to induce a protective response to sulphide exposure. Such a response has been demonstrated in molluscs from sulphidic habitats (Lee *et al.*, 1996), and also in other phyla such as vertebrates (Bagarinao and Vetter, 1993), which may indicate that there is a certain amount of conformity in how sublethal concentrations of sulphide are dealt with by different species. But this is the first time that such a phenomenon has been demonstrated in a bivalve that does not usually come into contact with sulphide in its natural habitat. This appears to add weight to the supposition that many benthic invertebrates have some sulphide detoxification ability, but it is better developed in animals from sulphidic environments (Vetter *et al.*, 1987; Bagarinao and Vetter, 1989).

In this series of experiments a number of points can be surmised about the acclimatisation process. As there is only a single tissue type present in the wall of the isolated ventricle, the changes must be taking place within the smooth muscle tissue cells. Furthermore, since it is low level exposure to sulphide for an extended time

period that is responsible for the acclimatisation, the sulphide must induce the intracellular production of the mechanism that is responsible for the ability to withstand sulphide toxicity.

At a cellular level, there are only 3 possible mechanisms by which the acclimatisation can occur. The production of a sulphide oxidase, responsible for oxidising the sulphide before it reaches the respiratory chain, an alternative sulphide-insensitive terminal oxidase induced in the acclimatised animals that by-passes cytochrome c oxidase, or simply the reliance on anaerobic pathways in the presence of sulphide (Powell and Somero, 1986a). Since there was an initial increase in heart rate in the presence sulphide (at all the concentrations), which is more pronounced in the sulphide acclimatised mussels, it is unlikely that this is due to an increasing anaerobic capability.

It has been suggested, however, that a sulphide oxidase or alternative terminal oxidase, exists in the lugworm, *Arenicola marina* (Völkel and Grieshaber, 1994), which can either oxidise sulphide or transfer electrons to oxygen during sulphide oxidation. Gamenick *et al.* (1998) found that there is a marked difference in the tolerance of sibling species of the polychaete *Capitella capitata* to sulphide. Gamenick *et al.* (1998) argued that if either of these oxidases exists in the most sulphide tolerant of the *C. capitata* sibling species, then abiotic stresses such as sulphide "can be a driving force in genetic differentiation, resulting in physiological divergence." But the crucial question raised by these findings is whether the ability to become acclimatised to sulphide affords *M. edulis* any physiological benefit by a modification in the detoxification mechanism used in the presence of sulphide, or by the use of an alternative terminal oxidase. Further research specifically aimed at metabolic end products of sulphide detoxification in *M. edulis*, as well as an

examination of the anaerobic end products in the presence of sulphide is needed to clarify these points.

Chapter 5

Metabolic end products of sulphide detoxification in *Mytilus edulis* and *Arctica islandica*

Introduction

The previous two chapters have demonstrated the existence of a mechanism or mechanisms at a cellular level that allow *Mytilus edulis* partially to negate the detrimental effects of sulphide exposure on aerobic respiration. The most likely avenues for sustaining a higher level of aerobic metabolism during exposure to sulphide are the existence of either an alternative terminal oxidase or a sulphide oxidase, as discussed in Chapters 1 and 4. If it is a sulphide oxidase that is induced in *M. edulis* by prior acclimatisation to low concentrations of sulphide, then there are only a fixed number of possible detoxification products. These products, often referred to as thiols, are sulphite, thiosulphate, sulphate, glutathione and L-cysteine.

L-cysteine is an amino acid containing a sulphur residue, and glutathione is a polypeptide that consists of glycine, cysteine and glutamic acid. Both are very active in cellular protection mechanisms (Tietze, 1969; Jaeschke, 1990; Reed, 1990; Locigno and Castronovo, 2001). L-Cysteine and glutathione have not received much attention as possible detoxification products of sulphide. However, cysteine can be hydrolysed to H_2S using L-serine sulphydrase or cysteine desulphurase in mammalian cells and in some micro-organisms (Thong and Coombs, 1985a, b), and is a rate limiting precursor of glutathione synthesis (Locigno and Castronovo, 2001).

Glutathione has been shown to be a sensitive indicator of oxidative stress in rat liver during hypoxia (Jaeschke, 1990) where it is involved with detoxification of some by-products of aerobic respiration. The two forms of glutathione are GSH and GSSG, the reduced and oxidised forms, respectively. The reduced form is very active in the control of reactive oxygen species (see Chapter 8). Bagarinao and Vetter (1989) reported that glutathione has some sulphide detoxifying ability, and becomes reduced in the process. Whether this is an important factor in detoxification or just a consequence thereof is unknown, but it makes both L-cysteine and glutathione interesting candidates for further examination.

There has been a large amount of research aimed at studying mechanisms of sulphide detoxification (for reviews see Vismann, 1991b; Grieshaber *et al.*, 1994; Grieshaber and Völkel, 1998). The main product of sulphide detoxification in marine animals is thiosulphate ($S_2O_3^{2-}$) formed from the oxidation of hydrogen sulphide (Gorodezky and Childress, 1994; Johns *et al.*, 1997). In thiobacilli, the oxidation of sulphide to sulphite (SO_3^{2-}) is well known, but in invertebrates sulphite has been shown to be the intermediate in the two step oxidation of sulphide to thiosulphate (O'Brien and Vetter, 1990). The formation of thiosulphate from sulphide has the lowest oxygen cost to the animal (Vismann, 1991a) since each thiosulphate molecule represents the detoxification of 2 sulphide molecules (Jahn *et al.*, 1996), which releases four electrons per mole for ATP production (Powell and Somero, 1986a; O'Brien and Vetter, 1990). Further oxidation of thiosulphate to sulphate is known to occur in the endosymbiotic bacteria found in the bivalve, *Solemya reidi.* These endosymbiotic bacteria utilise thiosulphate for carbon fixation (Powell and Arp, 1989). Oxidation of sulphide to sulphate releases eight electrons per mole in total, which are then available for entry into the respiratory chain (Doeller *et al.*, 1999a). Oeschger and Vismann (1994) have examined the potential energy gain due to the release of electrons during sulphide oxidation in polychaetes and found that sulphide stimulated ATP production may be possible in isolated mitochondria. Later work by Parrino *et al.*

(2000) showed that sulphide stimulated ATP production occurs in gill mitochondria of the ribbed mussel, *Geukensia demissa*, where the electrons enter the respiratory chain prior to complex III.

The site of detoxification of sulphide varies from species to species. In the hydrothermal vent crab, *Bythograea thermydron,* the site of detoxification is the hepatopancreas. In the thalassinidean crustacean, *Calocaris macandreae,* detoxification is not limited to one organ, but occurs in most tissues (Johns *et al.*, 1997). As a first line of defence against sulphide exposure the lugworm, *Arenicola marina*, oxidises sulphide in the body wall tissue (Völkel and Grieshaber, 1994), and subsequently via the "brown pigment" in the blood (Patel and Spencer, 1963). Thus there is the suggestion that haem-containing molecules have the ability to bind sulphide and may play an important role in the detoxification process (Smith and Gosselin, 1964; 1966; Bagarinao and Vetter, 1989; Powell and Arp, 1989; Oeschger and Vetter, 1992; Frenkiel *et al.*, 1996; Zal *et al.*, 1997, 1998; Cerda-Colon *et al.*, 1998; Silfa *et al.*, 1998; Cheeseman *et al.*, 2002; Rosado-Ruiz *et al.*, 2002).

A visible sign of sulphide oxidation activity is the blackening of the tissues involved (Oeschger and Vetter, 1992; Völkel and Grieshaber, 1994). The explanation given for such a phenomenon is the presence of Fe^{2+} in the tissues leading to the formation of FeS or Fe_3S_4. Some marine animals are known to concentrate iron in the hepatopancreas (Vismann, 1991a). Oeschger and Vetter (1992) found that *Halicryptus spinulosus* turned black in the presence of sulphide suggesting that the mucus coating the animal contains iron and is used as a first defence. The gills of *Calocaris macandreae* also turned black during exposure to sulphide (Johns *et al.*, 1997) lending further support to this hypothesis.

As discussed above, there is a range of mechanisms that are utilised by invertebrates to detoxify or oxidise sulphide. Chapter 3 highlighted the partial

resistance of *M. edulis* to sulphide. It is therefore possible that there are similar mechanisms present in *M. edulis*. However, any mechanisms present may not be strongly expressed, since these *M. edulis* do not normally come into contact with sulphide. The ocean quahog, *Arctica islandica*, has been shown to be very resistant to sulphide (Theede *et al.*, 1969; Chapter 2), and may display the mechanisms of sulphide oxidation more clearly than *M. edulis*.

The aim of these experiments was to examine the detoxification products (thiols) of sulphide in *M. edulis* and *A. islandica*. The thiols studied include sulphite, thiosulphate, glutathione and L-cysteine. The hypothesis tested was that if the acclimatisation process is increasing the ability of sulphide acclimatised *M. edulis* to deal with sulphide, the *M. edulis* that were not acclimatised to sulphide will have different levels of detoxification products from the acclimatised *M. edulis*. By comparing the sulphide detoxification products formed in *M. edulis* with those in a bivalve found in sulphidic sediments (*A. islandica*), the potential benefit of acclimatisation to *M. edulis* may be assessed. As there are a number of different possible tissues that may be involved in sulphide detoxification, it was essential that the study was not confined to a single tissue. Therefore, in order to examine the reaction of the whole bivalve to sulphide, the mantle, gill, heart, and foot tissues of *M. edulis* were examined, and the mantle, gill, heart, foot and blood of the sulphide tolerant (Chapter 2) bivalve *A. islandica*.

Materials and Methods

M. edulis were collected from the banks at the top of Loch Fyne (Strachur, Scotland) (56° 10` N; 5° 05` W), where the salinity ranged between 5 - 25°/$_{oo}$ due to the close proximity of a fresh water stream. The animals were then transported in seawater to the University Glasgow, where the mussels were maintained in re-circulating seawater aquaria (salinity = 34°/$_{oo}$; temperature = 10°C). Large *M. edulis* (size range

50 - 107 mm) were used to ensure that the amount of tissue available for sampling was as large as possible.

A. islandica from Irvine Bay, Firth of Clyde (55° 37` N; 4° 44` W), were collected from a depth of about 6m using a hydraulic dredge. Water is supplied from a deck pump at a rate of about 700 gallons a minute and a pump pressure of about 2 bar. The water passes down a 4 inch hose to the dredge and is squirted out of a blade which penetrates the seabed as the dredge is slowly pulled forward (Tuck *et al.*, 2000). The *A. islandica* were transported to the University Glasgow, where the clams (mean height 95.4 mm ± 9.4 mm) were maintained in re-circulating seawater under highly aerated, sulphide-free conditions. The experiments were completed within a week of collection.

The *M. edulis* were divided into two groups, the first group was kept in a highly aerated sulphide-free environment, and the second group was placed in an airtight aquarium connected to a flow through system and exposed to a constant concentration (200 µM) of sulphide. The required sulphide concentration was achieved by the manipulation of the flow from 2 peristaltic pumps, the first pumping seawater from the re-circulating system described above, and the second introducing sulphide stock solution (see below) to the aquarium. The mussels were allowed to acclimatise to this environment for 1 month prior to experimentation. None of the bivalves were fed during the experimental period but there may have been some particulate matter in the seawater from the re-circulating system that could have been of nutritional value to the bivalves.

The sulphide stock solution (100 mM) was prepared from $Na_2S.\ 9H_2O$ (Sigma, S-4766 Assay 100%) every day prior to use, and maintained under a nitrogen atmosphere to prevent oxidation. The concentration of the stock solution was determined using iodometric back titration (Parsons *et al.*, 1984), and colorimetric

determination of sulphide modified from Parsons *et al.* (1984) was used to monitor sulphide concentration in the exposure tank, and during experimentation (see Chapter 2). The pH of the sulphide solution was first adjusted to pH 8 using 6N HCl, and then maintained at pH 8 by the buffering properties of the seawater.

During experimentation, *A. islandica* (n = 5) and *M. edulis* from both the sulphide-free aquarium (n = 5) and those which had been acclimatised to sulphide (n = 5), were placed in a third aquarium. A lid was fitted, and the bivalves allowed to acclimatise for a period of 12 hours. Subsequently, the bivalves were exposed to a single concentration of sulphide for 30 minutes. The lid was larger than the exposure tank with an overhanging lip so that seawater pumped into the tank could overflow, displacing any air below the lid and preventing any interaction between the air and the seawater in the exposure tank as illustrated in Fig. 5.1.

Both the *A. islandica* and the two groups of *M. edulis* were exposed to one of 5 concentrations of sulphide. The concentrations used were 200 µM, 500 µM, 800 µM, 1000 µM and 1200 µM. The bivalves were exposed to sulphide for 30 minutes after which they were quickly dissected and samples of gill, mantle, foot and heart rapidly removed and frozen in liquid nitrogen. In addition, samples of blood were taken directly from the hearts of the *A. islandica*, as well as samples of mantle cavity water. Both sets of samples were immediately frozen in liquid nitrogen. The tissue samples were freeze dried whilst still frozen (Rauch, 1991) using an Edwards Freeze Drier (Modulyo). The freeze-dried tissue was ground to a powder using a mortar and pestle.

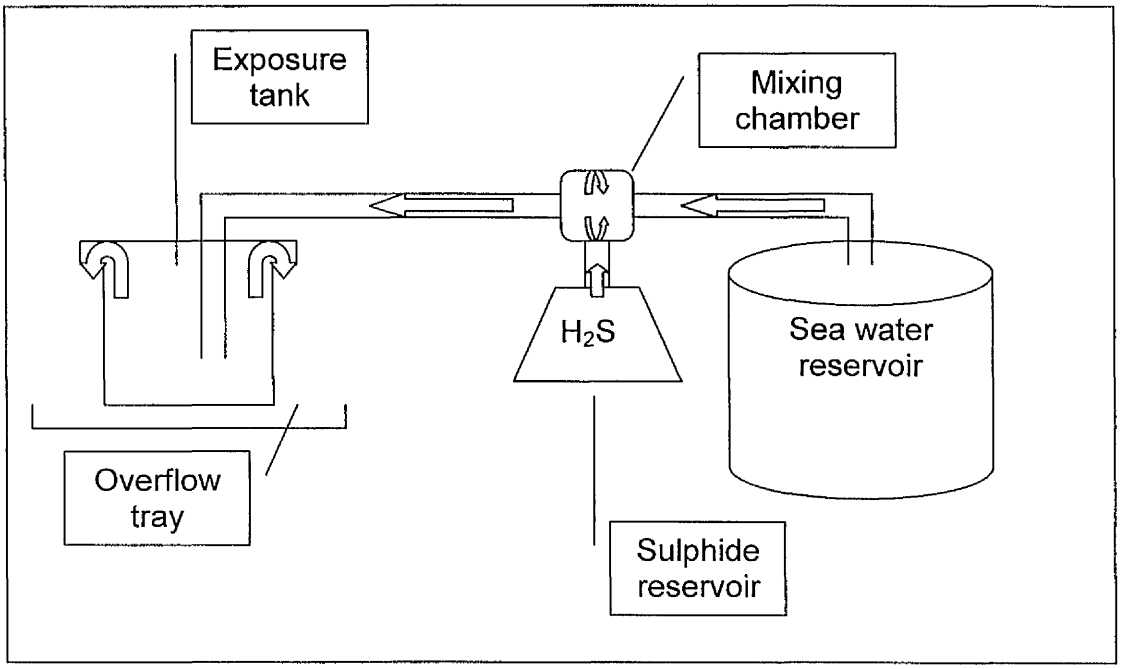

Figure 5.1: Diagrammatic representation of the closed system used to expose selected bivalve species to a constant concentration of sulphide whilst minimising sulphide oxidation caused by exposure to air.

To measure the concentrations of thiols (sulphite, thiosulphate, glutathione and L-cysteine) the frozen tissue and blood samples were derivatised using the method described by Völkel and Grieshaber (1992). The samples (either 50 µl blood sample or 10 mg powdered freeze dried tissue) were added to an Eppendorf tube (1.5 ml), containing 50 µl HEPES (160 mM) and EDTA (16 mM), and 50 µl Acetonitrile (HPLC grade). Following the addition of the blood or tissue sample, 10 µl bromobimane (46 mM) was added. The solution was mixed on a vortex mixer, and incubated at 4 °C for 30 minutes after which 100 µl methansulphonic acid (65 mM) was added. The solution was mixed again using a vortex mixer, and centrifuged at 13000 rpm for 10 minutes. The supernatant was separated from the pellet and stored at -80 °C. The samples were transported frozen to the Institut für Zoophysiologie, Heinrich-Heine Universität, Düsseldorf, Germany, where they were analysed for sulphur compounds using high pressure liquid chromatography (HPLC) as described by Völkel and Grieshaber (1992). The Eppendorf tubes containing the derivatised samples were

placed in a water bath for 3 minutes at 98 ± 1 °C to precipitate any protein, and centrifuged at 13 000 rpm for 10 minutes. The sample was separated using a Merck 50829 column and measured with a Shimadzu Florescence Spectromonitor FR530, with the excitation wavelength set at 380 nm, and the emission wavelength set at 480 nm at which sulphite, cysteine, glutathione, thiosulphate and sulphide are detectable. The column was calibrated using 5 step dilutions of authentic standards (100 µM) of each of the thiols. The standards were also used to test the initial repeatability of each run. A stepwise methanol and acetic acid (0.25% at pH 4) flow (1 $ml.min^{-1}$) was used to separate a 50 µl sample. The gradient was set up using a Merck/Hitachi L6200 pump as follows:

Table 5.1: The stepwise acetic acid and methanol gradient used to separate the samples from Mytilus edulis *and* Arctica islandica *for thiol quantification.*

Time (minutes)	**% Methanol**
0	12
2	12
7	20
20	40
21	60
22	100
30	100
30.1	12
35	12

Statistical analysis

All data were analysed using Sigma Plot 5.0 software. The data were normally distributed (normal scores > 0.917), and there was no significant heterogeneity of variance (Bartlett's value < 9.49; $p < 0.05$). Tukey's pairwise comparisons were used to show significant differences ($p < 0.05$) between the different measured parameters ($n = 5$). Since 95% confidence intervals (CI = 95%) are used as the error bars in the diagrams, error bars that do not overlap indicate significant differences between the

means. Unless otherwise stated, all statistical significances were quoted at a 95 % confidence level ($p < 0.05$).

Results

Pilot studies to test the methodology

Three separate pilot studies were carried out, the first to increase the accuracy of the HPLC method, and the others to test the reliability of the methodology.

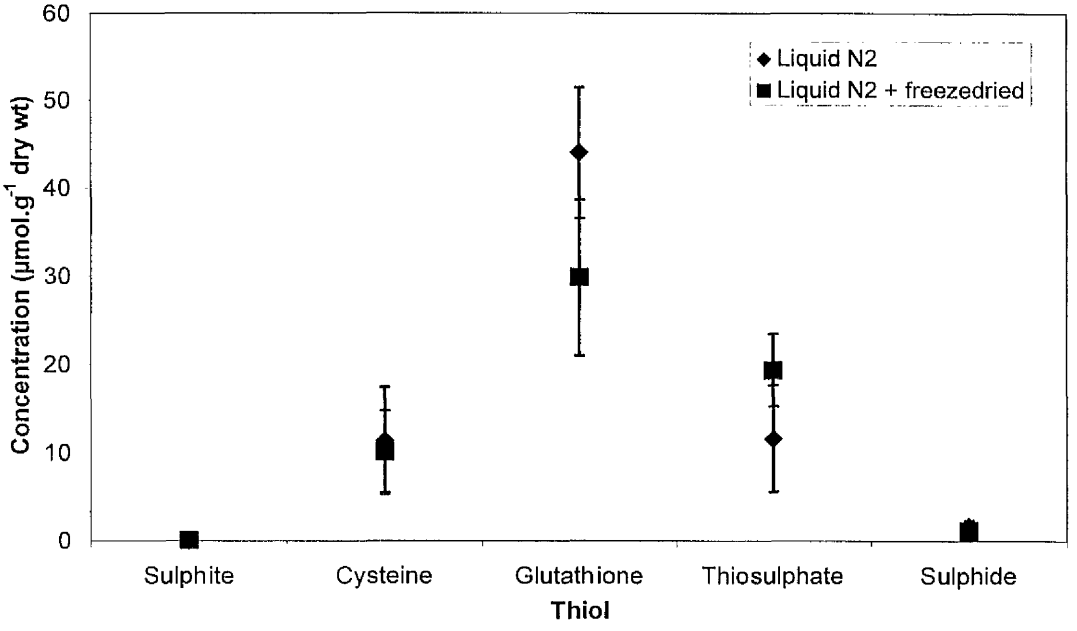

Figure 5.2: The concentration of thiols in the mantle tissue of Arctica islandica *exposed to 800 µM sulphide for 30 minutes. Data for those samples which were immediately frozen in liquid nitrogen, and those that were frozen and subsequently freeze dried are shown (n = 5). Values are means ± 95% CI.*

The derivatised samples left a large unidentified protein peak at the end of every run that made accurate determination of the sulphide peak difficult. To counteract this problem, all samples were first heated in order to precipitate any protein present. The

protein was then removed by centrifugation. There were no significant differences in the concentrations of the thiols between the samples that were heated to 98 ± 1°C and the samples that were not heated.

A further experiment was carried out to ensure that freeze drying the tissue did not affect the concentrations of thiols. Samples of mantle tissue from the same individual *A. islandica* that was exposed to 800 µM sulphide were analysed after freezing in liquid nitrogen, prior to freeze drying, and after freeze drying, as described above. The results of this experiment are shown in Fig. 5.2. There were no significant differences in the concentrations of the thiols in those samples which were immediately frozen in liquid nitrogen but not freeze dried, and those that were frozen and subsequently freeze dried.

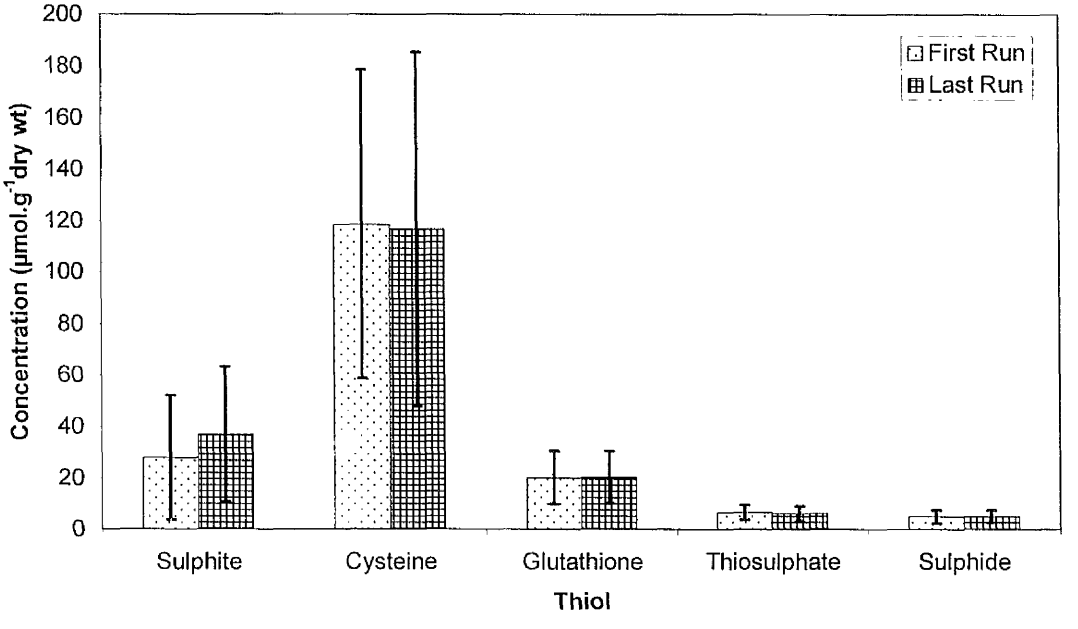

Figure 5.3: The thiols in the heart tissue of Arctica islandica *exposed to 200 µM sulphide for 30 minutes. The first run was the first of the samples separated by HPLC and the last run was the last samples separated by HPLC (n = 5). Values are means ± 95% CI.*

Due to the very large number of samples that were analysed by HPLC, the repeatability of the results was also tested. The concentrations of thiols in the tissues of *A. islandica* exposed to 200 µM sulphide for 30 minutes were separated and quantified (Fig. 5.3). The samples were analysed by HPLC as the first run of these experiments, and subsequently upon the completion of all the runs, approximately 2 years later. There were no significant differences in the concentrations of thiols in any of the tissues of *A. islandica* exposed to 200 µM sulphide for 30 minutes between the first HPLC run and the last HPLC run, hence only the results of the heart tissue are shown as an example.

The effects of sulphide on *M. edulis* and *A. islandica*

The concentrations of thiols in the foot, gill, mantle and heart of *M. edulis* acclimatised, and those not acclimatised to sulphide were quantified and compared to the concentrations of thiols in the foot, gill, mantle, heart, blood and mantle cavity water of *A. islandica*.

The concentrations of sulphite in the selected tissues from the acclimatised *M. edulis* and the *M. edulis* not acclimatised to sulphide, ranged between 5 – 20 $\mu mol.g^{-1}$ dry wt. Even though there were some significant differences between individual sulphide exposure concentrations, overall the concentrations of sulphite showed no significant differences between the acclimatised *M. edulis* and the *M. edulis* not acclimatised to sulphide. But the concentrations of sulphite in *A. islandica* exposed to similar concentrations of sulphide showed some significant increases in concentration of sulphite in different tissues (Fig. 5.4) with increasing sulphide exposure concentration. The concentrations of sulphite were also significantly lower in the tissues from *A. islandica*, than in the tissues from either the acclimatised *M. edulis* or the *M. edulis* not acclimatised to sulphide.

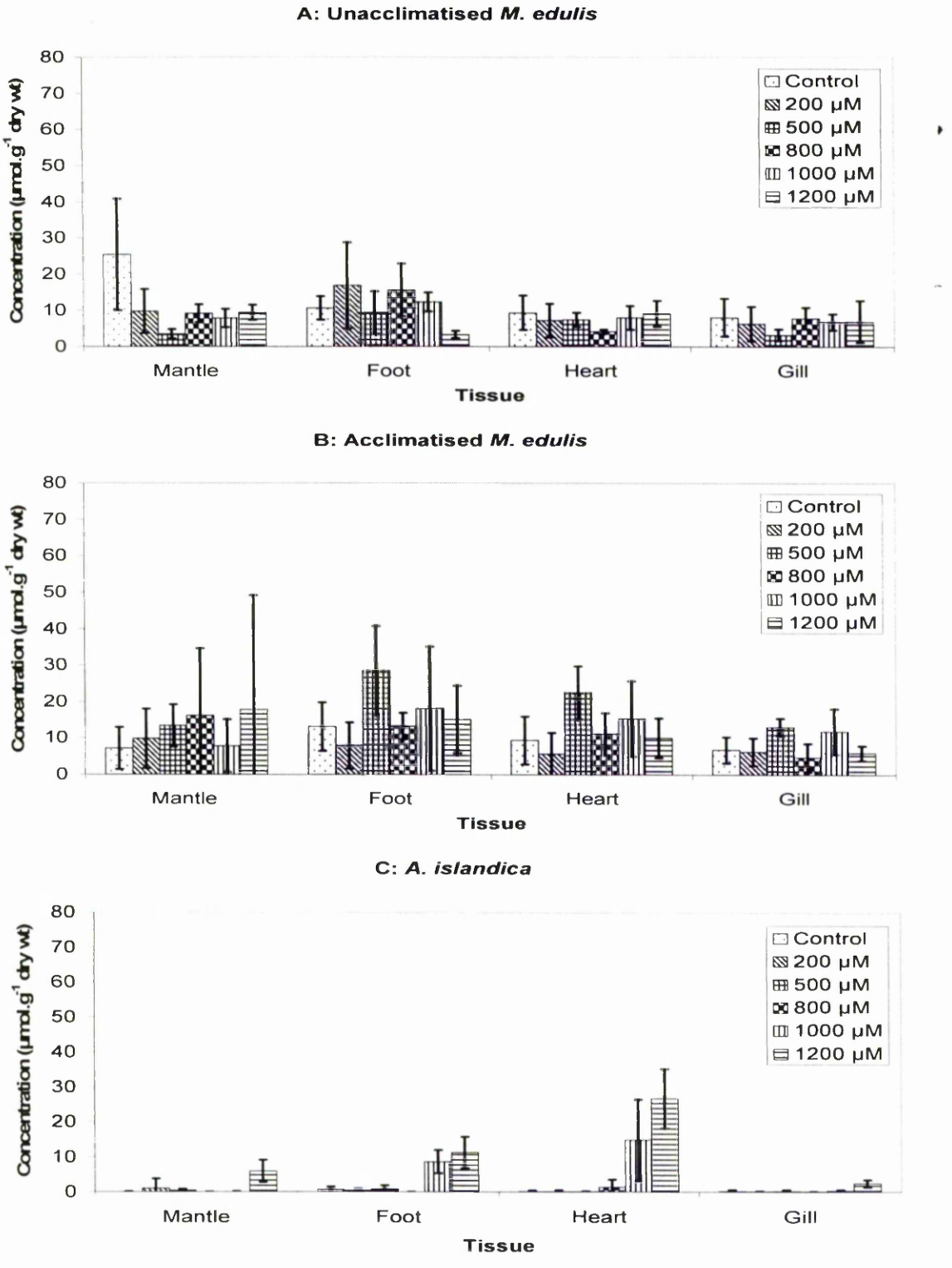

Figure 5.4: Concentrations of sulphite in selected tissues of bivalves exposed to increasing concentrations of sulphide (n = 5). Data for the tissues from the Mytilus edulis *not acclimatised to sulphide (A) and for sulphide acclimatised* Mytilus edulis *(B) are shown together with corresponding values for* Arctica islandica *(C). Values are means ± 95% CI.*

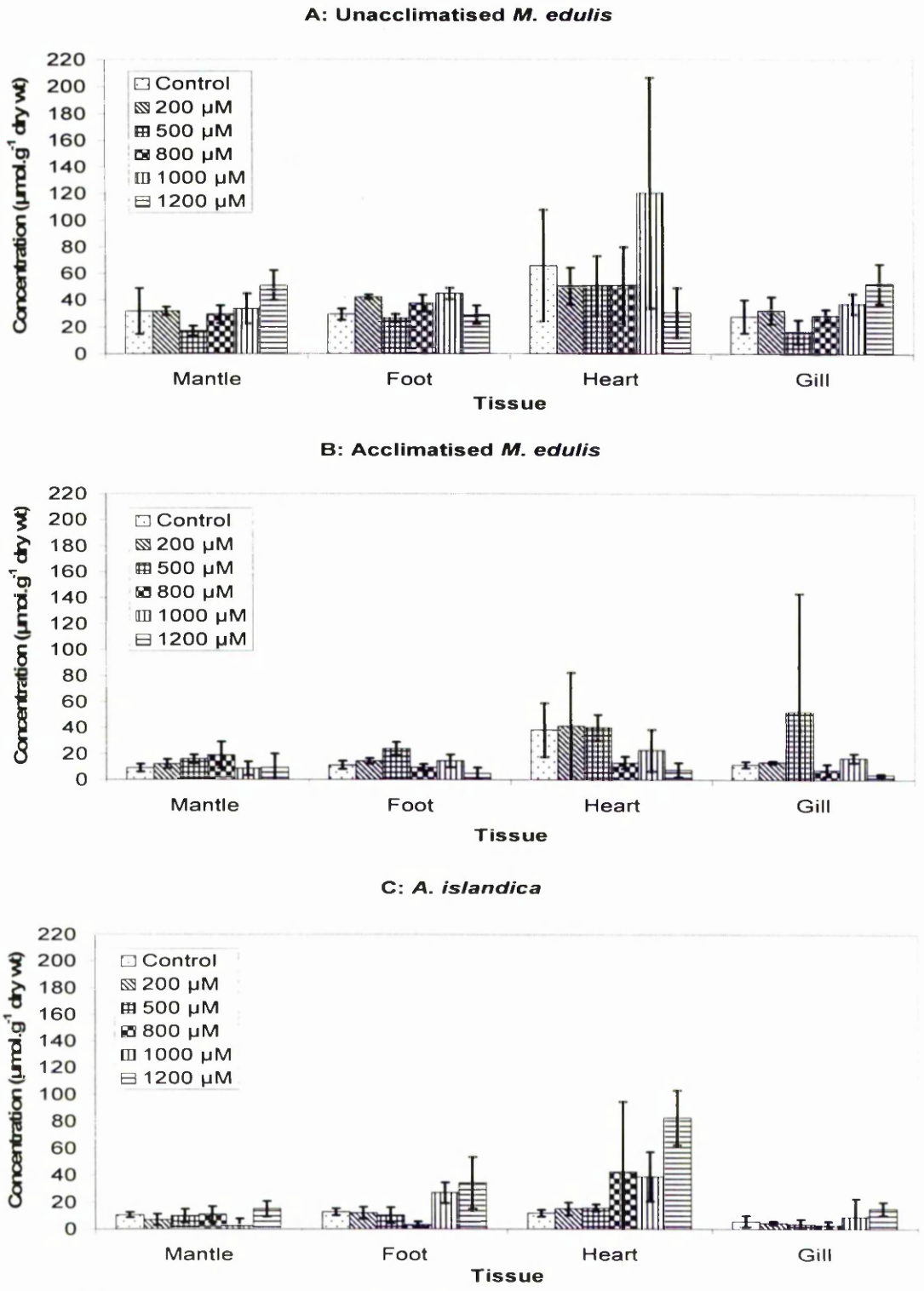

Figure 5.5: Concentrations of L-cysteine in selected tissues of bivalves exposed to increasing concentrations of sulphide (n = 5). Data for the tissues from the Mytilus edulis *not acclimatised to sulphide (A) and for sulphide acclimatised* Mytilus edulis *(B) are shown together with corresponding values for* Arctica islandica *(C). Values are means ± 95% CI.*

Concentrations of L-cysteine in each tissue examined remained relatively constant and showed no distinct trend with increasing sulphide exposure concentration (Fig. 5.5). The concentrations of L-cysteine were significantly higher in almost all the tissues of the *M. edulis* not acclimatised to sulphide, when compared to the tissues from the *A. islandica* and the sulphide acclimatised *M. edulis*. The only exception being the heart tissue samples from those *M. edulis* that were not acclimatised to sulphide. Although the concentrations of cysteine appear to be higher in the heart tissue of all the bivalves examined compared to the other tissues, these differences were not significant.

The concentrations of glutathione in the *M. edulis* not acclimatised to sulphide remained relatively constant and showed no distinct trend with increasing sulphide exposure concentration (Fig. 5.6). In general, the concentrations of glutathione were significantly higher in *M. edulis* not acclimatised to sulphide when compared to the *A. islandica* and the sulphide acclimatised *M. edulis*, even when not exposed to sulphide (controls). The concentrations of glutathione in *A. islandica* and the sulphide acclimatised *M. edulis* increased with increasing sulphide exposure concentration, unlike the *M. edulis* not acclimatised to sulphide, which maintained similar levels of glutathione as sulphide concentrations were increased.

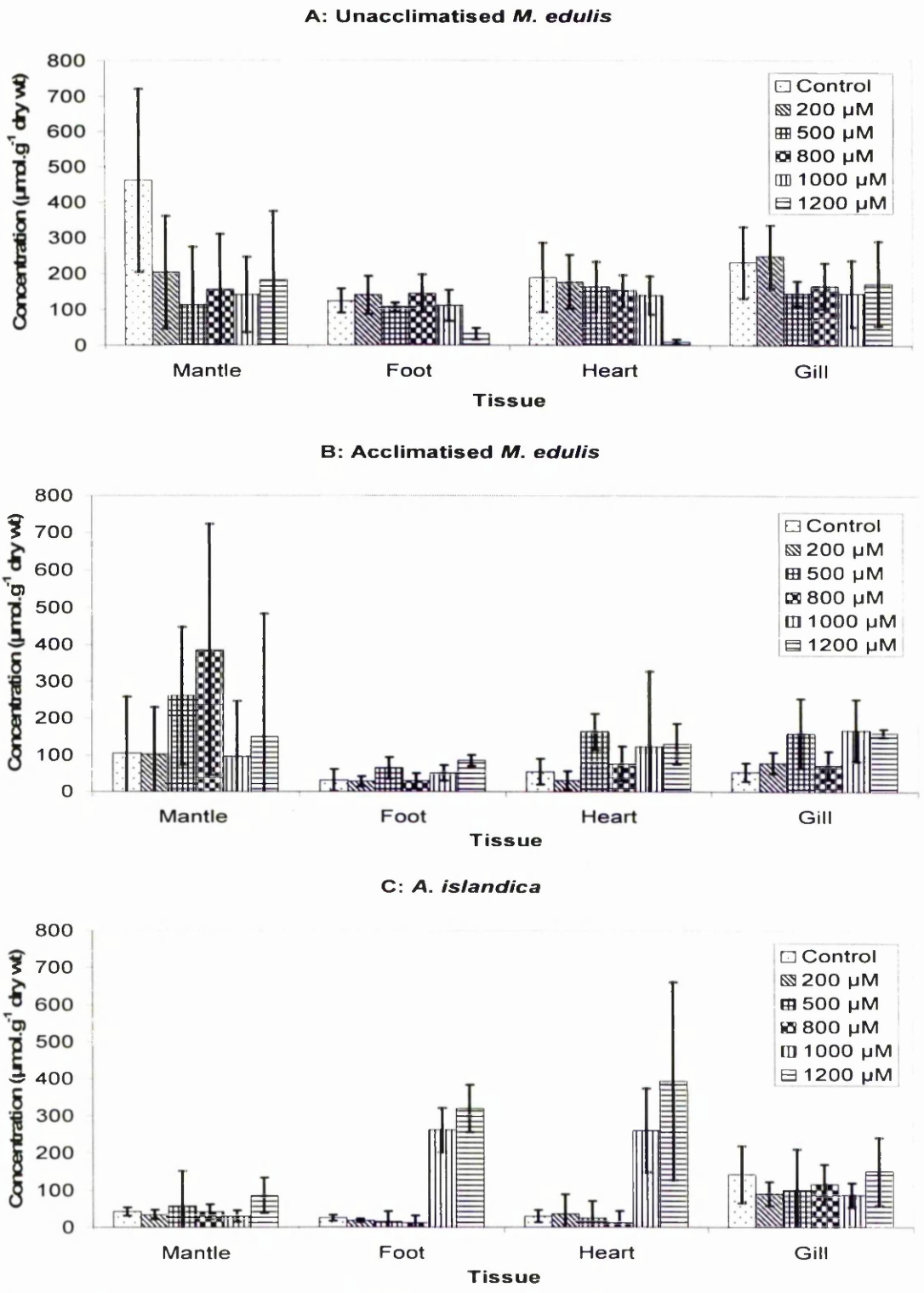

Figure 5.6: Concentrations of glutathione in selected tissues of bivalves exposed to increasing concentrations of sulphide (n = 5). Data for the tissues from the Mytilus edulis *not acclimatised to sulphide (A) and for sulphide acclimatised* Mytilus edulis *(B) are shown together with corresponding values for* Arctica islandica *(C). Values are means ± 95% CI.*

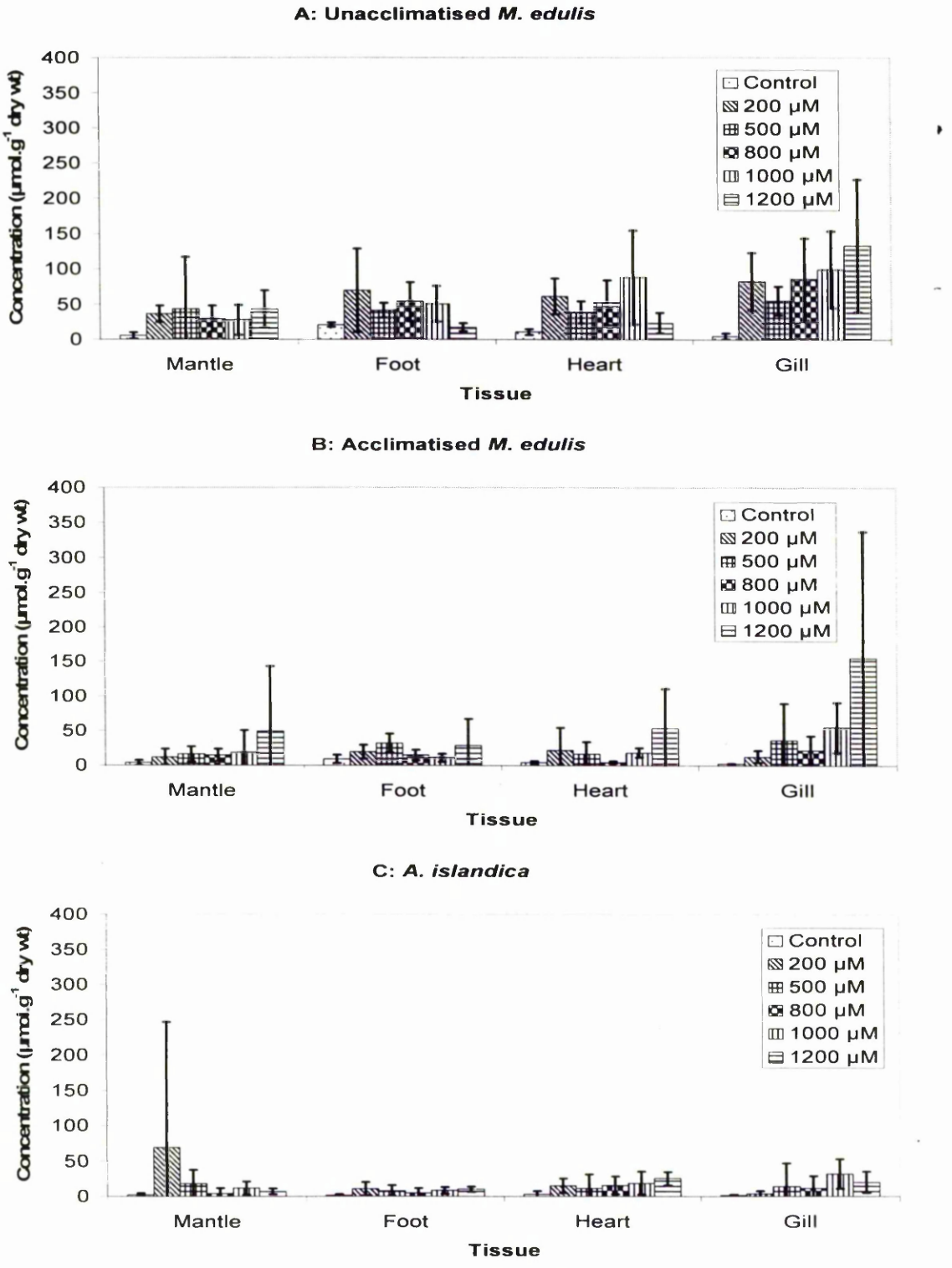

Figure 5.7: Concentrations of thiosulphate in selected tissues of bivalves exposed to increasing concentrations of sulphide (n = 5). Data for the tissues from the Mytilus edulis *not acclimatised to sulphide (A) and for sulphide acclimatised* Mytilus edulis *(B) are shown together with corresponding values for* Arctica islandica *(C) Values are means ± 95% CI.*

Although the concentrations of thiosulphate in all the tissues sampled appeared to increase with increasing sulphide exposure concentration, these changes were not significant (Fig. 5.7). The concentrations of thiosulphate were highest in the *M. edulis* not acclimatised to sulphide, with lower concentrations in the sulphide acclimatised *M. edulis*. The lowest concentrations were recorded in the *A. islandica*. The stepwise increase of thiosulphate concentration with increasing sulphide exposure concentration is best demonstrated by all the tissues from the acclimatised *M. edulis*, and the gill tissues from the *M. edulis* not acclimatised to sulphide.

The three groups consisting of *A. islandica*, the *M. edulis* not acclimatised to sulphide, and the acclimatised *M. edulis* all show the same trend upon exposure to sulphide (Fig. 5.8), with little difference between the groups. There was, however, a stepwise increase of tissue sulphide concentration with increasing sulphide exposure concentration as clearly demonstrated by the tissues from *A. islandica*, and the *M. edulis* not acclimatised to sulphide. This is less obvious in the sulphide acclimatised *M. edulis*.

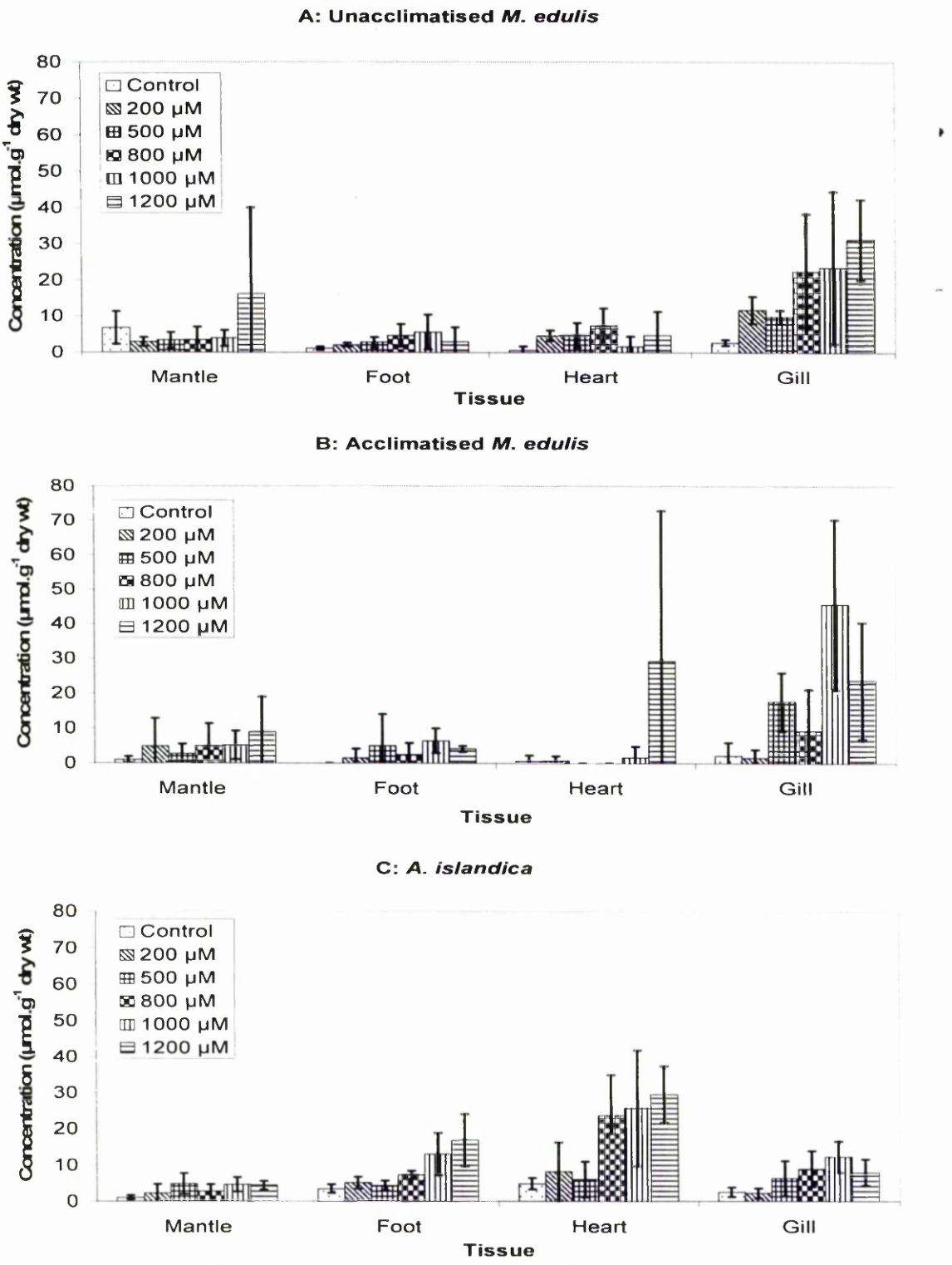

Figure 5.8: Concentrations of sulphide in selected tissues of bivalves exposed to increasing concentrations of sulphide (n = 5). Data for the tissues from the Mytilus edulis *not acclimatised to sulphide (A) and for sulphide acclimatised* Mytilus edulis *(B) are shown together with corresponding values for* Arctica islandica *(C). Values are means ± 95% CI.*

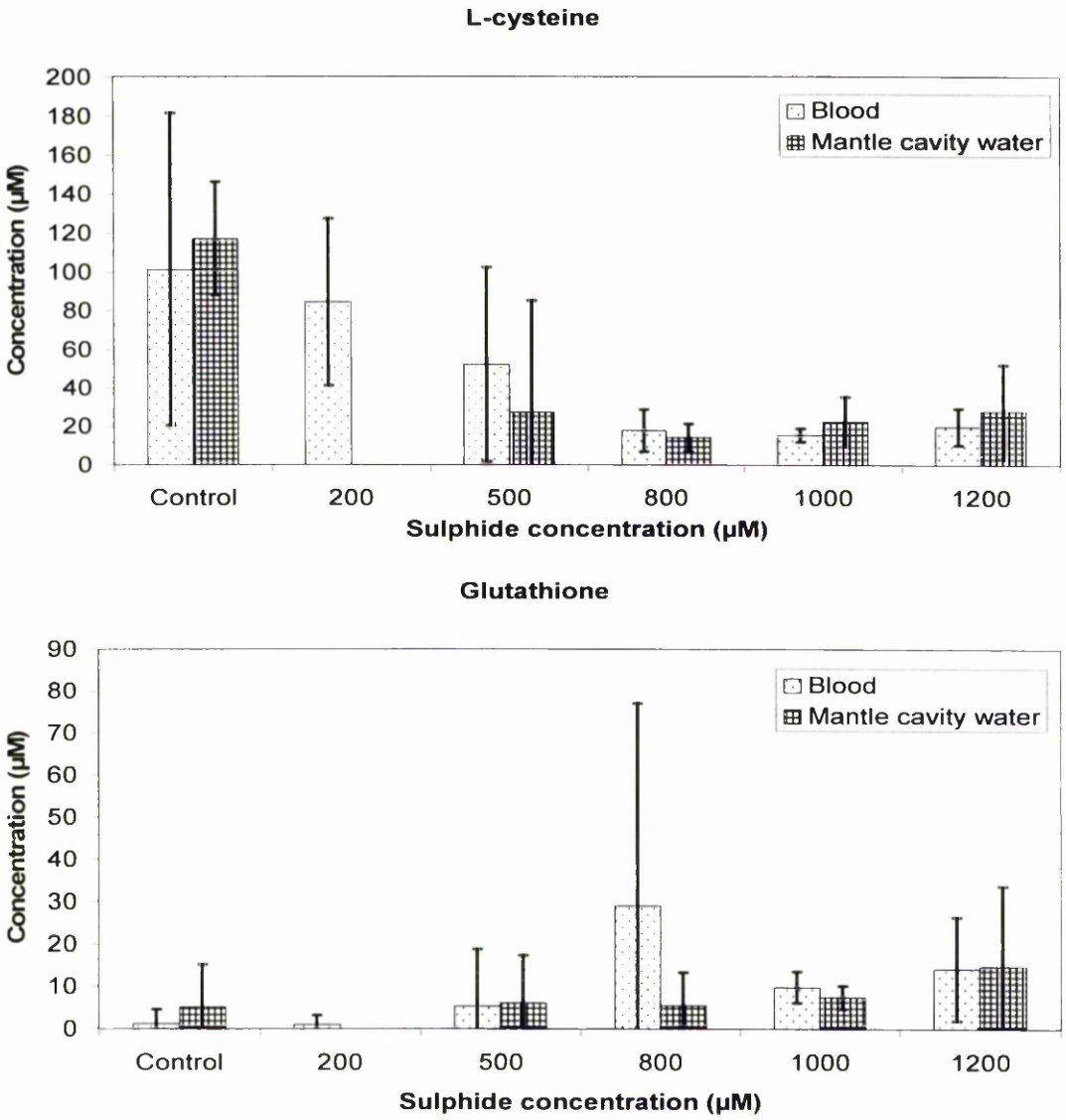

Figure 5.9: Concentrations of L-cysteine and glutathione in the blood and mantle cavity water of Arctica islandica *exposed to increasing concentrations of sulphide (n = 5). Values are means ± 95% CI.*

There was no sulphite found in either the blood or the mantle cavity water of *A. islandica*. L-cysteine concentrations in the blood and the mantle cavity water showed similar trends upon exposure to sulphide in both the blood and mantle cavity water (Fig. 5.9). However, there was a steady decrease in cysteine concentration with exposure to increasing concentrations of sulphide. The concentrations of glutathione showed the same trends upon exposure to sulphide in both the blood and mantle

cavity water, but the concentrations, although increasing slightly, did not change significantly with increasing sulphide exposure concentration (Fig. 5.9).

The concentrations of thiosulphate in the blood and mantle cavity water from the *A. islandica* varied from 60 µM – 500 µM, but showed no definite trends or differences when exposed to increasing levels of sulphide (Fig. 5.10).

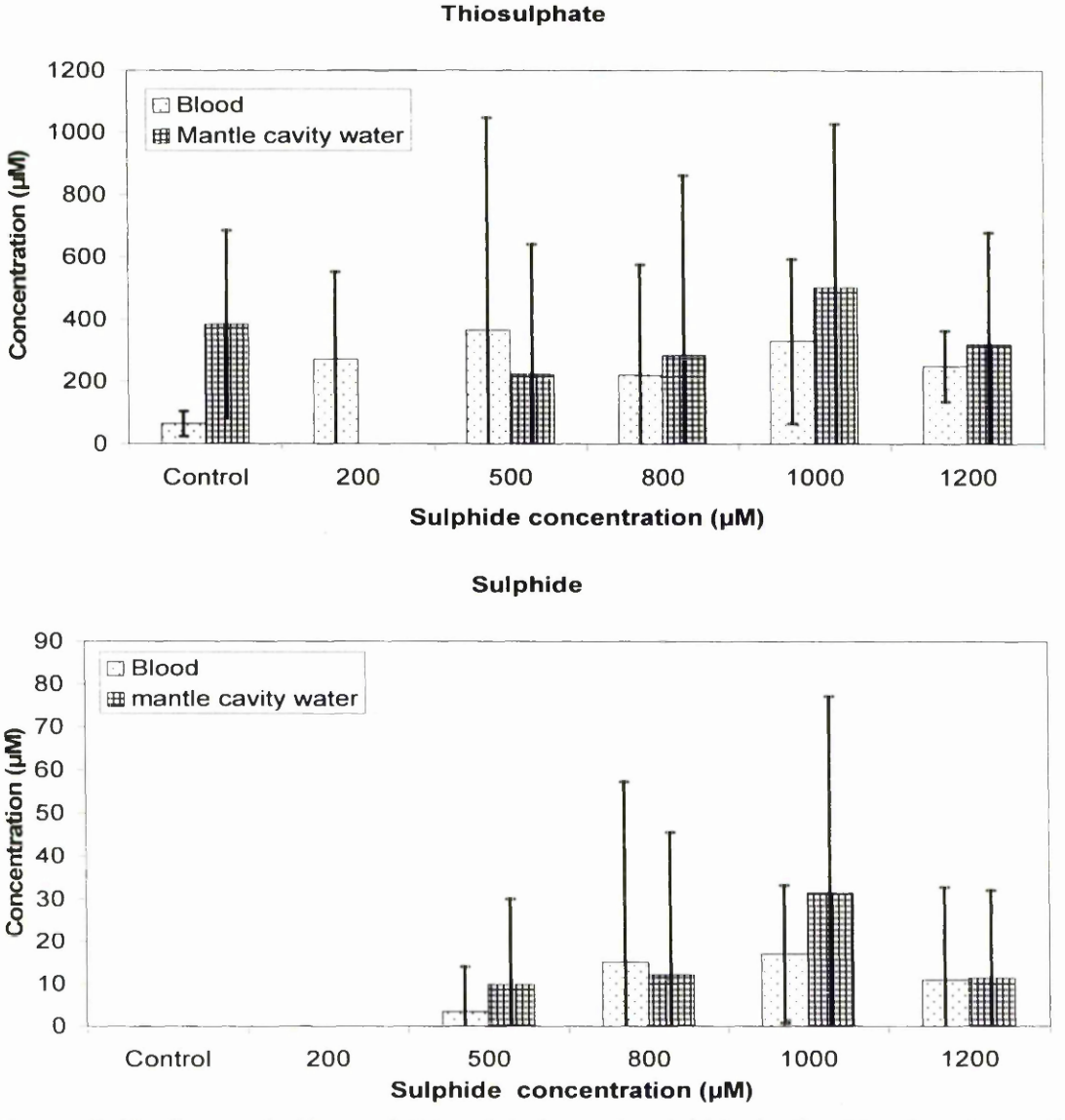

Figure 5.10: Concentrations of thiosulphate and sulphide in the blood and mantle cavity water of Arctica islandica *exposed to increasing concentrations of sulphide (n = 5). Values are means ± 95% CI.*

The concentrations of sulphide in the blood did appear to show an increase with increasing sulphide concentration; however this increase was not significant. Again, there was some correlation between the concentrations of sulphide (3 μM – 30 μM) in the mantle cavity water and in the blood (Fig. 5.10).

Discussion

Pilot studies to test the methodology

The first pilot study that was carried out prior to the main experimental run was to examine the effect of freeze drying on the concentrations of thiols in the samples. Due to the highly volatile nature of H_2S, the main concern when freeze drying the samples was the potential loss of the sulphide fraction from the samples. The pilot study demonstrated, however, that freeze drying the samples did not have a significant effect on the concentrations of thiols determined in the tissues

One of the biggest problems with the use of the bromobimane method on tissue samples from burrowing marine invertebrates is the amount of protein that is left on the column after only a small number of runs (M. K. Grieshaber *pers. comm.*). This results in the appearance of a large undefined peak at the end of the chromatogram that totally obscures the sulphide peak. Heating the derivatised sample prior to injection precipitates any protein, and this can subsequently be removed by centrifugation. The end result is a sample with no unidentified protein peaks distinguishable on the chromatogram.

Due to the large sample numbers and extensive time period over which the experiments were conducted, the repeatability of the results was tested. There were no significant differences in the concentrations of the thiols in any of these tissues from *A. islandica* exposed to 200 μM for 30 minutes between the first runs and the

last runs on the HPLC column. This demonstrated that not only are the results repeatable but that there was also no loss of thiols from the derivatised samples after storage at –80°C for up to 2 years.

The effects of sulphide on thiol production in *M. edulis* and *A. islandica*

The concentrations of sulphide in the tissues of both *M. edulis* and *A. islandica* range between 2 $\mu mol.g^{-1}$ dry wt and 40 $\mu mol.g^{-1}$ dry wt in the tissues (0 – 17 µM in the blood of *A. islandica*) of the control bivalves and those exposed to 1200 µM sulphide respectively. These concentrations are similar to those recorded in the coelomic fluid and tissue of the lugworm, *Arenicola marina* (1 – 7 µM) upon exposure to similar concentrations of sulphide for 8 hours (Völkel and Grieshaber, 1994). However the concentrations of thiosulphate in *A. marina* (2000 – 4000 µM) were approximately a factor of 40 higher than those described for *M. edulis* and *A. islandica* above (5 – 150 $\mu mol.g^{-1}$). In the priapulid worm, *H. spinulosus*, exposure to 200 µM sulphide for 24 h led to concentrations of approximately 30 µM sulphide and 1800 µM thiosulphate in the haemolymph (Oeschger and Vetter, 1992). Such a huge discrepancy in thiosulphate concentrations between *M. edulis* and *A. islandica* compared to *A. marina* and *H. spinulosus* when tissue sulphide concentrations are comparable could be due to the presence of a more effective sulphide oxidation system in the worms.

Concentrations of up to 340 µM sulphide have been found in the burrow water of *A. marina* (Völkel and Grieshaber, 1992), and laboratory experiments revealed that this polychaete worm is able to survive exposure to 10 mM sulphide for 90 days (Völkel and Grieshaber, 1994). Wilmot and Vetter (1992) exposed the gutless clam *Solemya reidi* to 100 µM sulphide for 2 hours and subsequently recorded concentrations of 1 ± 1 µM sulphide in the blood, which fits into the range of sulphide concentrations in the blood and mantle cavity water of *A. islandica* described above (0 – 17 µM). The same authors described thiosulphate concentrations of 300 ± 217 µM upon exposure to

100 µM sulphide, again similar to those recorded above for *M. edulis* and *A. islandica* (270 ± 281 µM) upon exposure to 200 µM sulphide.

As the concentrations of sulphide in the tissues and blood of *M. edulis*, *A. islandica*, *A. marina*, *H. spinulosus* and *S. reidi* are similar when exposed to comparable concentrations of sulphide (Oeschger and Vetter, 1992; Wilmot and Vetter, 1992; Völkel and Grieshaber, 1994) it seems likely that these invertebrates are not more able to exclude sulphide from their tissues than each other.

A steady increase in the concentration of sulphide in the tissues of *M. edulis* and *A. islandica*, as shown in this series of experiments and discussed above, is interesting as it indicates that these bivalves are unable to prevent sulphide diffusing into sensitive tissues. Sedentary bivalves such as *M. edulis* and *A. islandica* can exclude sulphide simply by closing the valves, a process which can be maintained for long periods if necessary as observed during periods of emersion (Baker, 1898; White, 1937; Brand, 1976; Taylor, 1976). As such it seems that *M. edulis* is unable to sense changes in sulphide concentration even when exposed to potentially lethal concentrations (Chapter 3), and cannot exclude sulphide. *M. edulis* may not have evolved a sulphide detection system as they are rarely exposed to sub-lethal concentrations of sulphide. But as only *M. edulis* were used in the ventilation experiments in Chapter 3, this evidence cannot be used to support the similar findings in *A. islandica*.

The concentrations of glutathione in the blood of *A. islandica* upon exposure to sulphide (1.2 – 28 µM) are comparable to those found by Wilmot and Vetter (1992) in *S. reidi* upon exposure to 100 µM sulphide (15 ± 5 µM). The concentrations of glutathione in the tissues of *M. edulis* and *A. islandica* were somewhat higher (9 – 461 $\mu mol.g^{-1}$ dry wt), and more comparable with those found in various species of shallow water marine fishes (170 – 530 µM) upon exposure to 0 – 2500 µM

(Bagarinao and Vetter, 1989). Unfortunately, the authors only mentioned glutathione for completeness, and so the individual species examined were not specified.

The efficiency with which sulphide is oxidised to thiosulphate is significantly higher in bivalves from sulphidic habitats (Doeller *et al.*, 1999a). Thus production of sulphite as a transient intermediate in thiosulphate production from sulphide (O'Brien and Vetter, 1990) would be reduced in bivalves from sulphidic habitats, such as *A. islandica,* due to the increased efficiency of sulphide oxidation. This was confirmed during the present study in which the concentrations of sulphite in the tissues of *A. islandica* were significantly lower than those in the tissues of the sulphide acclimatised *M. edulis* and the *M. edulis* not acclimatised to sulphide.

Production of L-cysteine is significantly higher in the mussels that had not been acclimatised to sulphide. Sulphide inhibits the terminal oxidase of the respiratory chain, which inhibits aerobic respiration (National Research Council, 1979). Hence the expected reaction of the mussels is to resort to anaerobic pathways for respiration (Grieshaber *et al.*, 1994). In an anaerobic system, L-cysteine has been shown to cleave disulphide bridges (Smith and Abbanat, 1966). During such an anaerobic episode, reducing the concentrations of L-cysteine in the tissues of the sulphide acclimatised *M. edulis* prevents protein denaturation during the acclimatisation period. Since the *A. islandica* inhabit anoxic sediment it is possible that this may explain why the concentrations of L-cysteine in *A. islandica* are lower than in *M. edulis*. Hydrogen sulphide also cleaves disulphide bridges (National Research Council, 1979; Grieshaber and Völkel, 1998). Disulphide bridges are the most important factor in the stability of protein tertiary structure, cleavage of which leads to a lack of stability in the folded protein which decreases specificity and can lead to partial denaturation (Choo and Klug, 1995). Thus increased levels of cysteine would add to the problem already caused by sulphide exposure. Lower production of cysteine in *A. islandica* and the sulphide acclimatised *M. edulis* is consistent with a

modification in metabolism that would be beneficial to bivalves exposed to sulphide. The effect of sulphide on disulphide bridges raise an interesting question: would this interaction not trigger the transcription of stress proteins, and if so, are they involved in the detoxifying process?

Cysteine production occurs in the tissues, and hence decreasing concentrations in the blood may be due to the interaction of cysteine with increasing amounts of partially denatured proteins as sulphide exposure concentration increases. The concentrations of cysteine in the blood and mantle cavity water of the *A. islandica* decreased with increasing sulphide concentrations. Since cysteine is not an excretory product, it is somewhat unexpected to find significant amounts in the mantle cavity water, especially as the concentrations recorded were almost identical to those found in the blood. The reasons for the similar concentrations of cysteine in the blood and mantle cavity water are unclear. However, as the mantle cavity water samples were taken as soon as a small section of the valve had been removed. It is unlikely to be due to bleeding into the mantle cavity.

Glutathione is a sensitive indicator of environmental hypoxia. It has been shown that decreased production of reactive oxygen species (ROS) during hypoxia leads to reduced glutathione concentrations (Jaeschke, 1990). Even in the *M. edulis* not acclimatised to sulphide, glutathione concentrations were significantly higher than in the *A. islandica* and the sulphide acclimatised *M. edulis* prior to the addition of sulphide for 30 minutes. This could suggest that either glutathione levels are being depleted during the sulphide acclimatisation period, or that the glutathione concentrations are lower in the *A. islandica* and in the acclimatised *M. edulis*, perhaps because of a modification of the glutathione synthesis mechanism. Such modifications in protein synthesis occur in the production of stress proteins specifically to allow large amounts of protein to be manufactured quickly as a cellular defence, when needed (Stryer, 1995). Differences in glutathione concentrations

during sulphide exposure have been attributed by Bagarinao and Vetter (1989), to sulphide reducing the limited supply of glutathione from GSSG to GSH (Chapter 8). Another explanation is that sulphide may inhibit glutathione peroxidase (the enzyme that catalyses the conversion of GSH to GSSG). But lower concentrations of glutathione could simply be due to only low levels of L-cysteine (as discussed above) being available for glutathione synthesis via y-glutamyl-cysteine synthetase and glutathione synthetase (Locigno and Castronovo, 2001). If the reduction of glutathione were a significant factor in the detoxification process of sulphide in *M. edulis*, those with higher concentrations of glutathione would be expected to have significantly lower concentrations of sulphide in their tissues, which is not the case.

As discussed earlier, the main product of sulphide detoxification in marine animals is thiosulphate. The increase in thiosulphate production with increasing sulphide exposure in *M. edulis* is confirmation that thiosulphate is the main detoxification product during sulphide oxidation in this species.

In conclusion, the *M. edulis* not acclimatised to sulphide have higher levels of glutathione and cysteine than the *A. islandica* and the sulphide acclimatised *M. edulis*, but whether these proteins are involved in a possible detoxification mechanism, or only with the control of reactive molecules, is as yet unclear. The *A. islandica* and the acclimatised *M. edulis* were both more efficient at thiosulphate production from sulphide than the *M. edulis* not acclimatised to sulphide. There were, however, no differences in the intracellular concentrations of sulphide between all 3 groups which suggest that the benefits of changes in glutathione and cysteine production remain vague.

Acclimatising *M. edulis* to sulphide (Chapter 4) appears not to affect the ability of *M. edulis* to oxidise sulphide, which may be one of the factors limiting *M. edulis* to sulphide-free habitats. There are three distinct possibilities. Firstly if acclimatisation

increases the ability of *M. edulis* to oxidise sulphide, the pathway that is induced is not particularly efficient in *M. edulis*, and no effect is apparent. Or secondly, it may not be the sulphide oxidation process that is affected, but the inhibition of cytochrome c oxidase that is bypassed (as discussed in Chapter 4); however this could not be examined during this experiment. The third possibility is that the *M. edulis* and *A. islandica* simply switch to anaerobic pathways in order to bypass the effect of sulphide on the respiratory chain. Further experiments specifically aimed at examining the end products of anaerobiosis in the presence of sulphide need to be pursued to elucidate the possible mechanisms used to deal with sulphide exposure in both *M. edulis* and *A. islandica*.

Chapter 6

Aerobic and anaerobic metabolism in *Mytilus edulis* and *Arctica islandica* in the presence of sulphide

Introduction

It is of vital importance, as discussed in Chapter 5, to resolve the question of whether *Mytilus edulis* and *Arctica islandica* are dependent on anaerobic pathways for ATP production in the presence of sulphide. Reliance on anaerobic pathways in the face of sulphide exposure appears to be a sound strategy, which would enable these two bivalves to avoid the most toxic effect of sulphide exposure, namely the inhibition of the terminal oxidase in the respiratory chain. Oeschger and Vetter (1992) hypothesised that animals use anaerobiosis "to passively outlast sulphide" but only as a last resort. Conflicting evidence was presented by Eaton and Arp (1993) who showed that respiration of the marine echiuran worm, *Urechis caupo* remains aerobic throughout exposure to environmental levels of sulphide. However, *U. caupo* was only exposed to relatively low concentrations of sulphide (mean = 27 μM), and therefore may not have needed to 'passively outlast' sulphide as suggested by Oeschger and Vetter (1992). Given the differences in the tolerance of individual species to sulphide (Chapter 2), both *M. edulis* and *A. islandica* would need to be examined specifically for evidence of anaerobic respiration in the presence of sulphide, before other alternatives (Chapters 5 and 7) are considered.

Early work on lamellibranchs showed that, unlike Crustacea, very little lactate was formed during anaerobiosis, but significant amounts of succinate were found

(Boyland, 1928). Unfortunately, the succinate was initially seen as a hindrance to the quantification of lactate during anaerobiosis, and authors continued to concentrate on quantifying lactate as the main anaerobic end product in lamellibranchs (Glaister and Kerly, 1936). Research over the last 30 years has led to a vastly improved understanding of anaerobic metabolism and we now know that there are a range of anaerobic metabolic end products, such as succinate, produced via a number of diverging pathways. Anaerobic metabolism has been examined in some depth in *M. edulis* but less so in *A. islandica.*

When emersed at low tide the valves of *M. edulis* close, in an attempt to prevent desiccation, which also prevents oxygen uptake and hence inhibits normal aerobic respiration (Coleman, 1973). As a result, the mussels utilise fermentative pathways for ATP production with succinate and propionate as end products (de Zwaan and Zandee, 1972; Kluytmans *et al.*, 1975; Brinkhoff *et al.*, 1983). Carbohydrates are the main energy source during anaerobiosis (de Zwaan and Wijsman, 1976), but fatty acid and amino acid conversion cannot be ruled out. In *M. edulis* the carbohydrate reserve in the mantle contributes 15 – 40% of the total body glycogen content, in the summer and winter, respectively. The differences between seasons are due to the demands of gametogenesis (de Zwaan and Zandee, 1972). The free fatty acids and mono-, di- and triglycerides form about 40 - 60 % of the lipid content, and the free amino acid content is about 20 $mg.g^{-1}$ wet wt in the adductor muscle (de Zwaan and Wijsman, 1976). Such high levels of free amino acids are common in marine bivalves because the free amino acids are involved with osmoregulation, which may be partially regulated by anaerobiosis (Zurberg and de Zwaan, 1981).

During anaerobiosis, glycogen mobilisation was restricted to the muscle tissue and the hepatopancreas in *M. edulis*, and the conversion of glucose into glycogen, lipids and protein decreased (de Zwaan and Zandee, 1972). In general, however, pool size

and turnover rate appear to dictate substrate availability for anaerobiosis, hence there is the possibility for seasonal variations in substrate availability to occur. Fatty acid mobilisation and conversion during anaerobiosis is only possible with the simultaneous mobilisation of carbohydrates via the Krebs cycle, both of which occur in the mitochondria (de Zwaan and Wijsman, 1976). Overall, carbohydrates are the main energy source during anaerobiosis but amino acids and fatty acids can contribute.

There are only a limited number of possible end-products or transient end-products of anaerobic metabolism, of which lactate, succinate, octopine, strombine, alanine, propionate, acetate and CO_2, amongst others, are commonly found in bivalves (de Zwaan and Wijsman, 1976). However, it is important to note that the formation of these organic acids and volatile fatty acids is somewhat dependent on season, size and spawning. This may explain the variation in the concentrations of these compounds recorded by different authors (Zuberg and de Zwaan, 1981; Shick *et al.*, 1983; Zange *et al.*, 1989). Succinate and acetate production show only weak seasonal fluctuations, but the conversion of succinate to propionate virtually ceases during winter due to lower average temperatures, although changes in salinity have also been implicated (Kluytmans *et al.*, 1981).

The concentrations of organic acids and volatile fatty acids are also time dependent. In *M. edulis*, lactate and alanine are initial end-products, but metabolic pathways change to produce alanine and succinate after 24 hours exposure to anoxia, and then succinate and propionate after a further 24 hours (de Zwaan and Marrewijk, 1973). Overall, the accumulation of alanine, succinate, lactate, and propionate in *M. edulis* accounts for the entire carbohydrate mobilisation during facultative anaerobiosis (Hochachka and Mustafa, 1972; de Zwaan and Wijsman, 1976). The change in the anaerobic end-products with time is due to a number of factors.

Initially, upon the onset of anaerobiosis, alanine may be produced by the transamination of aspartate with either pyruvate or α-oxoglutarate, which also produces glutamate, and as a result the free amino acid pool remains constant avoiding osmoregulatory problems (Kluytmans *et al.*, 1981; Zurberg and de Zwaan, 1981).

The production of organic acids from neutral compounds causes a reduction in pH that is buffered to some extent by calcium as a result of the breakdown of calcium carbonate from the calcareous shell. Nevertheless, the pH can still drop to 6.5 during emersion in *M. edulis* (de Zwaan and Wijsman, 1976). Since pH is the major regulating factor in the phosphoenolpyruvate (PEP) branchpoint, falling pH enhances phosphoenolpyruvate carboxykinase (PEP-CK) activity (Kluytman *et al.*, 1981) and increases oxaloacetate production. PEP-CK now competes with pyruvate kinase (PK) for PEP. At the same time, alanine production is reduced, due to decreasing pyruvate levels, because PK is inhibited by falling pH and feedback inhibition from alanine (de Zwaan and Marrewijk, 1973). The result of which is a switch from alanine production to succinate production before the aspartate pool is depleted.

In *M. edulis,* the Krebs cycle remains either fully or partially active under anaerobic conditions (de Zwaan and Marrewijk, 1973) due to fumarate or specific non-substrate electron acceptors replacing oxygen. This may also occur in other bivalves (Hochachka and Mustafa, 1972). The entry point for oxaloacetate, which is formed by glycogenolysis, into the Krebs cycle in the mitochondria, is via reduction to malate which probably enters the mitochondria utilising the malate-aspartate shuttle (de Zwaan *et al.*, 1983). Once in the mitochondria malate can be either dehydrated to fumarate, which is reduced to succinate via an anaerobic reversal of the Krebs cycle (de Zwaan and Marrewijk, 1973), or malate can be oxidised to oxaloacetate via aerobic steps in the Krebs cycle. Oxaloacetate can be oxidised to 2 keto-glutarate,

and then via glutamate dehydrogenase (present in *M. edulis*) to glutamate, which has been shown to accumulate during anaerobiosis (de Zwaan and Marrewijk, 1973). However, the activities of α-ketoglutarate dehydrogenase are high in facultative anaerobes and out-compete glutamate dehydrogenase for α-ketoglutarate, thereby channelling the majority of substrate through the Krebs cycle to succinate (Hochachka and Mustafa, 1972).

The reversal of the Krebs cycle is known to occur during anaerobiosis where the reduction of fumarate to succinate is via fumarate reductase (present in *M. edulis*) as described by Holwerda and de Zwaan (1980). Fumarate reductase and succinate dehydrogenase are both examples of Complex II, membrane bound enzyme complexes that consist of four subunits. These include a flavoprotein subunit containing a covalently bound FAD cofactor and the dicarboxylate binding site (2 – 4) and an iron–sulphur protein subunit containing three iron–sulphur clusters that are bound to the membrane anchor domain (Luna-Chavez *et al.*, 2000; Lemos *et al.*, 2001). Fumarate reductases rapidly reduce fumarate in the presence of electron donors of low redox potential and, at a somewhat slower rate, oxidize succinate in the presence of electron acceptors of high redox potential. In contrast, succinate dehydrogenase shows the opposite effect i.e. higher potential quinines (electron acceptors) demonstrating higher rates of succinate oxidation (Maklashina and Cecchini, 1999). Hence, although succinate dehydrogenase and fumarate reductase have somewhat different functions, their activities are complementary in the mitochondria. Fumarate reduction is coupled to ATP formation at the first step of the respiratory chain and results in the formation of ATP with every 2 electrons transferred to fumarate (Schöttler, 1977). Therefore, even though feedback inhibition occurs, anaerobic succinate production is more advantageous than glycolytic fermentation.

Formation of propionate from succinate is via succinyl CoA, the accumulation of which is a prerequisite for propionate formation leading to a time lag between the accumulation of succinate and the subsequent formation of propionate in *M. edulis* (Kluytmans and Zandee, 1983). Propionate can subsequently be excreted as a waste product (Saz, 1971).

Upon the cessation of hypoxic conditions, bivalves again utilise aerobic pathways. But there is some evidence that the energy production of aerobic pathways is still insufficient to re-establish levels of arginine phosphate, aspartate, glycogen and ATP. Additionally, anaerobic end products need to be removed. In order to facilitate rapid recovery, α-α'-iminodicarboxylic acids are formed via glycolytic fermentation (Eberlee *et al.*, 1983). In *M. edulis* the major product of glycolytic fermentation is strombine (Shick *et al.*, 1983).

Since it is aerobic respiration that is inhibited by H_2S (Chapter 1), animals exposed to significant levels of sulphide react by switching to anaerobic metabolism (Völkel and Grieshaber, 1992; Oeschger and Vetter, 1992). Recent reviews (Grieshaber *et al.*, 1992; Grieshaber *et al.*, 1994; Grieshaber and Völkel, 1998) reveal that animals with a high tolerance of hypoxia also seem to demonstrate a tolerance of sulphide. The aims of this experiment were to examine the concentrations of organic acids and volatile fatty acids in intact *M. edulis* and *A. islandica* during exposure to sulphide. In addition, isolated hearts were also used since it has been shown (Chapter 4) that isolated hearts of M. edulis can be acclimatised to sulphide. Finally, the oxygen consumption of isolated hearts from *M. edulis* was also examined to contrast aerobic and anaerobic metabolism upon exposure to sulphide. The hypothesis of these experiments was that both *M. edulis* and *A. islandica* remain aerobic during exposure to sub-lethal concentrations of sulphide, and therefore anaerobic end-products will

not accumulate, and oxygen consumption will increase with increasing concentrations of sulphide exposure.

Materials and Methods

M. edulis were collected from the banks at the top of Loch Fyne (Strachur, Scotland) (56° 10` N; 5° 05` W), where the salinity ranged between 5 - 25°/$_{oo}$ due to the close proximity of a fresh water stream. The animals were then transported in seawater to the University Glasgow, where the mussels were maintained in re-circulating seawater aquaria (salinity = 34°/$_{oo}$; temperature = 10°C). Large mussels (50 mm – 107 mm) were used to ensure that sufficient material was available for analysis.

A. islandica from Irvine Bay, Firth of Clyde (55° 37` N; 4° 44` W), were collected from a depth of about 6m using a hydraulic dredge as described in Chapter 5. The *A. islandica* were transported to the University Glasgow, where the clams (shell height 95.4 mm ± 9.4 mm) were maintained in re-circulating seawater aquaria (salinity = 34°/$_{oo}$; temperature = 10 °C). The *A. islandica* were kept in a highly aerated sulphide-free environment prior to experimentation. The experiments were completed within a week of the collection date.

The *M. edulis* were divided into two groups, the first group was kept in a highly aerated sulphide-free environment, and the second group was placed in an airtight aquarium connected to a flow-through system and exposed to a constant concentration of 200 µM sulphide. The required sulphide concentration was achieved by the manipulation of the flow from 2 peristaltic pumps, the first pumped seawater from the re-circulating system described above, and the second introduced sulphide stock solution to the aquarium. The *M. edulis* were allowed to acclimatise to these conditions for 1 month prior to experimentation. None of the bivalves were fed during

the experimental period but there was some particulate matter in the seawater from the re-circulating system that may have been of nutritional value to them.

The sulphide stock solution (100 mM) was prepared from $Na_2S.\ 9H_2O$ (Sigma, S-4766 Assay 100%) every day prior to use, and maintained under a nitrogen atmosphere to prevent oxidation. The concentration of the stock solution was determined using iodometric back titration (Parsons *et al.*, 1984), and colorimetric determination of sulphide modified from Parsons *et al.* (1984) was used to monitor sulphide concentration in the exposure tank, and during experimentation, as discussed in Chapter 2. The pH of the sulphide solution was first adjusted to pH 8 using 6N HCl, and then maintained at pH 8 by the buffering qualities of the seawater.

Anaerobic end products in M. edulis and A. islandica

During experimentation, *A. islandica* (n = 5), the sulphide acclimatised *M. edulis* (n = 5) and the *M. edulis* that were not acclimatised to sulphide (n = 5) were placed in a third aquarium (10 °C). A lid was fitted, and the bivalves allowed to settle for 12 hours prior to exposure to a single concentration of sulphide for 30 minutes. The lid was larger than the exposure tank with an overhanging lip so that seawater pumped into the tank could overflow, displacing any air below the lid and preventing any interaction between the air and the seawater in the exposure tank (Fig. 5.1). The *A. islandica,* the sulphide acclimatised *M. edulis* and the *M. edulis* not acclimatised to sulphide were exposed to one of 5 concentrations of sulphide. The concentrations used were 200 µM, 500 µM, 800 µM, 1000 µM and 1200 µM. The exposure period was 30 minutes after which samples of gill and adductor muscle from *M. edulis*, and mantle, foot and gill tissue from the *A. islandica* were rapidly removed and frozen in liquid nitrogen. The foot tissue from *M. edulis* that was used in these experiments was analysed for thiol content (Chapter 5). Thus, due to constraints in available

tissue, there was insufficient tissue to subsequently analyse the foot tissue for anaerobic end product content. Therefore the adductor was utilised from the same *M. edulis* as a sample of muscle tissue.

Further experiments were carried out on isolated hearts from sulphide acclimatised *M. edulis*, and *M. edulis* not acclimatised to sulphide, to examine the accumulation of anaerobic end products upon exposure to sulphide. Hearts were isolated and prepared as described by Carlson (1906). These hearts (n = 10) were then perfused with one of 5 concentrations of sulphide utilising the same methodology and apparatus described in Chapter 4. The concentrations used were 500 µM, 800 µM, 1000 µM and 1200 µM for the *M. edulis* not acclimatised to sulphide, and 1000 µM and 1200µM for the acclimatised *M. edulis* each for a 30 minute exposure period, and subsequently frozen rapidly in liquid nitrogen. Due to the small size of the isolated hearts, it was necessary to pool ten hearts to provide sufficient material for analysis.

The frozen tissue samples were placed in a mortar of liquid nitrogen and ground to a powder using a pestle. 50 mg of the powdered tissue was transferred to a 1.5 ml Eppendorf tube, and 500 µl chilled PCA (0.3 M) added to precipitate protein. After thorough mixing on a vortex mixer, the mixture was centrifuged at 13 000 rpm for 10 minutes and the supernatant decanted. The supernatant was neutralised (pH = 7 $\pm$ 0.2) using potassium bicarbonate (2 M). The pH was monitored using a Russell Type combination pH electrode which facilitated very accurate monitoring of the neutralisation process. The neutralised supernatant was kept on ice for 20 minutes and the pH measured again to ensure that there had been no change due to the slow formation of the resultant potassium perchlorate precipitate. If the supernatant was still neutral the samples were centrifuged at 13 000 rpm for 10 minutes to remove the precipitate, and the resulting supernatant stored at -20 °C.

The derivatised samples were transported frozen to the Institut für Zoophysiologie, Heinrich-Heine Universität, Düsseldorf, Germany where they were analysed for organic acids using high pressure liquid chromatography (HPLC) as described by Wormersley *et al.* (1985). Degassed sulphuric acid (0.004 N) was set up at a flow of 0.05 $ml.min^{-1}$ using a Merck/Hitachi L6200 pump through an Animex column (HPX-87H 300mm x 7.8mm) encased in a column oven (Merck/Hitachi) at 65 °C. The organic acids were measured with a UV detector (Merck/Hitachi) at a wavelength of 215 nm at which oxalate, citrate, malate, succinate, fumarate, lactate, acetate and propionate are all detectable. Prior to injection onto the column the samples were again centrifuged at 13 000 rpm for 20 minutes to limit the amount of particulate matter injected onto the column. To calculate the concentrations of the organic acids in the samples, calibration curves were constructed from multiple (n = 5) stepwise dilutions of known concentrations of each of the organic acids. The concentrations of the organic acids were calculated as a function of the area of each peak on the chromatogram using D-7000 HPLC Manager software.

One problem encountered was that lactate elutes at 16.05 minutes, and causes a double peak with fumarate (elutes at 15.54 minutes), if there are significant concentrations of both organic acids present in the same sample. However, an enzymatic digestion of suspected lactate peaks was carried out by the addition of lactate dehydrogenase. Lactate dehydrogenase digests lactate and produces pyruvate as a product. Hence, both the change in the lactate/fumarate peak could be monitored and the chromatogram examined for the possible appearance of a pyruvate peak (elutes at 8.82 minutes).

The oxygen consumption of isolated hearts from *M. edulis*

Measurements were carried out to determine the effect of sulphide on the oxygen consumption of isolated hearts from *M. edulis* not acclimatised to sulphide. Hearts were dissected out of *M. edulis* that had not been acclimatised to sulphide, using the method described by Carlson in 1906 (Chapter 4). Oxygen consumption of isolated *M. edulis* ventricles was monitored using a dual chamber Oxygraph (Anton Paar, Austria), and monitored on a PC by use of Datlab software from the same source.

The dual chamber system consists of two identical respiratory chambers with adjustable volumes, in a temperature controlled unit. The oxygen concentrations were continuously recorded by means of two identical oxygen electrodes, one in each chamber. The solution was continually mixed by means of a magnetic stirrer in each chamber. There was an injection port in each of the chambers to facilitate the addition of solutions without compromising the oxygen concentrations in the chambers. The advantages of the dual chamber system are that a blank or control can be simultaneously recorded with each experimental run and the differences between the two respirometers accurately calculated using the Datlab software.

An isolated heart was placed in one of the chambers of the dual chamber respirometer, and the oxygen consumption recorded prior to and after the addition of sulphide. Subsequently the oxygen consumption of the sulphide blank from the second chamber was subtracted from that of the sulphide and isolated heart in the first chamber, to allow differences in oxygen concentration due to spontaneous oxidation of sulphide in the medium around the isolated heart to be quantified.

At 100% oxygen saturation (10 °C), a single concentration of sulphide was injected into each of the respirometer chambers and the change in oxygen consumption recorded for 20 minutes. Both chambers of the respirometer were subsequently

flushed with sulphide-free oxygenated seawater, and the next concentration of sulphide added. Each heart (n = 12) was exposed to all the concentrations of sulphide. The concentrations used were 200 µM, 300 µM, 400 µM, 500 µM, 800 µM, 1000 µM and 1200 µM sulphide. To monitor the post exposure effect of sulphide on the oxygen consumption of the isolated hearts, the post-sulphide exposure oxygen consumption of the isolated hearts in sulphide free seawater was compared to the pre-sulphide exposure (control) oxygen consumption. The differences in oxygen consumption of each heart (n = 10) have been calculated at 80% oxygen saturation of the surrounding medium. Data are presented as weight specific oxygen consumption (ml $O_2.mg^{-1}.h^{-1}$) to account for any differences in wet weight of the hearts.

Statistical analysis

All raw data pertaining to the organic acids were analysed using Sigma Plot 5.0 software, and the oxygen consumption rates were analysed using Microsoft Excel version 7 and Minitab version 11. Statistical significance was examined using a two way ANOVA ($p = 0.05$). The data were normally distributed ($0.922 <$ Normal scores < 0.969), and there was no significant heterogeneity of variance (Bartlett's value = 17.12; $p = 0.017$). Tukey's pairwise comparisons were used to show significant differences ($p < 0.05$) between the different measured parameters. 95% confidence intervals (CI = 95%) are used as the error bars in the diagrams, hence error bars that do not overlap indicate significant differences. Since the succinate concentrations in the isolated hearts were obtained using pooled tissue samples, regression analysis was used to show differences between the sulphide acclimatised *M. edulis* and the *M. edulis* not acclimatised to sulphide. Unless otherwise stated, statistical significance is quoted at a 95% confidence level ($p < 0.05$).

Results

The chromatograms of the derivatised tissue samples from *M. edulis* and *A. islandica* showed a number of peaks which represent the organic acids that are detectable at 215 nm as demonstrated in Fig. 6.1. Not all of these organic acids are relevant in the context of this experiment, but for completeness any significant differences will be mentioned. As discussed in the methodology, lactate and fumarate can form a double peak if both organic acids are present in significant concentrations. However enzymatic digestions of these peaks revealed that there were no detectable concentrations of lactate present in the samples from either *M. edulis* or *A. islandica*. Furthermore, there were no detectable concentrations of pyruvate in the samples to which LDH had been added to digest any lactate.

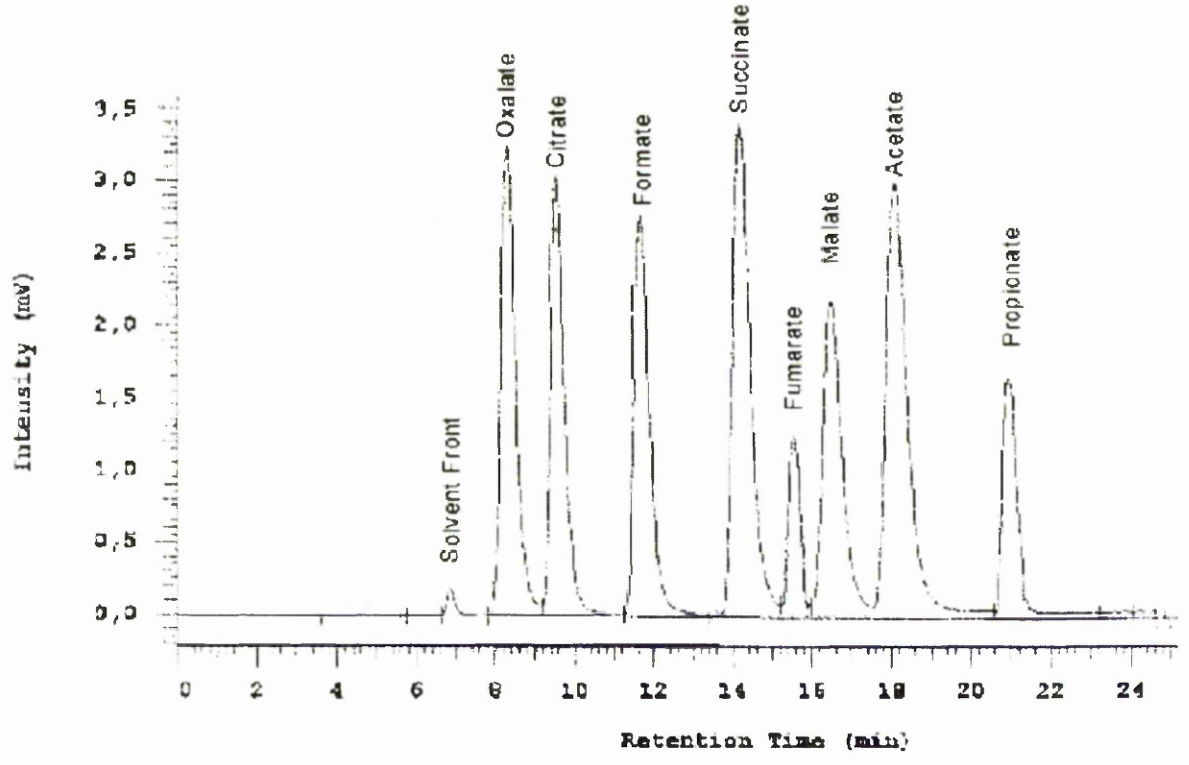

Figure 6.1: An HPLC chromatogram showing the peaks of organic acids eluted by sulphuric acid (0.004N). The peak at 6.34 minutes is the injection peak, followed by the peaks for the organic acids and volatile fatty acids.

Anaerobic end products in *M. edulis* and *A. islandica*

The concentrations of succinate in the gills, mantle and foot of *A. islandica* and in the adductor muscle and gills of *M. edulis* were quantified. Since succinate is the main indicator of anaerobic metabolic activity in *M. edulis* and in most other bivalves, the concentration of succinate in the tissues of the *A. islandica*, the *M. edulis* not acclimatised to sulphide, and the sulphide acclimatised *M. edulis* are summarised graphically in Fig. 6.2.

The concentrations of succinate in the tissues from the control bivalves showed no significant differences from the concentrations of succinate in the tissues of the bivalves exposed to increasing concentrations of sulphide. Furthermore, there were no significant differences in the concentrations of succinate between the different tissues from the *A. islandica*, the *M. edulis* not acclimatised to sulphide, and the sulphide acclimatised *M. edulis*. However, the gills of the sulphide acclimatised *M. edulis* contained significantly higher concentrations of succinate than the adductor muscle.

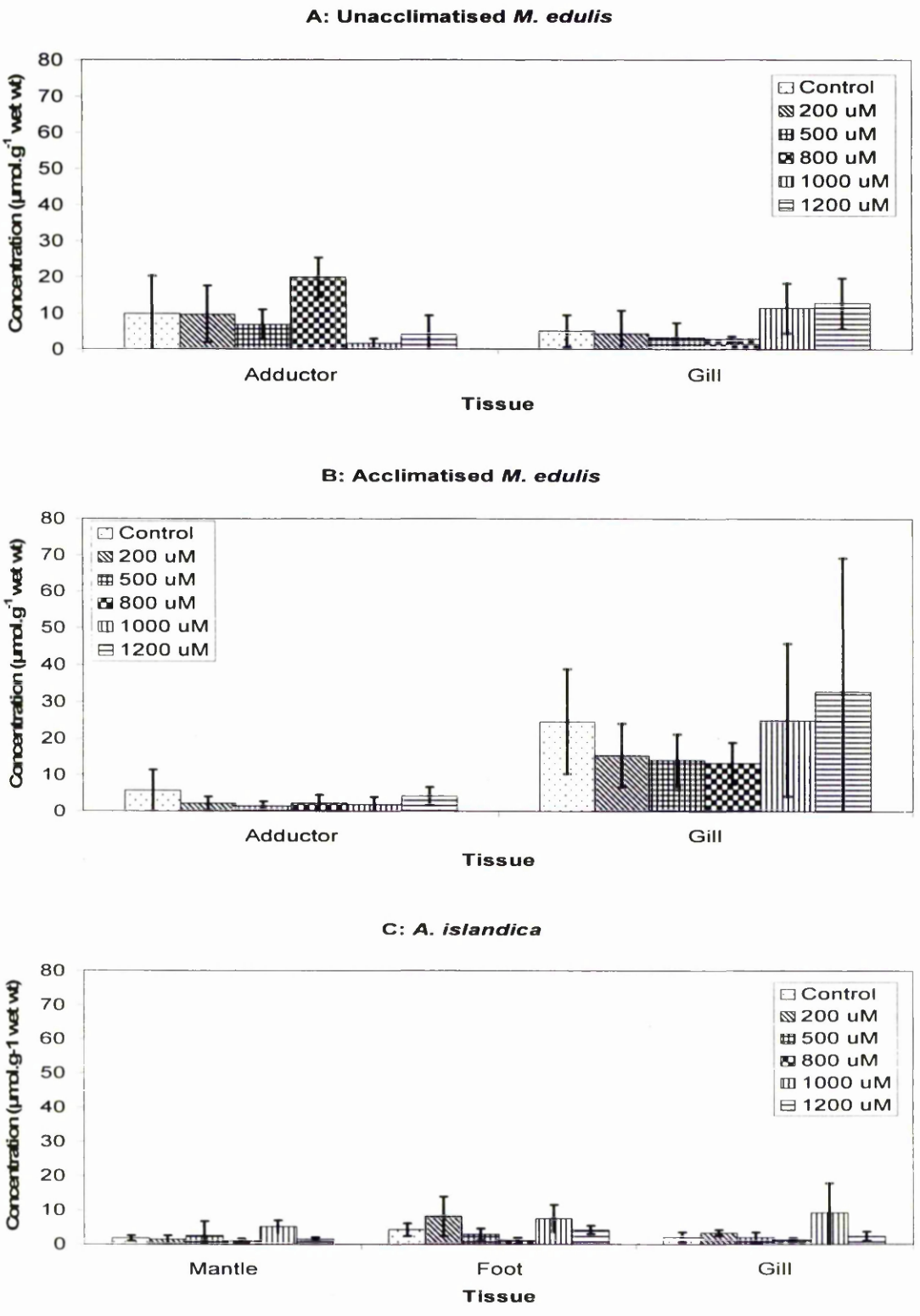

Figure 6.2: The concentrations of succinate in selected tissues from Mytilus edulis *not acclimatised to sulphide (A) sulphide acclimatised* Mytilus edulis *(B) and* Arctica islandica *(C). Values are means ± 95% CI. (n = 5).*

The concentrations of fumarate in the different tissues from the *A. islandica*, the *M. edulis* not acclimatised to sulphide, and the sulphide acclimatised *M. edulis* are presented graphically in Fig. 6.3. In each of the three groups, the muscle tissues (adductor in *M. edulis* and the foot in *A. islandica*), had significantly higher concentrations of fumarate than the gills or mantle. The best example of which is shown by *A. islandica* in which the concentrations of fumarate in the foot are higher by a factor of approximately ten than in the gills and mantle. There was no significant difference in the concentrations of fumarate between the foot muscle of *A. islandica* and the adductor muscles of the *M. edulis* not acclimatised to sulphide, and the sulphide acclimatised *M. edulis*. The concentrations of fumarate in the gills and mantle of the *A. islandica* were significantly lower than the concentrations of fumarate in the gills and adductor muscle from the *M. edulis* not acclimatised to sulphide, and from the sulphide acclimatised *M. edulis*. There is some indication that fumarate concentrations increase with increasing sulphide exposure concentration in the gills and mantle from all three groups but these differences were not significant.

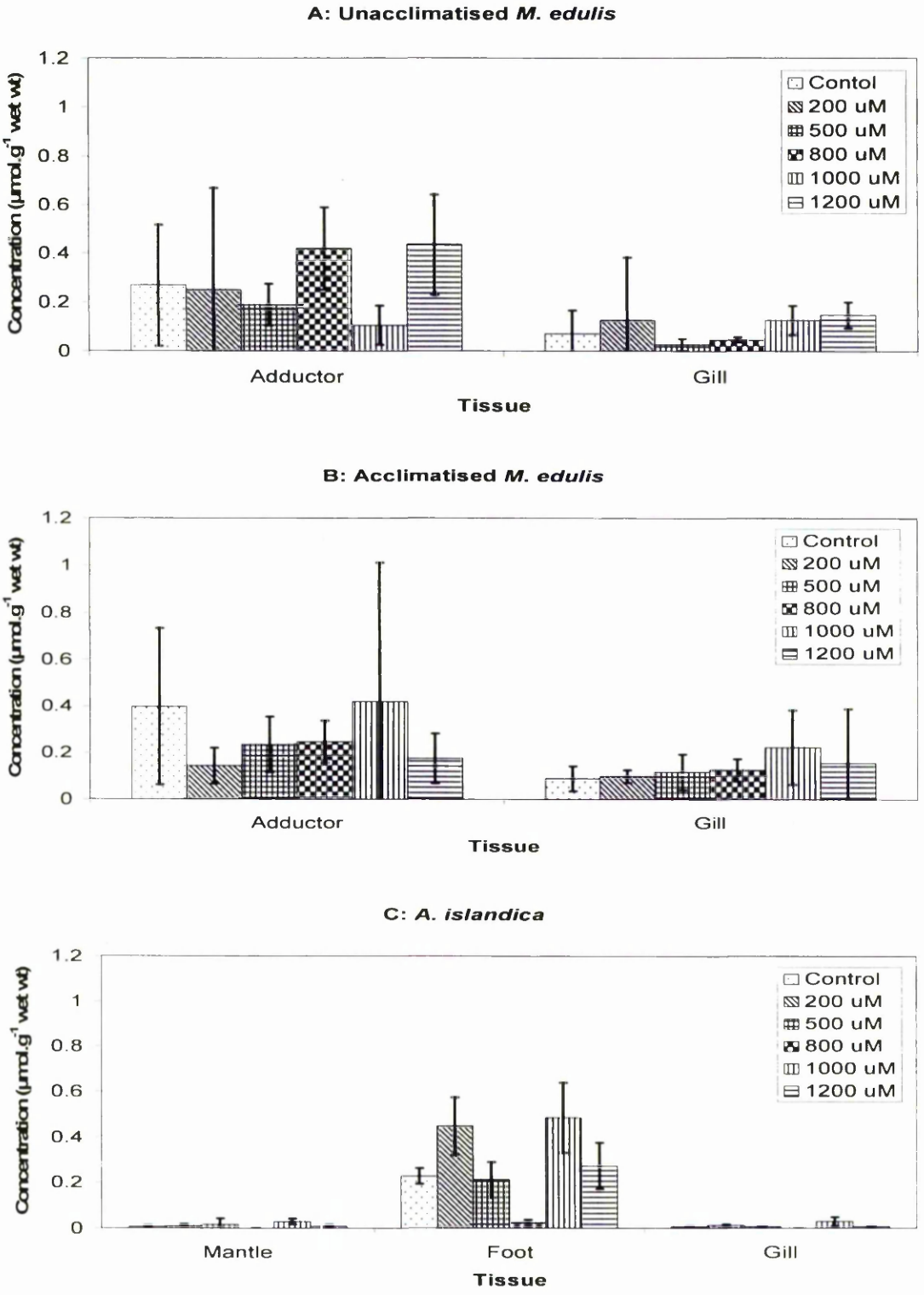

Figure 6.3: The concentrations of fumarate in selected tissues from Mytilus edulis *not acclimatised to sulphide (A) sulphide acclimatised* Mytilus edulis *(B) and* Arctica islandica *(C). The values are means ± 95% CI (n = 5).*

The concentrations of oxalate, formate, acetate and propionate in the tissues of *A. islandica* and *M. edulis* are summarised in Table 6.1. There were no significant differences in oxalate concentrations between the different tissues from the *A. islandica*, the *M. edulis* not acclimatised to sulphide, and the sulphide acclimatised *M. edulis*. Similarly, there were no significant differences in the concentrations of formate between the different tissues from the *A. islandica*, the *M. edulis* not acclimatised to sulphide, and the sulphide acclimatised *M. edulis*.

Table 6.1: The minimum and maximum concentrations of organic acids and volatile fatty acids in selected tissues from Mytilus edulis *and* Arctica islandica *exposed to sulphide for 30 minutes (n = 5).*

Organic Acids	**Concentration ($\mu mol.g^{-1}$)**
Oxalate	0.003 – 0.05
Formate	0.7 – 37
Volatile Fatty Acids	
Acetate	0.01 – 0.3
Propionate	0.006 – 0.15

Very low concentrations of the volatile fatty acid acetate were found in all the tissues, however propionate was found only in the gill, mantle and foot of *A. islandica*. There were no significant differences in the concentrations of either acetate or propionate in any of the tissues from the *A. islandica*, the *M. edulis* not acclimatised to sulphide, and the sulphide acclimatised *M. edulis*.

The concentrations of organic acids and volatile fatty acids in the blood of *A. islandica* exposed to 200 – 1200 µM sulphide were also quantified and are summarised in Table 6.2. There were, however, no significant differences in the

concentrations of either the organic acids or volatile fatty acids at any of the sulphide exposure concentrations used.

Table 6.2: The minimum and maximum concentrations of organic acids and volatile fatty acids in the blood of Arctica islandica *exposed to varying concentrations of sulphide for 30 minutes (n = 5).*

Organic Acids	**Concentration (μM)**
Oxalate	0.5 – 1.8
Formate	0.02 – 0.3
Succinate	47 – 297
Fumarate	0.5 – 2
Volatile Fatty Acids	
Acetate	0.05 – 9
Propionate	3 - 103

The concentrations of succinate in isolated hearts from *M. edulis* not acclimatised to sulphide, and the sulphide acclimatised *M. edulis* were also quantified and are presented in Fig. 6.4. The concentrations of succinate in the isolated hearts from the *M. edulis* not acclimatised to sulphide increased with increasing sulphide exposure concentration from a value of 0.17 to 4.9 $\mu mol.g^{-1}$ wet weight (pooled values).

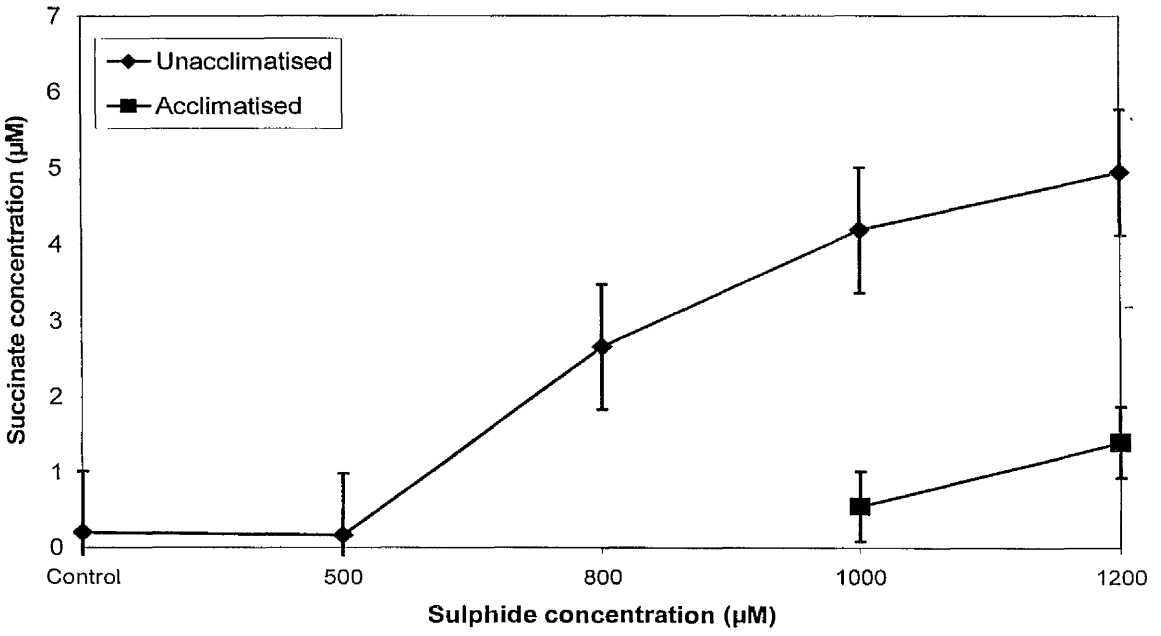

Figure 6.4: The concentrations of succinate in isolated hearts (n = 10) from sulphide acclimatised Mytilus edulis *and* Mytilus edulis *not acclimatised to sulphide, exposed to increasing concentrations of sulphide for 30 minutes. The values are means ± standard deviation (n = 5).*

In contrast, the isolated hearts from the sulphide acclimatised *M. edulis* contained significantly lower concentrations ($F_{1;1}$ = 5825.73; p = 0.008) of succinate after exposure to 1000 µM and 1200 µM sulphide than the isolated hearts from the *M. edulis* not acclimatised to sulphide, and exposed to similar concentrations of sulphide.

Oxygen consumption of isolated hearts from *M. edulis*

Isolated hearts from *M. edulis* not acclimatised to sulphide were exposed to a stepwise increase in sulphide. However, even though the chambers of the dual chamber respirometer were flushed between sulphide exposure concentrations, there was the possibility that there could be an additive effect of sulphide on the rate of oxygen consumption. To this end, the recovery of the oxygen consumption of the

isolated hearts was monitored after sulphide exposure in sulphide free oxygenated seawater. The oxygen consumption of the isolated hearts recovered immediately to similar rates to those recorded prior to sulphide exposure. Hence it is possible to assume that there is no additive effect of increasing sulphide exposure concentration on the oxygen consumption of the isolated hearts. As mentioned in the methodology the advantage of using a dual chamber respirometer is that the rate of spontaneous oxidation of sulphide could be quantified from the reduction in the percentage oxygen saturation in the chamber not containing an isolated heart. Subsequent calculation of the difference between the reduction in percentage oxygen saturation in the two chambers facilitated accurate measurements of oxygen consumption of the isolated hearts.

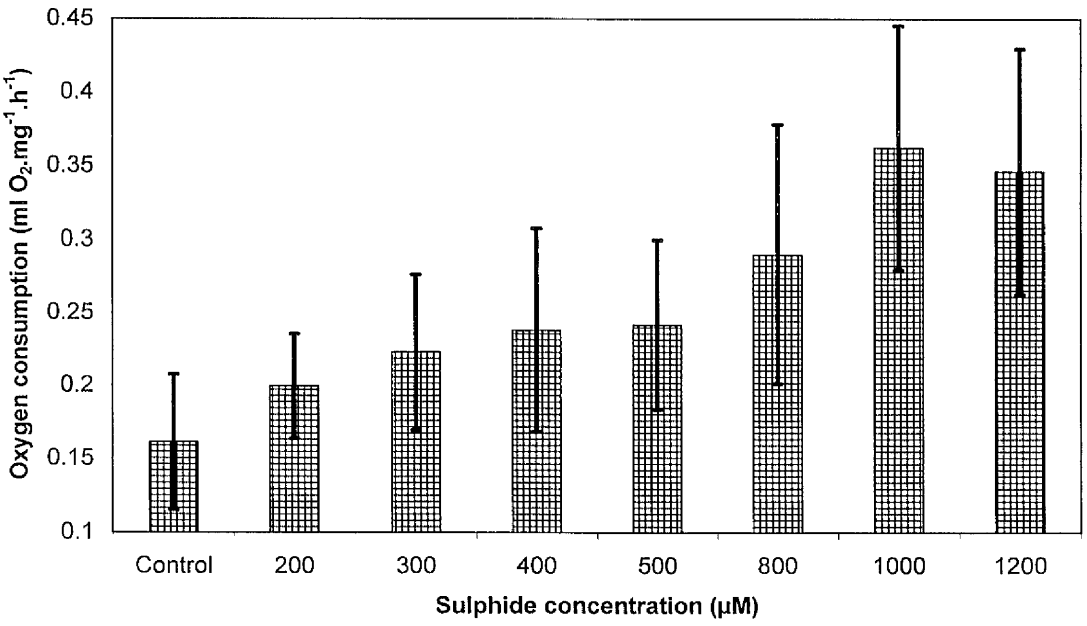

Figure 6.5: Changes in oxygen consumption of isolated hearts from Mytilus edulis *not acclimatised to sulphide and exposed to increasing concentrations of sulphide. The values are means ± 95% CI (n = 12).*

There was considerable variation between the oxygen consumption of the individual isolated hearts upon exposure to sulphide. The isolated hearts from *M. edulis* that were not acclimatised to sulphide exhibited a significant increase in oxygen consumption with increasing sulphide exposure concentration.

Discussion

Anaerobic end products in *M. edulis* and *A. islandica*

The concentrations of succinate in the *A. islandica*, the *M. edulis* not acclimatised to sulphide, and the sulphide acclimatised *M. edulis* ranged between 2 - 10 $\mu mol.g^{-1}$ wet weight in all the control tissues with the exception of the gills from the sulphide acclimatised *M. edulis*, which were consistently higher. The concentration of succinate in the isolated hearts from the *M. edulis* not acclimatised to sulphide was even lower at 0.2 $\mu mol.g^{-1}$ wet weight. The concentrations of succinate in the gills and adductor muscle of *M. edulis* are higher than those found by other authors. Kluytmans and Zandee (1983) reported succinate concentrations of 2.3 $\mu mol.g^{-1}$ dry weight in *M. edulis* maintained in aerated seawater (13 – 14 °C), and Shick *et al.* (1983) also recorded higher levels of 1.25 $\mu mol.g^{-1}$ wet weight after 3 hours of anoxia (15 °C). Other authors, however, have recorded concentrations of succinate in *M. edulis* that are closer to the concentrations found in the isolated hearts from the *M. edulis* not acclimatised to sulphide. Brinkhoff *et al.* (1983) reported concentrations of 0.31 ± 0.24 $\mu mol.g^{-1}$ wet weight under normoxic conditions (17°C), and Zange *et al.* (1989) found 0.1 $\mu mol.g^{-1}$ wet weight in isolated anterior byssus retractor muscle incubated under normoxic conditions (15 °C). Overall, there is some variation in the reported concentrations of succinate in *M. edulis* which may be due to a number of environmental parameters or differences between separate populations and methods of determination.

The concentrations of succinate in the tissues from *A. islandica* are similar to those reported by Oeschger and Storey (1993) who found succinate concentrations of 0.5 and 1.5 $\mu mol.g^{-1}$ wet weight under normoxic conditions (9.5 °C) in the adductor and foot, respectively. Although results of the whole bivalve incubations with sulphide seem to indicate that there was an increase in succinate within each tissue as sulphide exposure concentration increased, these increases were not significant. It is also evident that there are some differences in the concentrations of succinate between individual tissues within the same species. Since succinate is the main end-product of anaerobiosis in *M. edulis* and in *A. islandica* (de Zwaan and Zandee, 1972; Kluytmans *et al.*, 1975; Brinkhoff *et al.*, 1983), it appears that these bivalves remain aerobic in the presence of sub-lethal concentrations of sulphide.

The concentration of succinate in the isolated hearts from *M. edulis* not acclimatised to sulphide, increased with increasing sulphide concentration. Unfortunately, because the hearts were pooled (n = 10), it is not possible to show significant differences in the increase in succinate. The concentrations recorded are, however, within the same range as those found in both the adductor and gill tissues in the whole *M. edulis* incubations. The isolated hearts from the sulphide acclimatised *M. edulis,* however, have significantly lower concentrations of succinate than the isolated hearts from the *M. edulis* not acclimatised to sulphide and exposed to similar concentrations of sulphide. The significant differences in succinate concentration between the isolated hearts from the *M. edulis* not acclimatised to sulphide and the sulphide acclimatised *M. edulis*, support the findings of Chapter 4 where the ability of *M. edulis* to acclimatise to sulphide was examined.

Previous studies have not reported the concentrations of fumarate during anaerobiosis since fumarate is not an end product of anaerobic metabolism in bivalves. Although in the gill tissues of both *A. islandica* and *M. edulis* there was a

tendency for the concentration of fumarate to increase as sulphide exposure concentration increases, this trend is not significant.

The concentrations of fumarate were low (0.01 – 0.4 $\mu mol.g^{-1}$ wet weight) in the tissues from the control *M. edulis* and *A. islandica*. Such low concentrations are expected because during both aerobic and anaerobic metabolism the Krebs cycle intermediates would only be present in low concentrations due to the continued functioning of that cycle (de Zwaan and Marrewijk, 1973). Fumarate concentrations in the gills and mantle from *A. islandica* are significantly lower than those in the gills from *M. edulis*, which, because invertebrates with higher anaerobic capacity have higher levels of fumarate reductase activity (Grieshaber *et al.*, 1994), is not surprising. Bivalves that are better adapted to anaerobiosis have more active fumarate reductase activity, which in some cases can even surpass that of succinate dehydrogenase (Hammen and Lum, 1966).

Accumulation of the Krebs cycle intermediate fumarate in bivalves with very active fumarate reductase would be less than in bivalves with lower fumarate reductase activity, simply due to the more efficient reduction of fumarate to succinate. The most striking differences in fumarate concentration in all groups tested are that the muscle tissues (adductor in *M. edulis* and foot in *A. islandica*) have significantly higher concentrations of fumarate than the gills or mantle. The reasons for differences in fumarate concentration between muscular and non-muscular tissues remain obscure. A possible explanation is the presence of differing concentrations of fumarate reductase or differences in the ratio of fumarate reductase to succinate dehydrogenase in different tissues, relating to the functional divergence of the tissues, but detailed information on the concentrations of these enzymes in different bivalve tissues is lacking.

Early work using enzyme assays showed that succinate dehydrogenase is inhibited by sulphide under normoxic conditions (Bergstermann and Lummer, 1947). H_2S is a strong reducing agent with E_o = -0.114 – 0.402 V (Kelly, 1982). If succinate dehydrogenase is exposed to H_2S it has the potential to change the normal direction of succinate dehydrogenase in the Krebs cycle and to reduce fumarate to succinate due to the effect of high redox potential donors on the activities of complex II enzymes (see Introduction). But since O_2 is a strong oxidising agent (E_o = 0.816 V) the net turnover of succinate dehydrogenase would be oxidising succinate to fumarate. Simultaneously, if fumarate reductase was exposed to H_2S it has the potential to reduce some fumarate to succinate. However, again due to the presence of O_2, the net turnover of fumarate reductase would be the oxidation of succinate to fumarate. It is also important to note that at higher sulphide concentrations the activities of the two Complex II enzymes will be more affected and *vice versa* at lower sulphide concentrations. These interactions between succinate dehydrogenase, fumarate reductase and H_2S are therefore dependent on O_2 to maintain the aerobic direction of electron flow through the Krebs cycle. If ambient oxygen tensions fall, sulphide would increase fumarate reduction, simply due to the redox potential of the molecule, and not anaerobic metabolism. As such, succinate production is not a reliable measure of anaerobic metabolism in the presence of sulphide.

Oxygen consumption of isolated hearts from *M. edulis*

The values for oxygen consumption of the isolated hearts from the *M. edulis* not acclimatised to sulphide (at 10 °C) determined during this study (0.16 ± 0.046 ml $O_2.mg^{-1}.h^{-1}$) were similar to the values reported by Glaister and Kerly (1936), who recorded 0.22 ml $O_2.mg^{-1}.h^{-1}$ at 15°C in unbuffered seawater. In the present study, there was a significant increase in oxygen consumption when the hearts were exposed to increasing concentrations of sulphide. Since the terminal oxidase of the

respiratory chain is inhibited by sulphide (National Research Council, 1979) there are two possible explanations. Either an alternative sulphide insensitive terminal oxidase is present that is active whilst cytochrome c oxidase is inhibited by sulphide, or the increase in oxygen consumption is due to intracellular sulphide oxidation mediated by a possible sulphide oxidase.

The hypothesis of this series of experiments was that *M. edulis* and *A. islandica* maintain aerobic metabolism during exposure to sulphide. In this context it is important to differentiate between aerobic metabolism and aerobic respiration. Aerobic respiration refers to the activity of the respiratory chain whereas aerobic metabolism refers to the activity of core metabolic processes in the presence of oxygen, such as the aerobic cycling of the Krebs cycle. During aerobic metabolism, the entry point for glycolysis into the Krebs cycle is acetyl-COA, not malate as under anaerobic metabolism (de Zwaan *et al.*, 1983). When exposed to a sulphide concentration of 200 µM, cytochrome c oxidase in *M. edulis* is 70 % inhibited (Lee *et al.*, 1996), hence aerobic respiration is inhibited but, as the results of this chapter show, aerobic metabolism continues.

In summary, these experiments have shown that upon exposure to sulphide *M. edulis* and *A. islandica* do not simply rely on anaerobic pathways. Instead, the Krebs cycle continues in the aerobic direction, although probably at a slower rate due to the partial reversal of succinate dehydrogenase activity by sulphide.

Succinate dehydrogenase is also the enzyme complex responsible for the first electron transfer of the respiratory chain. Hence the first step of the respiratory chain catalysed by succinate dehydrogenase is also partially inhibited by sulphide as described above. Therefore, both the first and terminal enzyme complexes of the respiratory chain are inhibited to some extent by sulphide, not only the terminal

enzyme complex as previously believed (National Research Council, 1979). Parrino *et al.* (2000) reported that electrons from sulphide enter the respiratory chain prior to Complex III for sulphide stimulated ATP production, and hypothesised that it may occur via succinate dehydrogenase. In both *M. edulis* and *A. islandica* it now seems likely that if there is an alternative terminal oxidase that is sulphide insensitive, there must also be an alternative oxidase which is sulphide insensitive at the Complex II branch point. However, the question of the existence of a detoxification mechanism that is inducible and which oxidises sulphide to less toxic substances, as introduced in Chapter 4, remains unanswered.

Chapter 7

Metabolic end products of sulphide detoxification in *Arctica islandica* from two different habitats

Introduction

Previous studies have shown that *Arctica islandica* is very resistant to both hypoxia and hydrogen sulphide, with LD_{50} survival times of 800 - 1000 hours when exposed to 200 µM sulphide and 0.15 ml $O_2.l^{-1}$ (Theede *et al.*, 1969). Calorimetric measurements have shown that after prolonged anoxia, energy release in *A. islandica* is less than 1% of the aerobic rates (Oeschger, 1990). Such high resistance to both sulphide and hypoxia has been attributed to a variety of mechanisms, both behavioural and physiological. These include detoxification of sulphide to thiosulphate, avoidance of H_2S by valve closure and a reliance on very efficient anaerobiosis when aerobic respiration is no longer possible due to either low concentrations of O_2 or inhibition of the respiratory chain by high concentrations of H_2S (Oeschger and Storey, 1993).

Subsequent experiments have shown that *A. islandica* appears to maintain aerobic metabolism during periods of sulphide exposure (Chapter 6). Furthermore, when the concentrations of some thiols were quantified after a sulphide challenge, there were significant differences between *A. islandica* and the blue mussel, *Mytilus edulis* both of which had not been previously acclimatised to sulphide (Chapter 5). Hence *A. islandica* may be able to utilise mechanisms, when exposed to sulphide, that allow not only aerobic metabolism to continue (Chapter 6), but also enable the oxidation of

sulphide to less toxic substances to take place (Chapter 5). There is also the possibly that *A. islandica* is able to gain an energy benefit from this process (Chapter 2).

Two possible mechanisms that may exist in invertebrates to deal with sulphide exposure are the proposed existence of an alternative terminal oxidase or the existence of a sulphide oxidase as discussed in Chapter 5. Early work on *Tubifex* spp. using enzyme kinetics to search for an alternative terminal oxidase, showed that it is absent in this species although there is still a discrepancy between the lower sensitivity of the intact animal to sulphide, and that of its isolated mitochondria (Degn and Kristensen, 1981). Subsequent work by Powell and Somero (1986a) showed that sulphide oxidation is coupled to oxidative phosphorylation in the mitochondria of the bivalve, *Solemya reidi.* Similar work on the lugworm, *Arenicola marina* indicates that in this polychaete sulphide oxidation in the mitochondria is uncoupled from oxidative phosphorylation (Völkel and Grieshaber, 1996). Such differences between species appear to indicate that the sulphide detoxification story is more complex than it first appears, and that there might be more than one strategy exploited by an individual species.

Previous experiments have shown that the Krebs cycle functions aerobically during sulphide exposure in *M. edulis* and in *A. islandica*, and that succinate dehydrogenase is inhibited by sulphide (Chapter 6). Thus, if there is an alternative terminal oxidase active in *A. islandica* during sulphide exposure that allows aerobic respiration to continue, then there must be an additional sulphide insensitive enzyme present to replace succinate dehydrogenase as the first electron carrier of the respiratory chain. The other alternative is the existence of a sulphide oxidase or other protein that mediates sulphide oxidation at a cellular level. The results of previous experiments to examine the effect of sulphide on the production of thiols (Chapter 5) revealed that glutathione and L-cysteine concentrations are lower in *A. islandica* and in sulphide acclimatised *M. edulis* than in *M. edulis* not acclimatised to sulphide (acclimatisation

refers to pre-treatment with low levels of sulphide for long periods in order to desensitise the bivalves to the toxic effects of sulphide). These differences suggest that both cysteine and glutathione may be involved in sulphide metabolism; however the importance of each is unknown. Unfortunately, it appears that any mechanism that is present is not strongly expressed in *M. edulis* (Chapter 5), as it is a bivalve not usually associated with sulphidic habitats.

To examine sulphide resistance in more detail in a single species, the metabolic end products of sulphide detoxification, and associated thiol compounds in *A. islandica* dredged from two different habitats were determined. By careful selection of the collection sites, the first a sulphide rich habitat, and the other a habitat where sulphide does not occur, the differences in thiol production between *A. islandica* from the two sites may be apparent. Because *A. islandica* is very resistant to sulphide and hypoxia (Chapter 2) the cellular reaction induced by sulphide exposure may be sufficiently intense to highlight the mechanisms behind sulphide oxidation.

Two areas which support populations of *A. islandica* are the Skagerrak, and the Kattegat. Although the two areas are close geographically, the environmental conditions at each site differ considerably. Some research has already been done in the Southern Kattegat on the seasonal occurrence of hypoxic conditions. Here mass mortalities due to hypoxia and the accumulation of H_2S in the sediment are a seasonal occurrence that results from the stratification of the Baltic Sea during the summer months. The halocline acts as a barrier, preventing the exchange of O_2 which quickly becomes deficient in the bottom water due to the high O_2 load created by eutrophication processes (Baden *et al.*, 1990). Such mass mortalities are very important, as they are indicative of an increasing organic load in the sediments of a terrestrial origin. In some cases, an influx of high density cold water can cause the hypoxic, sulphide-rich bottom waters to rise to the surface and this can result in high mortalities of the local fauna (Theede *et al.*, 1969). One such area, Laholm Bay, acts

as the drainage basin of two rivers, the Lagan and the Nissan. The sediment in the Bay is a fine mud, rich in organic matter from the two rivers. Laholm Bay is characterised by a shallow shelf that is only slightly below the summer halocline. During the summer months a tongue of water from below the halocline intrudes into the Bay which makes the bottom waters especially susceptible to hypoxia caused by seasonal stratification (S. Baden *pers. comm.*).

The aim of these experiments is to examine possible mechanisms of sulphide detoxification in more detail by comparing the end products of sulphide oxidation, and associated thiol compounds, in *A. islandica* dredged from two different habitats, one where sulphide is present in significant amounts, and one without sulphide.

Materials and Methods

A. islandica were collected using a towed ring dredge from Laholm Bay (56° 34` N; 12° 43` E; Ca 17m), a sulphide-rich hypoxic site, and from Kockholmen (58° 13` N; 11° 21` E; Ca 17m), a sulphide-free, well oxygenated site. The bivalves were transported to Kristineberg Marine Research Station (KMF) on the Gullmars fjord in Sweden where all experiments were undertaken. Bottom water samples and sediment samples from both dredge sites were also analysed for oxygen and hydrogen sulphide concentration. The bottom water samples were collected by means of a Nansen bottle, and the sediment samples using a Van Veen grab. The interstitial water was sampled by centrifuging a sample of sediment in a 1.5 ml sample tube (Eppendorf) for a few seconds, and subsequently removing the supernatant using a 1 ml syringe. To fix the sulphide in the bottom water and sediment samples, 500 µl of 0.12 M zinc acetate and 125 µl of 0.6% NaOH were added to a 50 µl sample. The samples were subsequently stored at -20 °C. The bivalves were then maintained in tanks at 10°C in seawater pumped directly from the bottom of the

Gullmars fjord. The seawater was allowed to overflow from the holding tanks. None of the bivalves were fed during the experimental period but there was particulate matter in the seawater that may have been of nutritional value to the bivalves. All experiments were completed within a week of capture.

The sulphide stock solution (100 mM) was prepared from $Na_2S.\ 9H_2O$ (Sigma, S-4766 Assay 100%) every day prior to use and maintained under a nitrogen atmosphere to prevent oxidation. The concentration of the stock solution was determined using iodometric back titration (Parsons *et al.*, 1984), and colorimetric determination of sulphide modified from Parsons *et al.* (1984) was used to monitor sulphide concentration in the exposure tank, and during experimentation, as discussed in Chapter 2. The pH of the sulphide solution was first adjusted to pH 8 using 6N HCl, and then maintained at pH 8 by the buffer qualities of the seawater.

To examine the thiol concentrations in the *A. islandica* from the two sites, samples of gill, mantle, foot, heart and blood (n = 10) were taken immediately after collection and frozen in liquid nitrogen. In a second experiment, bivalves from both sites were exposed to one of 5 concentrations of sulphide. The sulphide concentrations used were 200µM, 500µM, 800µM, 1000µM and 1200µM. During the experiments, *A. islandica* (n = 5) were placed in a third aquarium and a lid was fitted. The bivalves were allowed to acclimatise for 12 hours prior to exposure to a single concentration of sulphide for 30 minutes. The lid was larger than the exposure tank with an overhanging lip so that seawater pumped into the tank could overflow, displacing any air below the lid and preventing any interaction between the air and the seawater in the exposure tank as illustrated in Fig. 5.1. The required sulphide concentration was achieved by the manipulation of the flow from 2 peristaltic pumps, the first pumping seawater from the system described above, and the second introducing sulphide stock solution to the aquarium. The pre-exposure or control *A. islandica* were sampled prior to the addition of sulphide to the exposure tank.

Detoxification of hydrogen sulphide in *A. islandica* from Laholm Bay (hypoxic/sulphidic environment) with time was scrutinised by exposing the bivalves to 1000 µM sulphide for 30 minutes in the same experimental set-up described above. Subsequently, samples of gill, mantle, foot, heart and blood were removed and frozen in liquid nitrogen (n = 5), at 15 minute intervals over a period of 60 minutes.

The tissue and blood samples were then transported frozen, in a mixture of dry ice and liquid nitrogen, to the University Glasgow. Here tissue samples were freeze-dried using an Edwards Freeze Drier (Modulyo) whilst still frozen. Subsequently, all samples were prepared for HPLC analysis using bromobimane (Chapter 5). The prepared samples were transported frozen to the Institut für Zoophysiologie, Heinrich-Heine Universität, Düsseldorf, Germany, where they were analysed for thiols using high pressure liquid chromatography (HPLC) as described in Chapter 5 (Völkel and Grieshaber, 1992). Bottom water and sediment samples were fixed and analysed at KMF, Sweden using the colorimetric method (Chapter 2).

In summary, three parameters were examined. Firstly, the differences in thiol concentrations in freshly caught *A. islandica* from a normoxic/sulphide-free site (Kockholmen) were compared to the thiol concentrations in freshly caught *A. islandica* from a hypoxic/sulphide-rich site (Laholm Bay). Secondly, changes in thiol concentrations during sulphide exposure and during a recovery period under sulphide-free conditions were quantified in *A. islandica* from a hypoxic/sulphide-rich site (Laholm Bay). And lastly, the accumulations of thiols in *A. islandica* from both Kockholmen and Laholm Bay, after 30 minutes of sulphide exposure were compared.

Statistical analysis

All raw data were analysed using Sigma Plot 5.0 software. The data were normally distributed (normal scores > 0.917), and there was no significant heterogeneity of

variance (Bartlett's value < 9.49; $p < 0.05$). Tukey's pairwise comparisons were used to show significant differences ($p < 0.05$) between the different measured parameters ($n = 5$). As 95% confidence intervals (CI = 95%) are used as the error bars in the diagrams; error bars that do not overlap indicate significant differences. Unless otherwise stated, statistical significance is quoted at a 95% confidence level ($p < 0.05$).

Results

The concentrations of sulphide in the bottom water and in the sediment together with the oxygen concentration of the bottom water from the two sample sites (Kockholmen and Laholm Bay) are shown in Table. 7.1.

Table 7.1: The concentrations of total sulphide and of oxygen in the bottom water, a mixture of bottom water and sediment, and in the sediment, from the two sample sites, Kockholmen and Laholm Bay. The values are means ± 95% CI (n = 6).

Sample site	**Bottom water H_2S (µM)**	**Sediment/bottom water H_2S (µM)**	**Sediment H_2S (µM)**	**Bottom water O_2 ($ml.l^{-1}$)**
Kockholmen	0	0	0	6.45
Laholm Bay	41.7 ± 1.0	41.8 ± 1.6	41.6 ± 3.4	1.0

The sediment from the Kockholmen site was coarse grained sand, with no anoxic black coloration. The Kockholmen site, situated near the entrance to the Gullmars fjord, was a high-energy area due to wave action, characterised by high oxygen levels and no sulphide. Conversely, Laholm Bay was characterised by low levels of oxygen in the bottom water and thick black anoxic mud.

Thiol concentrations in the *A. islandica* sampled immediately upon collection

It was observed that the average size of the individual *A. islandica* from Laholm Bay (48.0 mm ± 6.8 mm) was less than that of animals from Kockholmen (98.3 mm ± 7.9 mm). Although the concentrations of thiols in the blood of the Kockholmen *A. islandica* sampled immediately after collection were almost double those of the *A. islandica* from Laholm Bay, these differences were not significant (Fig. 7.1).

The mean concentrations of sulphite in the different tissues were significantly higher in the foot and gill tissue of the *A. islandica* from Kockholmen (normoxic, sulphide-free site), than in the foot and gill of the *A. islandica* from Laholm Bay (hypoxic, sulphide-rich site). No sulphite was found in the blood of the Laholm Bay *A. islandica*, and no significant concentrations were found in the blood of the Kockholmen bivalves.

L-Cysteine was present at significantly higher concentrations (double) in the heart of the Kockholmen bivalves, as opposed to those from Laholm Bay, but not in the other tissues. Although the mean concentration of L-cysteine in the blood of the Kockholmen bivalves was again approximately double the concentration found in the Laholm Bay bivalves, these differences were not significant. Glutathione was present in significantly higher concentrations in all the tissues of the Laholm Bay bivalves excluding the mantle. In the mantle, concentrations were approximately double those of the Kockholmen bivalves, but these differences were not significant. The concentrations of glutathione in the blood appear to be approximately three times higher in the Kockholmen *A. islandica*, than those from Laholm Bay, however these differences were not significant.

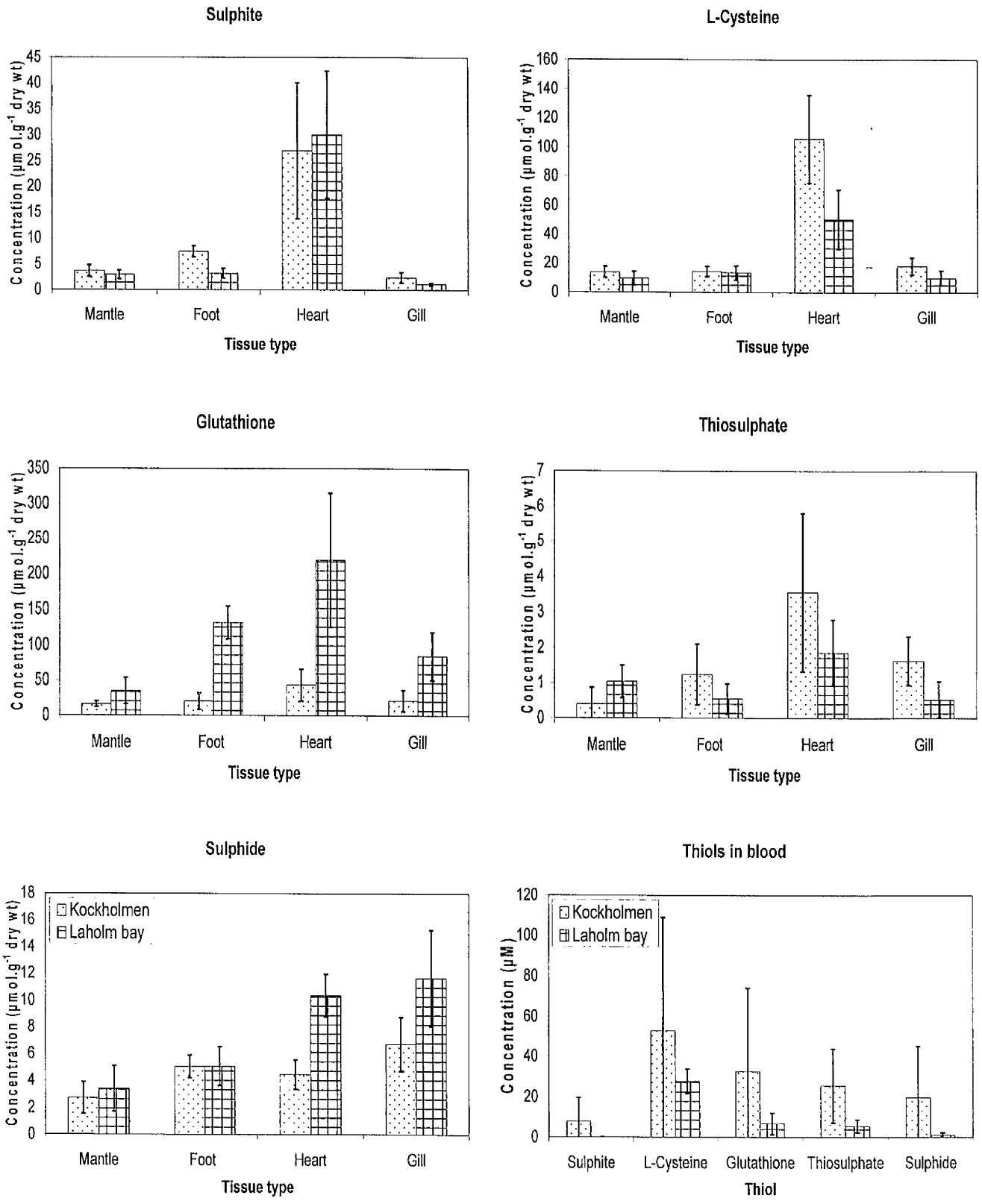

Figure 7.1: The concentrations of thiols in selected tissues and in the blood from Arctica islandica *collected at Kockholmen and Laholm Bay. Values are means ± 95% CI (n = 10).*

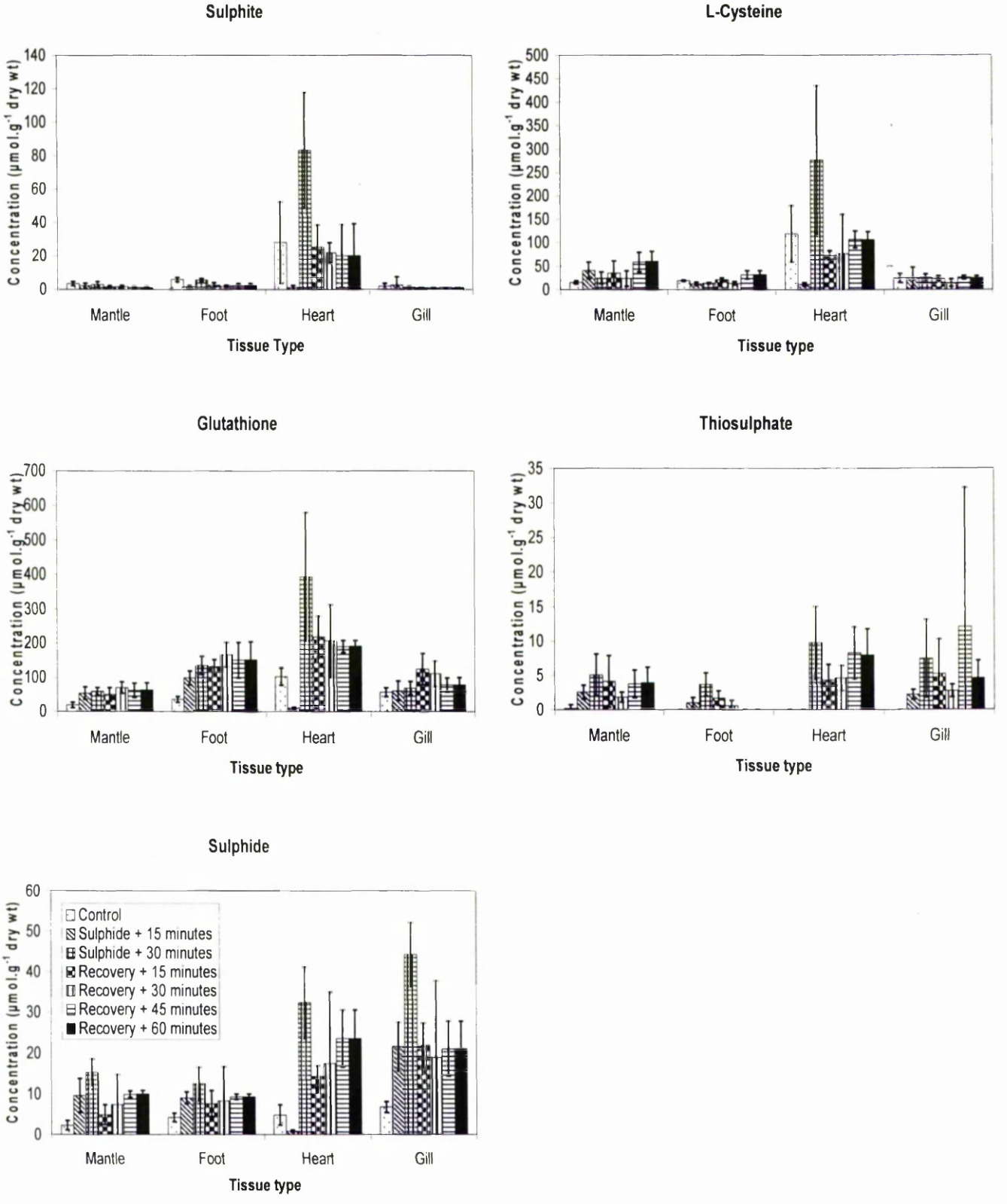

Figure 7.2: Thiol concentrations in selected tissues from Arctica islandica *exposed to 1000µM sulphide for 30 minutes and subsequently left to recover in sulphide-free seawater for 60 minutes. Values are means ± 95% CI (n = 5).*

Thiosulphate, the main detoxification product of sulphide oxidation in marine invertebrates (Chapter 5), was not present in significantly different concentrations in the tissues or the blood of the bivalves from the two areas. The thiosulphate concentrations were very low in the tissues of the bivalves from both sites (max > 4 $\mu mol.g^{-1}$ dry wt), but somewhat higher in the blood (max < 25 μM). The concentration of sulphide in each tissue was higher in the *A. islandica* from Laholm Bay, but these differences were only significant for the heart tissue.

The mean concentration of sulphide in the blood of the Kockholmen Bay *A. islandica* appeared to be approximately five times higher than in those from Laholm Bay, however these differences were not significant. It is noteworthy that the concentrations of different thiols, excluding sulphide, were highest in the heart tissue of all the *A. islandica*, whereas sulphide was present at the highest concentrations in the gill.

The accumulation of thiols in *A. islandica* during recovery from sulphide exposure

In order to examine the production of thiols by *A. islandica* during the period of recovery following sulphide exposure, *A. islandica* from Laholm Bay, as the example of sulphide/hypoxia adapted individuals, were exposed to 1000 μM sulphide and subsequently allowed to recover for 60 minutes in sulphide-free water. Tissue and blood samples were taken every 15 minutes, from different animals, and the concentrations of thiols quantified (Fig. 7.2).

Upon sulphide exposure and during the recovery period, the sulphite concentrations in the tissues of *A. islandica* all displayed a similar trend. There was an increase in the concentration of sulphite during the exposure period, after which the concentration of sulphite fell significantly within 15 minutes of the beginning of the recovery period, subsequently stabilising at similar concentrations to those in the pre-

exposure concentrations (controls). Concentrations of sulphite are significantly higher in the hearts than in any other tissue. The concentrations of L-cysteine did not alter significantly in any of the tissues during either the exposure period, or the recovery period. Glutathione concentrations showed a significant increase during the sulphide exposure period, after which the concentration became stable during the recovery period. Thiosulphate concentrations were very low in the tissues both during the exposure period, and the recovery period (max < 15 $\mu mol.g^{-1}$ dry wt).

In general, the same trend was shown by the concentrations of thiosulphate in each of the tissues examined. There was an increase in thiosulphate concentration during the exposure period, to concentrations significantly higher than the pre-exposure (control) concentrations. Subsequently, thiosulphate concentrations decreased during the recovery (post-exposure) period. Sulphide concentrations also increased significantly during the exposure period, and decreased during the recovery period, during which the concentrations of sulphide eventually stabilised. The concentrations of sulphite, thiosulphate and sulphide all showed similar trends during sulphide exposure, and during the subsequent recovery period. The concentrations of sulphite, cysteine and glutathione were significantly higher in the heart tissues than in any other tissue.

The blood samples from the *A. islandica* from Laholm Bay that were exposed to 1000 µM sulphide for 30 minutes and allowed to recover in sulphide-free seawater for 60 minutes were also analysed for thiol content. No significant concentrations of glutathione and sulphite were found, therefore only the concentrations of cysteine, thiosulphate and sulphide are summarised graphically (Fig. 7.3).

Cysteine concentrations ranged between 27 µM and 65 µM, but did not vary significantly during the sulphide exposure period from the values recorded in control animals maintained in sulphide-free sea water. However, during the recovery period

concentrations of cysteine significantly higher than the control concentrations were determined. The concentrations of thiosulphate increased significantly upon exposure to sulphide, and remained significantly higher than those in the pre-exposure (control) animals during the recovery period.

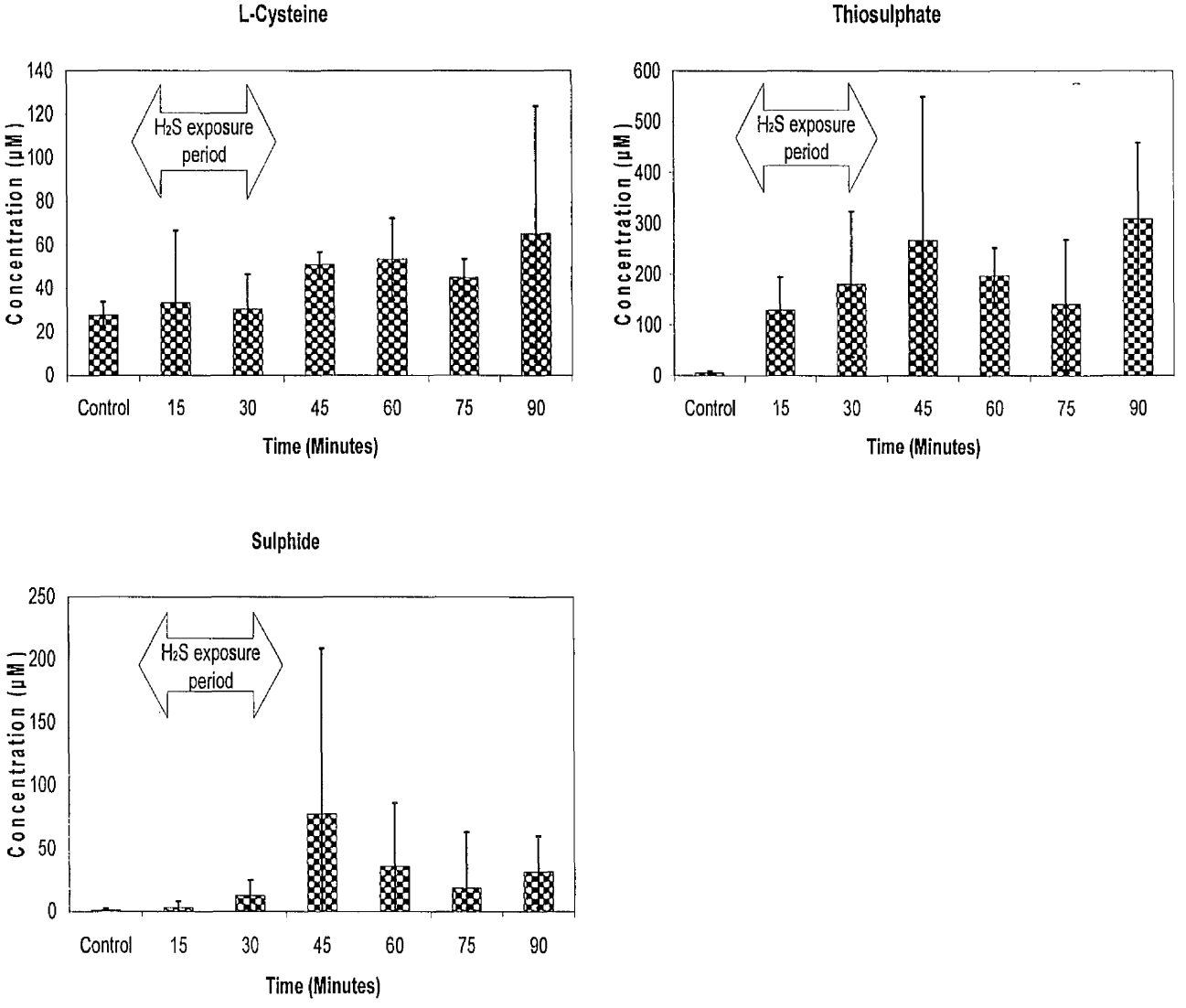

Figure 7.3: Cysteine, thiosulphate and sulphide concentrations in the blood of Arctica islandica *exposed to 1000 µM sulphide for 30 minutes and then allowed to recover for 60 minutes in sulphide-free water. Values are means ± 95% CI (n = 5).*

The concentrations of sulphide showed no statistical difference with either sulphide exposure or during the recovery period from the control concentrations; however both thiosulphate and sulphide displayed similar trends of changing concentration with

time. Concentrations of thiosulphate were approximately three times higher than those of sulphide at each of the time intervals sampled.

The accumulation of thiols in *A. islandica* from Kockholmen

A. islandica from Kockholmen, the high energy site characterised by sulphide-free normoxic conditions, were exposed to varying concentrations of sulphide for thirty minutes prior to having selected tissues sampled to examine the effect of sulphide on *A. islandica* that do not come into contact with sulphide in their natural habitat. The concentrations of the thiols in the tissues are presented in Fig. 7.4.

The concentration of both sulphite and cysteine in the tissues from the Kockholmen *A. islandica* displayed very little change with increasing sulphide exposure concentration. However, the concentration of sulphite and cysteine tended to decrease as sulphide concentration increased. Sulphite and cysteine concentrations were significantly higher in the heart tissues compared to the mantle, foot and gill tissues. Glutathione concentrations increased significantly in all the tissues with increasing sulphide concentration. All the glutathione concentrations (max = 371 $\mu mol.g^{-1}$ dry wt) were high compared with those of sulphite and cysteine (max < 100 $\mu mol.g^{-1}$ dry wt), and approximately a factor of ten higher than the sulphide concentrations in the same tissues. Thiosulphate concentrations also increased very sharply with increasing sulphide concentration (max = 107 $\mu mol.g^{-1}$ dry wt). Surprisingly, the thiosulphate concentration upon exposure to 1200 µM sulphide decreased to a lower concentration than that of the *A. islandica* exposed to 500 µM sulphide. As would be expected, there was a significant increase in the concentration of sulphide in each tissue as the exposure concentration increased.

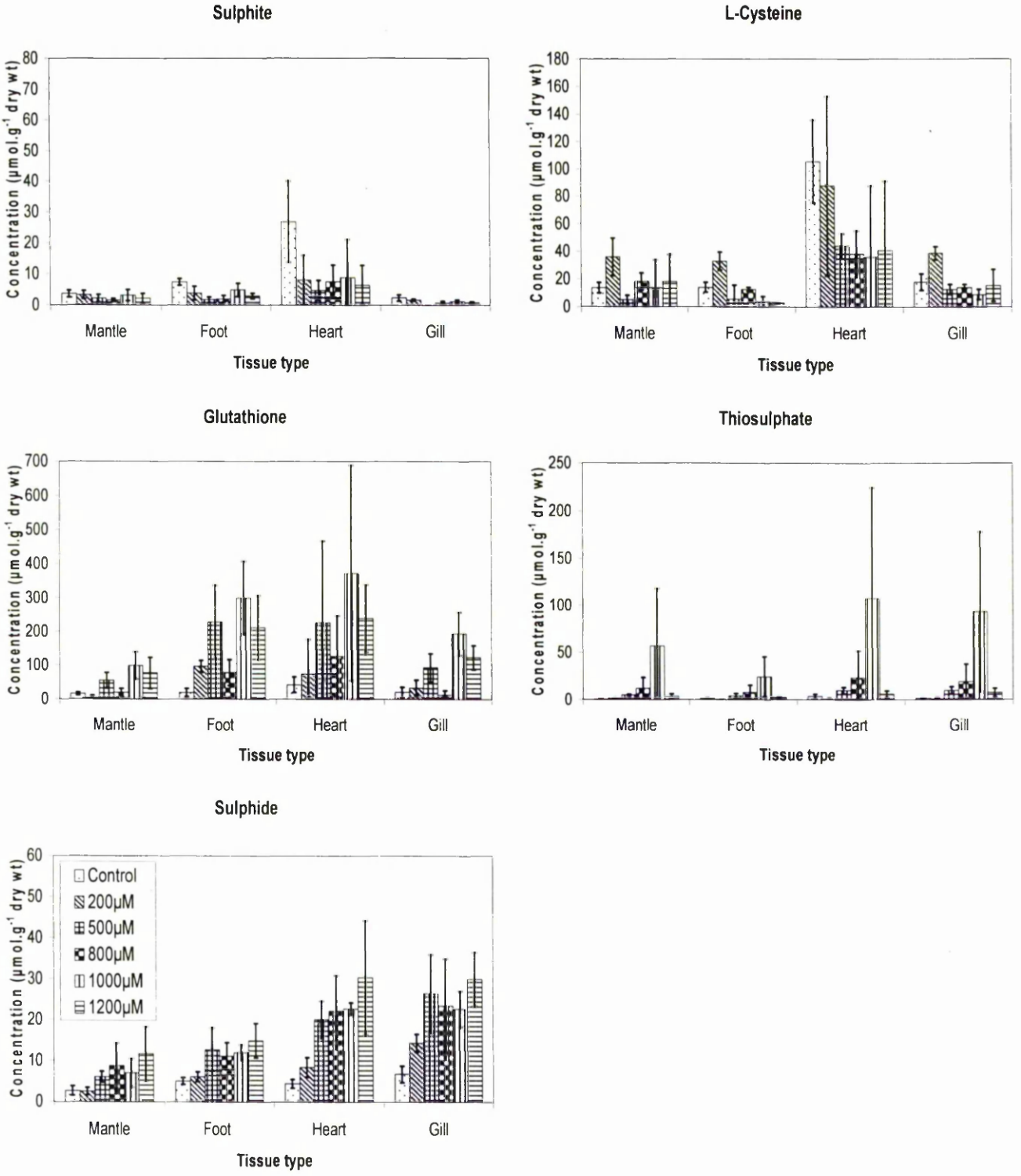

Figure 7.4: Thiol concentrations in selected tissues from Arctica islandica *collected at Kockholmen and exposed to varying concentrations of sulphide for 30 minutes. Values are means ± 95% CI (n = 5). The legend concentrations displayed on the graph represent the sulphide exposure concentrations.*

The accumulation of thiols in *A. islandica* from Laholm Bay

A. islandica dredged from Laholm Bay were exposed to varying concentrations of sulphide to examine the effect of sulphide on bivalves that come into contact with sulphide in their natural habitat. The differing concentrations of the thiols in the tissues are presented in Fig. 7.5.

Neither the concentrations of sulphite nor cysteine in the tissues of *A. islandica* from Laholm Bay differed significantly from the pre-exposure concentrations, as sulphide exposure concentration increased. However, the concentrations of sulphite and cysteine were significantly higher in the heart tissue compared to the mantle, foot and gill tissues. Glutathione concentrations were not significantly different from the pre-exposure (control) concentrations as sulphide concentration was increased. However, the concentrations of glutathione were again a factor of ten higher than the concentrations of sulphide in the tissues. Thiosulphate concentrations were very low, and although there was some increase in thiosulphate with increasing sulphide exposure concentration, it was neither consistent nor significant. The concentrations of sulphide in the tissues of *A. islandica* from Laholm Bay again increased significantly as sulphide exposure concentrations increased.

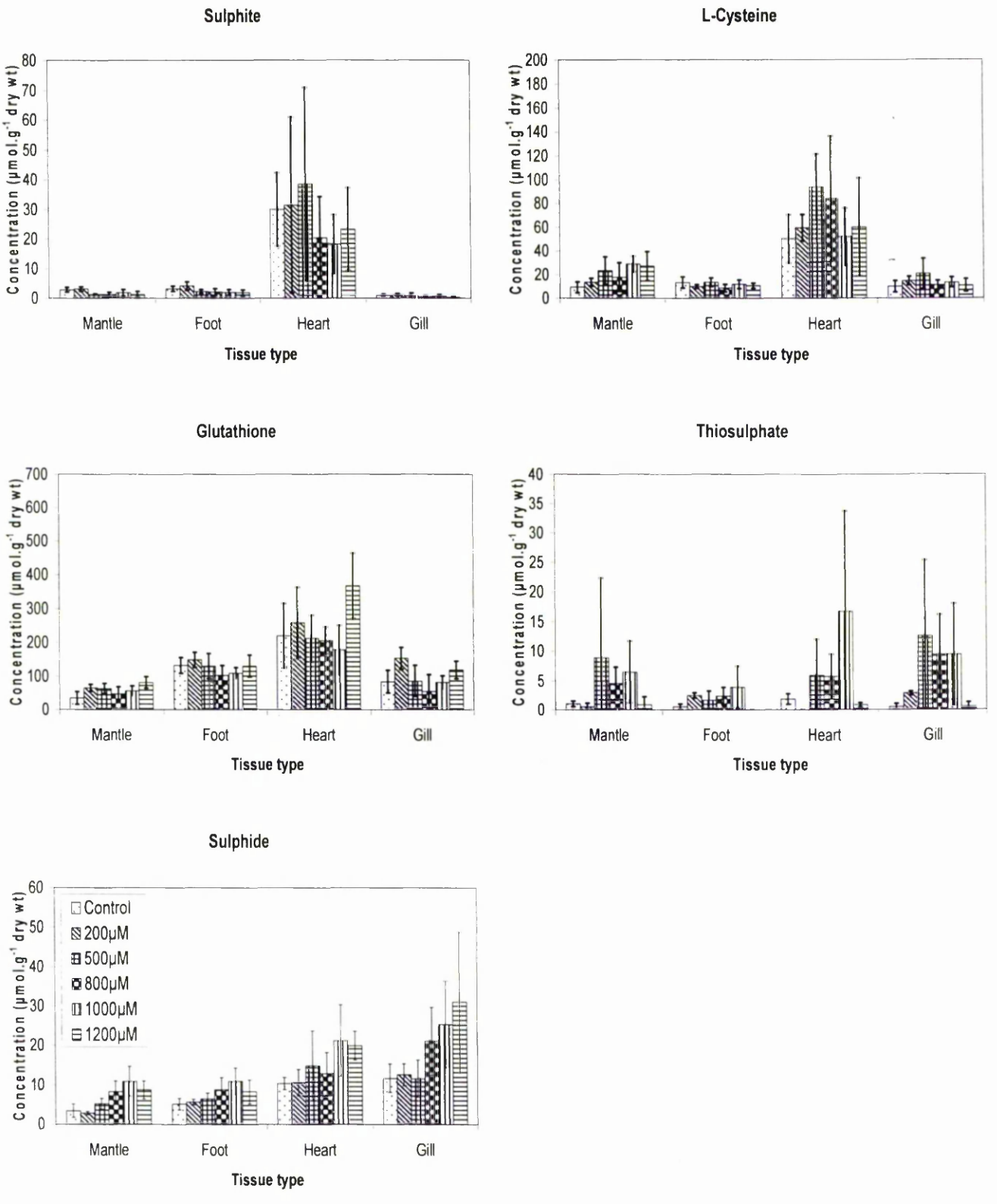

Figure 7.5: Thiol concentrations in selected tissues from Arctica islandica *collected at Laholm Bay and exposed to varying concentrations of sulphide for 30 minutes. Values are means ± 95% CI (n = 5). The legend concentrations displayed on the graph represent the sulphide exposure concentrations.*

A comparison of the accumulation of thiols in A. islandica from Kockholmen and Laholm Bay

A comparison of the *A. islandica* collected at Kockholmen (sulphide-free, normoxic site), and the *A. islandica* from Laholm Bay (sulphide-rich, hypoxic site), subsequently exposed to differing concentrations of sulphide, revealed that for animals from both sites, the concentrations of all the thiols were comparable upon exposure to sulphide. Sulphite and cysteine concentrations were not significantly different in the tissues of the *A. islandica* from the two sites. The concentration of glutathione was initially significantly lower in the control *A. islandica* from Kockholmen compared to the *A. islandica* from Laholm Bay. But as sulphide exposure concentrations increased, glutathione concentrations increased in the bivalves from Kockholmen, to concentrations similar to those found in *A. islandica* from Laholm Bay. Conversely, the concentrations of thiosulphate in the tissues of the *A. islandica* from Kockholmen were significantly higher than those in the specimens from Laholm Bay, by an approximate ratio of 5:1. Sulphide concentrations in the tissues from both sites were almost identical.

Blood samples were also taken from the *A. islandica* from both sites following exposure to differing concentrations of sulphide, and the concentrations of thiols in the blood quantified. Sulphite concentrations in the *A. islandica* from both areas showed the same general trends (Fig. 7.6). Initially, there was almost no sulphite in the blood of the *A. islandica* maintained under sulphide-free conditions. Upon exposure to sulphide, sulphite concentrations increased significantly from the control concentrations, but did not vary with increasing sulphide exposure concentration.

Concentrations of cysteine in the blood were very similar in the control *A. islandica* from both sites. But upon sulphide exposure the *A. islandica* from Kockholmen displayed a steady and significant increase in cysteine concentration with exposure to increasing sulphide concentrations. Conversely, the concentration of cysteine in the

animals from Laholm Bay remained similar to the control concentrations throughout, and significantly lower than the concentrations found in the Kockholmen *A. islandica*.

The concentrations of glutathione in the blood of the bivalves from both areas were similar in the pre-exposure (control) samples, and there was no significant change upon exposure to sulphide.

Prior to sulphide exposure, thiosulphate concentrations in the blood were similar in *A. islandica* from both areas. Upon exposure to sulphide, the concentrations of thiosulphate in the blood of the Kockholmen *A. islandica* did not vary significantly from those in the blood of the control animals. However, the concentrations of thiosulphate in the blood of the Laholm Bay *A. islandica* increased significantly and were higher by a ratio of approximately 10:1 from those found in the *A. islandica* from Kockholmen.

Initially, there was no sulphide in the blood of the pre-exposure (control) *A. islandica* from both areas, but upon exposure to sulphide there was a significant increase in sulphide in the blood with increasing sulphide exposure concentration in the *A. islandica* from both areas.

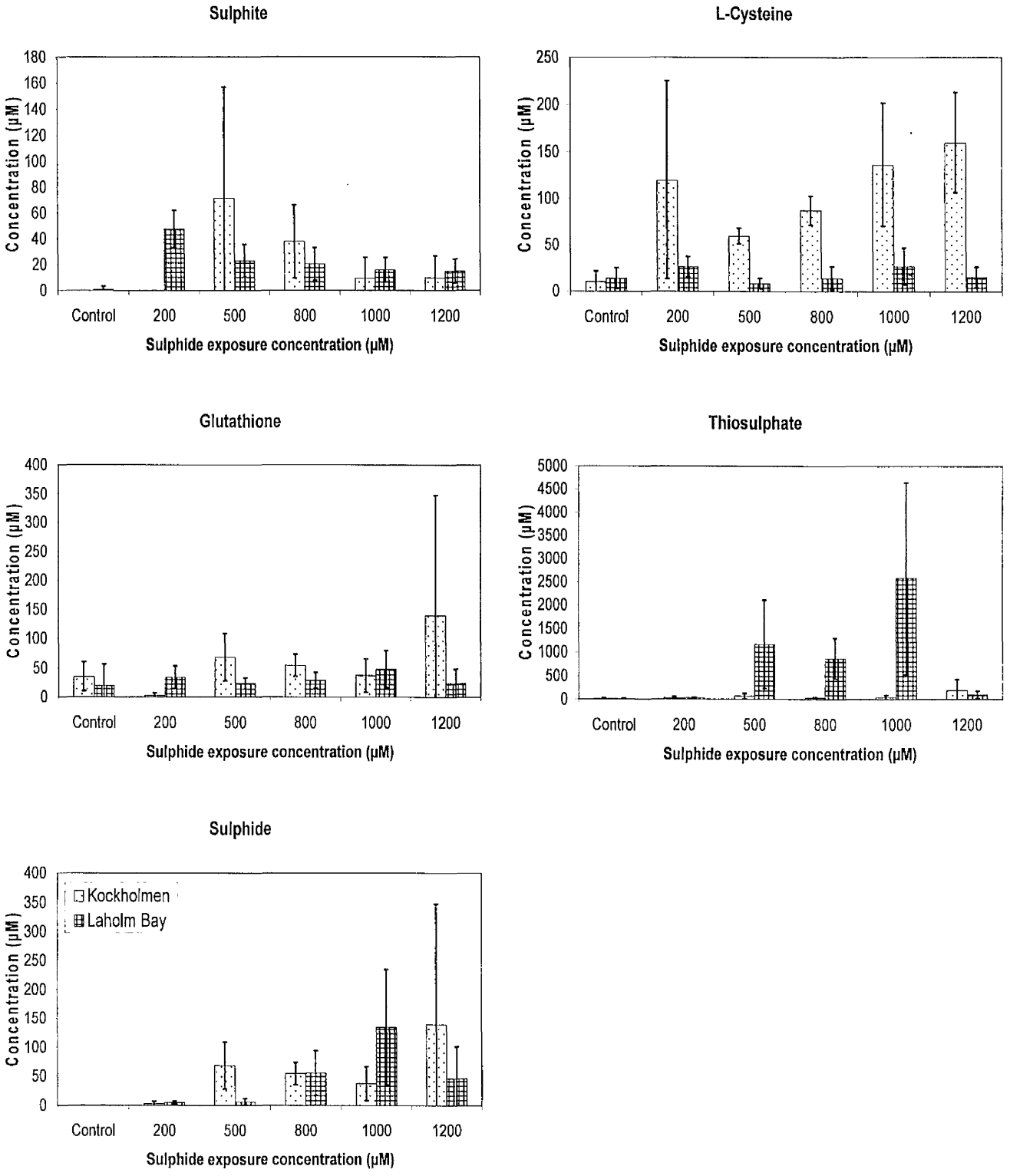

Figure 7.6: Thiol concentrations in the blood of Arctica islandica *collected at Kockholmen and Laholm Bay and subsequently exposed to varying concentrations of sulphide for 30 minutes. Values are means ± 95% CI (n = 5).*

A comparison of the accumulation of thiols in the blood and tissues of *A. islandica*

The concentrations of the thiols in the blood were compared with those in the tissues of the *A. islandica* from the two collection sites. The concentrations of sulphite were low in both the tissues and the blood, and showed very little variation with increasing sulphide exposure concentration. Cysteine concentrations, however, increased significantly in the blood of the Kockholmen *A. islandica* (normoxic/sulphide free site) as sulphide exposure concentrations increased. The concentrations of cysteine in the blood of the *A. islandica* from Laholm Bay (hypoxic/sulphide rich site) did not change significantly. Similarly, the concentrations of cysteine in the tissues of the *A. islandica* from both collection sites also remained constant. Glutathione concentrations in the blood of the *A. islandica* from the two sites did not vary significantly with increasing sulphide exposure concentration. However, the concentrations of glutathione in the tissues were consistently higher (by a ratio of 10:1) than the sulphide concentrations in the tissues of the bivalves from both Kockholmen and Laholm Bay.

The concentrations of thiosulphate increased significantly in both the tissues and in the blood of all the *A. islandica* upon exposure to increasing levels of sulphide. In the Kockholmen *A. islandica*, the tissues had significantly higher concentrations of thiosulphate than the tissues of the Laholm Bay *A. islandica*. Conversely, the blood sampled from the Laholm Bay *A. islandica*, had significantly higher concentrations of thiosulphate than in the blood from the Kockholmen *A. islandica*. All the sulphide concentrations in both the tissues and the blood of the bivalves from both Kockholmen and Laholm Bay displayed a significant increase in concentration with increasing exposure concentrations.

Discussion

The results of the experiments to examine the effects of sulphide in *A. islandica*, sulphide acclimatised *M. edulis*, and in *M. edulis* that had not been acclimatised to sulphide were reported in Chapter 5. The results showed that there were some differences in cysteine and glutathione production between the three groups of bivalves, however the mechanisms that were modified upon exposure to sulphide were not strongly expressed, thus characterisation of these mechanisms was not possible. However, the *A. islandica* that inhabit Kockholmen and Laholm Bay presented a unique opportunity to utilise these sulphide tolerant bivalves (Chapter 2) as a model species, and to examine differences in thiol production in animals from two habitats that differ in oxygen and sulphide content.

The most obvious difference in the *A. islandica* from the two sites was size. The Laholm Bay *A. islandica* were approximately half the size of those from Kockholmen, and were the only living macrofauna apparent in the dredge, the rest of the catch consisted of empty valves. This is typical of Laholm Bay, and it has been known by local fisherman to be impoverished in terms of fauna for approximately thirty years (*pers. comm.*). A severe hypoxic event, a number of years ago, may have caused a mass mortality of all living macrofauna in the Bay, and the population of *A. islandica* found is derived from subsequent spat settlements. The recruitment of *A. islandica* is known to be low, and a number of years may pass when no recruitment takes place (Kennish and Lutz, 1995). However, there was also no evidence of substantial settlement by any other species, so it is likely that the conditions in Laholm Bay do not encourage settlement. Unfortunately, the reason for the difference in size of *A. islandica* from the two sample sites remains elusive.

Analyses of the heart tissue samples taken immediately after collection of the bivalves from the two sites showed that there were higher concentrations of sulphite,

L-cysteine and glutathione present in these tissues. However, the concentrations of thiosulphate and sulphide are more or less consistent from tissue to tissue. Early work suggested that ultrafiltration occurs across the ventricle wall of bivalves, but Tiffany (1972) disproved these findings and showed that the osmotic pressure gradient in bivalves is from the pericardial fluid to the blood. In view of this, it is unlikely that the heart of *A. islandica* is involved in filtration. A more likely explanation is that the hearts contained a significant volume of blood when isolated, and therefore the concentrations of thiols in the heart actually reflect a mixture of blood and heart tissue.

Sulphite was found in relatively low concentrations, compared to the other thiols, in all the *A. islandica* examined. There were some significant increases in sulphite concentration between the pre-exposure (control) concentrations, and upon exposure to sulphide. These changes in sulphite concentration mirrored changes in thiosulphate and sulphide concentration, but at much lower concentrations. In invertebrates, sulphite has been shown to be the intermediate in the two step oxidation of sulphide to thiosulphate (O'Brien and Vetter, 1990). It is possible to surmise that if the main product of sulphide oxidation in invertebrates is thiosulphate (Johns *et al.*, 1997; Völkel and Grieshaber, 1992), sulphite levels, as the intermediate in the two step oxidation of sulphide to thiosulphate, will always remain low. This is consistent with the current findings. The higher concentrations of sulphite in the blood upon exposure to sulphide, compared to the tissue concentrations, could be due to the spontaneous oxidation of sulphide caused by oxygen present in the blood.

L-Cysteine concentrations in the tissues of *A. islandica* varied very little upon exposure to sulphide when compared to pre-exposure concentrations, with the exception of the heart tissue. The concentrations of L-cysteine in the heart tissue may better reflect those in the blood as discussed above. A number of tissue and blood

samples were taken from the *A. islandica* immediately after collection. The concentrations of L-cysteine in the blood and the heart tissue of the *A. islandica* from Kockholmen (normoxic, sulphide-free site), were consistently and significantly higher than those found in the *A. islandica* from Laholm Bay (hypoxic, sulphide-rich site). Furthermore, upon the addition of increasing concentrations of sulphide, the concentration of L-cysteine in the blood of the *A. islandica* from Kockholmen increased significantly in an approximately linear manner. Unfortunately, only the *A. islandica* from the Laholm Bay area were exposed to 1000 µM sulphide for 30 minutes and subsequently allowed to recover for 60 minutes. Thus, it is not possible to say if there would also be significant differences in the L-cysteine concentrations in the *A. islandica* from Kockholmen during the recovery or post-exposure period.

The Kockholmen *A. islandica* are from a well aerated sulphide-free habitat. The experiments were carried out under aerobic conditions at all times, so the only variable was sulphide. In the presence of oxygen, H_2S inhibits succinate dehydrogenase, the enzyme that catalyses the oxidation of succinate to fumarate in the Krebs cycle (Chapter 6). Under the same conditions, L-cysteine restores the activity of succinate dehydrogenase completely (Bergstermann and Lummer, 1947), and it has also been speculated that the hydrosulphide (HS^-) anion may interact in a similar manner with other enzymes inhibited by sulphide. Alternatively, in an anaerobic system L-cysteine has been shown to cleave disulphide bridges (Smith and Abbanat, 1966). H_2S also has a detrimental effect on disulphide bridges (National Research Council, 1979; Park *et al.*, 1984). Under anaerobic conditions, such as those found in the Laholm Bay habitat, higher levels of L-cysteine in *A. islandica* would add to the detrimental effect of H_2S on disulphide bridges. Supporting evidence was presented in Chapter 5. Significantly higher concentrations of cysteine were recorded in *M. edulis* not acclimatised to sulphide, when compared to those that had been acclimatised to sulphide (Chapter 5). It seems logical, therefore, that there

should be significantly lower concentrations of L-cysteine in the Laholm Bay bivalves, both under experimental conditions and in their natural habitat. The difference in L-cysteine concentrations between the *A. islandica* of Kockholmen and Laholm Bay is only one adaptive strategy to life in sulphidic/hypoxic sediments, and highlights the importance of the interaction between hypoxia, and sulphide concentration on benthic invertebrates.

The concentrations of glutathione in the tissues of the Laholm Bay *A. islandica* were significantly higher than in those in the freshly collected animals from Kockholmen. Since glutathione has been shown to be a sensitive indicator of oxidative stress in rat liver during hypoxia (Jaeschke, 1990), the occurrence of higher levels in the Laholm Bay *A. islandica* sampled immediately after collection is surprising. Due to the hypoxic conditions, lower concentrations of glutathione would be expected (Chapter 5), because lower levels of reactive oxygen species (ROS) during hypoxia lead to lower concentrations of glutathione.

The *A. islandica* from Laholm Bay exposed to sulphide already had high concentrations of glutathione in the tissues prior to sulphide exposure. However, in the tissues of the *A. islandica* from Kockholmen, significant increases in glutathione reaching concentrations similar to those found in the Laholm Bay *A. islandica*, were observed upon exposure to sulphide. The differences in the initial concentrations of glutathione and the subsequent changes in glutathione concentration upon exposure to sulphide in *A. islandica* from both sites, is therefore not dependant on hypoxia. Since the only experimental parameter that was manipulated in these experiments was sulphide concentration, it is likely that sulphide exposure stimulates glutathione production.

Although thiosulphate oxidation is known to be glutathione dependant (Sörbo, 1964), the role of glutathione in sulphide detoxification has generally been reported as a minor one. High concentrations of glutathione have been observed to be present in fish exposed to sulphide (Bagarinao and Vetter, 1989). These were attributed to sulphide either reducing the limited supply of glutathione from the disulphide form (GSSG), to the sulphydryl form (GSH), which is the most dominant form (90% of total), or the inhibition of glutathione peroxidase by sulphide (National Research Council, 1979). Unfortunately, the authors present no statistical analysis, which makes any comparison impossible.

The concentrations of glutathione in the tissues of *A. islandica* recorded in this study were more than 10 times those of sulphide in the tissues. Smith and Abbanat (1966) proposed that 1 mole of oxidised glutathione can detoxify approximately 0.5 mole of sulphide, based on shifts in LD_{50} curves of mice protected by GSSG injection against sulphide. If the approximate1:9 ratio between GSSG and GSH forms of glutathione is also taken into consideration, then the glutathione concentration in the tissues of *A. islandica* appears to be sufficient to detoxify the amount of sulphide present. As mentioned earlier, there is a definite discrepancy between the lower sensitivity of the isolated animal tissues to sulphide, and that of the isolated mitochondria (Degn and Kristensen, 1981). This may possibly be due to the action of GSSG on sulphide, protecting the sulphide sensitive mitochondria.

The low and relatively constant concentration of glutathione in the blood of the *A. islandica* from both habitats indicates that GSSG detoxification of sulphide occurs mainly at an intracellular level. As mentioned earlier, thiosulphate oxidation is glutathione dependant (Sörbo, 1964), and in *Solemya reidi*, a bivalve that contains intracellular symbiotic bacteria that are chemoautotrophic sulphur oxidisers, whole animal incubations in the presence of sulphide have produced sulphate as the main

oxidation product. The low concentration of glutathione in the blood of *A. islandica*, however, seems to indicate that this is not the case in this species.

Analyses of tissues sampled immediately after capture showed that there were no significant differences in the concentration of thiosulphate between the *A. islandica* from the two sites. Predictably, in the time recovery experiments the thiosulphate concentrations peaked after 30 minutes of sulphide exposure. But there were some differences in thiosulphate concentration between the bivalves from both areas when exposed to increasing concentrations of sulphide. There was an increase in thiosulphate concentration with sulphide exposure until the exposure concentration reached 1000 µM, but at higher concentrations (1200 µM) the thiosulphate production fell drastically in the blood and tissues of *A. islandica* from both sites. The reason for this discrepancy between the 1000 µM and the 1200 µM exposure concentrations is possibly that the bivalves are overwhelmed by exposure to 1200 µM sulphide, and the sulphide detoxification process breaks down (M.K. Grieshaber, pers. com).

The sulphide exposed *A. islandica* from Kockholmen had approximately five times as much thiosulphate in the tissues as that in the Laholm Bay *A. islandica*. However, the Laholm Bay *A. islandica* had about ten times as much thiosulphate in the blood, as those from Kockholmen. But there was no significant difference in the amounts of thiosulphate in the bivalves sampled immediately upon collection. Furthermore, upon exposure to sulphide, the concentrations of sulphide present in the tissues and blood were similar in the bivalves from both areas. Therefore either the thiosulphate is actively taken up in the tissues of the Kockholmen *A. islandica* upon exposure to sulphide, as has been demonstrated in *S. reidi* (O'Brien and Vetter, 1990), or the differences between the tissue and blood thiosulphate concentration could be due to different mechanisms of sulphide detoxification within the *A. islandica* from the two sites. The Laholm Bay *A. islandica* appear to have higher rates of sulphide oxidation

in the blood, and the Kockholmen *A. islandica* appear to have higher rates of sulphide oxidation in the tissues. This suggests that there could be two mechanisms at work. The first in the Kockholmen bivalves, which do not usually come into contact with sulphide, is a detoxification mechanism at an intracellular level, and the second, found in the Laholm Bay *A. islandica*, detoxifies as much sulphide as possible in the blood before it reaches the tissues. Differences in sites of sulphide conversion to thiosulphate have been reported in different invertebrates, the hepatopancreas detoxifies sulphide in crustaceans (Johns *et al.*, 1997), and the lugworm, *Arenicola marina* detoxifies sulphide in the body wall (Völkel *et al.*, 1995). But differences in the site of sulphide detoxification have not previously been described in the same species as a result of changing environmental parameters. The most attractive explanation is that *A. islandica* that do not usually encounter sulphide can still detoxify significant amounts at a cellular level, but at some metabolic cost. If the bivalves are acclimatised to sulphide, a different method of detoxification is induced which operates in the blood at a lower metabolic cost.

There were higher concentrations of sulphide present in the Laholm Bay *A. islandica* sampled immediately upon collection, due to habitat differences, but upon the addition of sulphide the levels increased significantly in the bivalves from both areas. However, there were no significant differences between intracellular concentrations of sulphide in the *A. islandica* from the two habitats. Furthermore, there were no significant differences between the blood sulphide levels of the bivalves from the two habitats. This seems to indicate that both mechanisms of sulphide oxidation are efficient, but perhaps have a different metabolic cost.

Sulphide is known to interact with haem bodies in the blood. *A. marina* oxidises sulphide via the "brown pigment" in the blood (Patel and Spencer, 1963). Bagarinao and Vetter (1989) showed that shallow-water marine fish have sulphide oxidising

activity in the blood, spleen, kidney, liver and gills that correlates to haem content. In light of this, it is interesting that Oeschger and Vetter (1992) reported on the binding of sulphide to hemerythrin in the priapulid, *Halicryptus spinulosus*, but speculated that the capacity of the hemerythrin to bind sulphide may be dependent on the oxygen carrying capacity of that molecule. Similarly, methemoglobin has also been shown to inactivate sulphide in armadillos, rabbits, rats and mice (Smith and Gosselin, 1964; 1966). Studies on the bivalves *S. reidii*, *Calyptogena magnifica* and *Lucinoma annulata* discovered non-hemoglobin hematins (brown hematins) that appear to oxidise sulphide (Powell and Arp, 1989). Thus it is possible that haem-containing molecules have the ability to bind sulphide and may play an important role in the detoxification process. Further examination of *A. islandica* blood compounds in the presence of sulphide is necessary for clarification.

In summary, there appears to be a number of mechanisms at work in *A. islandica*, each contributing to the overall detoxification of sulphide. It is a combination of these mechanisms that allows this bivalve to survive in sulphidic sediments. At a cellular level, glutathione production is stimulated by sulphide exposure and subsequently oxidises sulphide. In the blood, L-cysteine reactivates enzymes that are inhibited by sulphide, but only in those bivalves not acclimatised to sulphide. The sulphide acclimatised *A. islandica* appear to use another sulphide detoxification mechanism that is active in the blood and possibly operates at a lower metabolic cost than the use of glutathione alone.

Chapter 8

Glutathione mediated sulphide oxidation in vitro during normoxia and hypoxia

Introduction

The role of glutathione in the cell has drawn interest from a wide range of toxicologists as can be seen by the numerous reviews available, one of the most recent of which is by Locigno and Castronovo (2001). In order to place the significance of sulphide-stimulated glutathione production into perspective, the role of glutathione in the cell needs to be investigated.

Glutathione was discovered in yeast during the 19th century, but only in the latter part of the 20th century was the importance of glutathione in cellular reductive processes examined in depth (Reed, 1990). There are two predominant forms of glutathione, the reduced or sulphydryl form, γ-glutamyl-cysteinyl-glycine (GSH), and the oxidised or disulphide form (GSSG) that exist simultaneously in cellular compartments (Tietze, 1969). Glutathione cycles between these two forms, catalysed by glutathione peroxidase, a selenium dependant enzyme, and glutathione reductase (Mize and Langdon, 1962). The cycle is known as the glutathione redox cycle. GSH is oxidised to GSSG via glutathione peroxidase at a rate of approximately 40 μmoles.min^{-1}.g^{-1} in

rat liver (Jaeschke, 1990). Glutathione peroxidase fails to display saturation with respect to GSH therefore the maximum velocity for infinite peroxide (H_2O_2) concentration is a linear function of GSH concentration (Reed, 1986). GSH is the predominant form accounting for 95% of the total glutathione in the cell (Jaeschke, 1990). GSH is also an important antioxidant, acting as a scavenger of free radicals such as the superoxide ion O_2^{-0}, involved in the formation of H_2O_2, and of the hydroxyl radical OH^0 (Locigno and Castronovo, 2001). These free radicals or reactive oxygen species (ROS) are formed as a by-product of aerobic respiration. Hence accumulation of GSSG as a result of GSH mediated reduction of ROS is dependant on ambient oxygen concentration. Therefore, GSSG accumulation is a sensitive indicator of oxidative stress during hypoxia (Jaeschke, 1988).

As aerobic respiration occurs mainly in the mitochondria of eukaryotes, most oxidative stress occurs in this cellular compartment. The mitochondria contain no catalase, and consequently rely solely on GSH and glutathione peroxidase to detoxify peroxide (Reed, 1990). The reduction of GSSG is via glutathione reductase with NADPH providing the energy for this reaction. NADPH becomes reduced to $NADP^+$ in the process. The maximum rate of NADPH reduction via glutathione reductase is 8 – 10 $\mu moles.min^{-1}.g^{-1}$ in rat liver, 5 – 10 times the rate of fatty acid synthesis, mixed function oxidase and *t*-butyl hydroperoxide reduction (Jaeschke, 1990). Maximum rates of GSSG reduction therefore place a regulatory effect on these other NADPH cytochrome reductase activities in the rat liver. This monopolisation of NADPH means that at maximal rates of glutathione reductase activity, the NADPH/ $NADP^+$ pool turns over once per minute (Jaeschke, 1990). Consequently, in the glutathione redox cycle the rate limiting step is the reduction of GSSG to GSH due to the comparatively low enzyme activity rate of glutathione reductase compared to glutathione peroxidase, and the limited availability of the energy source NADPH to drive glutathione reductase activity.

GSH, as mentioned above, is a substrate for H_2O_2 detoxification via glutathione peroxidase and also via glutathione S-transferases (GSTs). GSTs, in conjunction with GSH, play a central role in the strategy used by researchers when developing methods of drug targeting during treatment of a range of ailments including cancers (for a full review of GSTs see Salinas and Wong, 1999). Any deficiencies in GSH concentrations lead to accumulations of ROS. Increased levels of ROS have been implicated as a major cause of the influenza virus, hepatitis, the development of AIDS from HIV, Alzheimer and Parkinson's diseases, cancer and premature ageing and even some allergies (Locigno and Castronovo, 2001). During GSH mediated destruction of hydroperoxides, a GSH depletion of 20 – 30 % can lead to cell injury and death (Reed, 1990). ROS cause lipid peroxidation of the polyunsaturated fatty acids that give membranes their fluidity of movement. The consequence of lipid peroxidation is a change in the fluidity of membranes and hence a change in function of membrane-bound enzymes (Kretzschmar and Müller, 1993).

Glutathione is synthesised as GSH via two enzymatic steps. Firstly, the formation of γ-glutamylcysteine is catalysed by γ-glutamylcysteine synthase from L-glutamate and L-cysteine. Subsequently, GSH synthase adds L-glycine to form GSH. The first step is rate limiting as it is dependent on cysteine availability and negative feedback by GSH competes for the L-glutamate binding site on γ-glutamylcysteine synthase (Locigno and Castronovo, 2001).

In Chapter 7, sulphide-stimulated glutathione production in *Arctica islandica* was demonstrated. The results showed that intracellular glutathione concentrations increased as sulphide exposure concentration increased. GSSG injected into rats prior to an H_2S challenge significantly reduced mortality when compared to control animals pre-treated with saline. However, it was demonstrated that under anaerobic

conditions the GSSG/sulphide mixture was still toxic, suggesting that oxygen is a prerequisite of irreversible oxidation of sulphide (Smith and Abbanat, 1966). The relationship between glutathione and H_2S was investigated by Vismann (1991a), but unfortunately the effects of sulphide on glutathione were not separated from the effects of hypoxia, and hence the results were ambiguous.

The aim of this experiment was to investigate the oxidation of sulphide by GSSG *in vitro* under normoxic and hypoxic conditions. The hypothesis was that GSSG mediates chemical oxidation of H_2S under normoxic conditions, and at a slower rate under hypoxic conditions.

Materials and Methods

The sulphide stock solution (1.2 g per 100 ml; ± 50 mM) was prepared from $Na_2S.9H_2O$ (Sigma, S-4766 Assay 100%) every day prior to use and maintained under a nitrogen atmosphere to prevent oxidation. The exact concentration of the stock solution was determined using iodometric back titration (Parsons *et al.*, 1984), and colorimetric determination of sulphide modified from Parsons *et al.* (1984) was used to monitor the oxidation of sulphide with time during experimentation, as discussed in Chapter 2. The pH of the sulphide solution was adjusted to pH 8 prior to use, using 6N HCl. Although pH 8 is a little higher than intracellular pH values, it was used since glutathione was found not only in the tissues and in the blood, but also in the mantle water of *A. islandica*, (Chapter 5). The choice of pH 8 therefore represented a compromise between the intracellular pH, blood pH and seawater pH. All experiments were conducted at room temperature.

Since there were no protocols available that were applicable to these experiments, it was necessary to develop a new protocol that fitted the needs of this series of experiments, which would not be beyond budget. Due to the prohibitive cost of both GSH and GSSG free acids, it was necessary to keep the volumes used for each experiment as small as possible. Glass HPLC auto-sampler tubes (1.5 ml) with a flat bottom were utilised throughout. A small magnetic flea was inserted into the tube and covered with 0.5 ml liquid paraffin. A small rubber bung pierced with two plastic capillary lines was fitted to the HPLC tube. The diameter of the capillary lines was sufficient to allow a hypodermic needle (Microlance 3; $25G^{5}/_{8}$ 0.5 x 16) to pass into the HPLC tube.

A latex glove was attached to a Tygon thick walled tube (Diameter = 0.3mm) and filled with nitrogen. A hypodermic needle (Microlance 3; $25G^{5}/_{8}$ 0.5 x 16) was securely attached to the Tygon tube, and passed into the HPLC tube through the bung. The HPLC tube was then fixed onto a magnetic stirrer using Blue-tack, and nitrogen bubbled at a very slow rate for 10 minutes through the paraffin in order to displace all oxygen in the HPLC tube. The latex glove was used because the flow control from the nitrogen cylinder was not sufficiently sensitive to ensure the delivery of very low flow rates of nitrogen. A diagrammatic representation of the apparatus that was used is shown in Fig. 8.1.

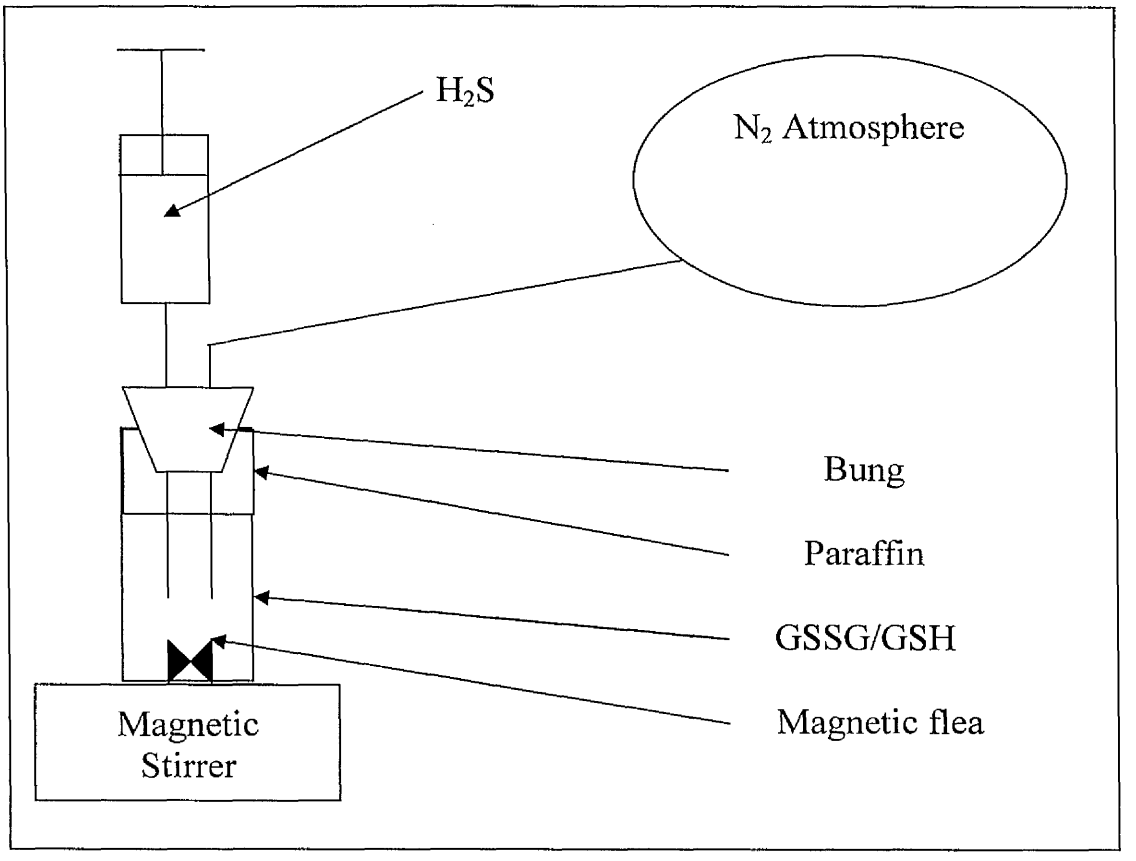

Figure 8.1: Diagrammatic representation of the apparatus used to measure the oxidation of sulphide under normoxic and hypoxic conditions in the presence of GSSG and GSH.

For the experiments under hypoxic conditions, either GSSG (Sigma G-6654 Assay 98%) or GSH (Sigma G-6529 Assay 98 – 100%) was added to oxygen-free distilled water under a nitrogen atmosphere, to obtain a concentration of 60 µM. In order to use biologically relevant concentrations of GSSG and GSH, the average dry weight concentrations of glutathione in the heart, mantle, foot and gill upon exposure to 1000 µM sulphide was used (Chapter 7). Hence 300 $\mu mol.g^{-1}$ dry weight, as described in Chapter 7, is approximately 60 $\mu mol.g^{-1}$ wet weight or 60 µM.

After mixing on a magnetic stirrer, 1 ml of the oxygen free distilled water and GSSG or GSH solution was removed with a hypodermic syringe and slowly injected into the

bottom of the HPLC tube in order to displace the paraffin. The magnetic stirrer was then started at a slow rate to mix the oxygen free distilled water and glutathione solution, but not disturb the water/paraffin interface. Nitrogen was bubbled through the solution at a very slow rate to ensure a positive pressure inside the HPLC tube and prevent interaction with atmospheric oxygen. For the experiments under normoxic conditions, oxygenated distilled water was used instead of the oxygen free distilled water.

Sulphide stock solution was injected into the HPLC tube containing the glutathione and distilled water solution to make an initial sulphide concentration of 1000 μM. A sample (50 μl) was immediately taken and analysed for sulphide content using the colorimetric method modified from Parsons *et al.* (1984) as described in Chapter 2. Samples (50 μl) were taken at 15 second intervals for 2 minutes and the changes in sulphide concentration determined as described above. Unfortunately, H_2S is highly unstable in the presence of oxygen. Therefore, an H_2S blank was run under both hypoxic and normoxic conditions so that any spontaneous oxidation could be calculated and subtracted from oxidation mediated by glutathione.

Under normoxic conditions the oxidation of sulphide in the presence of GSSG (60 μM, 120 μM and 180 μM) and GSH (60 μM and 120 μM) was examined. Under hypoxic conditions the oxidation of sulphide in the presence of GSSG (60 μM) and GSH (60 μM), was examined.

Statistical Analysis

All raw data were analysed using SPSS Version 9. An analysis of covariance was used to display significant differences ($p < 0.05$) in the glutathione-mediated oxidation of sulphide under differing experimental conditions.

Results

Initially, the effect of sulphide oxidation in the presence of GSH (60 μM and 120 μM) was examined (Fig. 8.2). The log rate of sulphide oxidation in the presence of 60 μM GSH, and 120 μM GSH decreased linearly when plotted against log time. There was no significant difference in the effect of the log velocities of sulphide oxidation with log time (slope) at either GSH concentration ($F_{1, 8}$ = 4.84; p = 0.06). Similarly, the initial log velocities (y-intercept) did not vary significantly between the two concentrations of GSH ($F_{1, 9}$= 1.8; p = 0.21). Thus there was no significant effect of GSH concentration on the rate of sulphide oxidation.

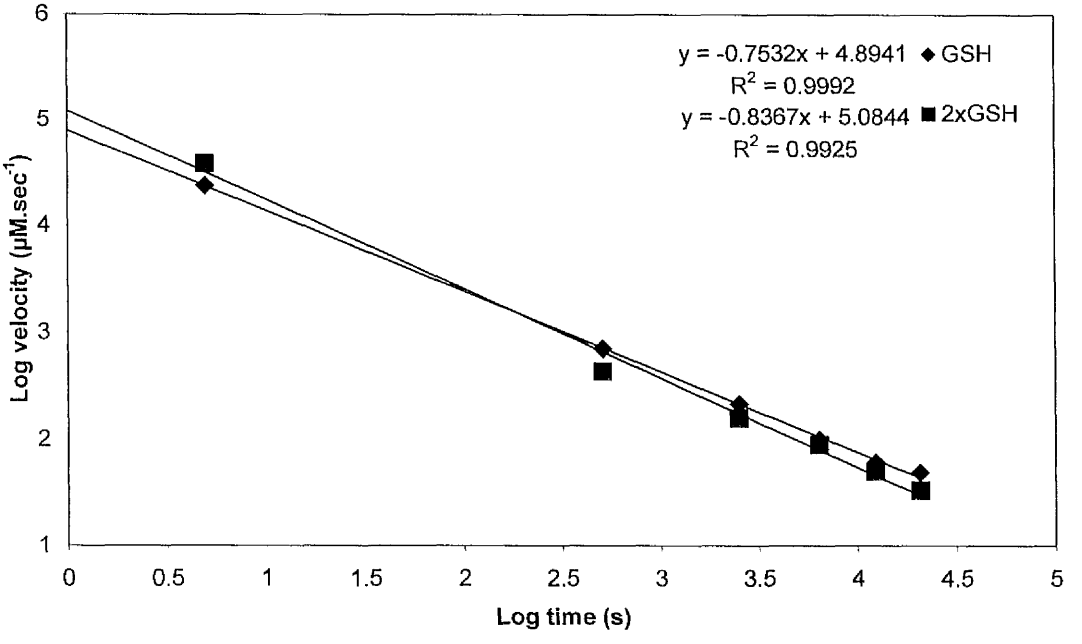

Figure 8.2: The velocity of sulphide oxidation in the presence of 60 μM GSH, and 120 μM GSH. The regression equations and R^2 values for each line are displayed on the figure.

The rate of sulphide oxidation in the presence of 60 μM, 120 μM and 180 μM GSSG was quantified. A linear relationship between the log rate of sulphide oxidation in the presence of all three concentrations of GSSG and log time was obtained (Fig. 8.3).

There was a significant increase in the log velocities of sulphide oxidation with log time (slope) between the addition of 60 μM GSSG and 120 μM GSSG ($F_{1, 8} = 26.53$; $p = 0.001$), and also between the addition of 60 μM GSSG and 180 μM GSSG ($F_{1, 8} = 23.23$; $p = 0.001$). There was no significant change in the log velocities of sulphide oxidation with log time between the addition of 120 μM GSSG and 180 μM GSSG ($F_{1, 8} = 0.90$; $p = 0.37$). However, the initial log velocities (y-intercepts) increased significantly between the addition of 120 μM GSSG and the addition of 180 μM GSSG ($F_{1, 9} = 896.22$; $p < 0.001$). As the slopes varied between the addition of 60 μM GSSG and both 120 μM GSSG and 180 μM GSSG it is not possible to say that the lower initial velocity (y-intercept) is statistically significantly different upon the addition of 60 μM GSSG. But visual inspection indicates that the initial velocity upon the addition of 60 μM GSSG and both 120 μM GSSG and 180 μM GSSG is greater than the difference between the addition of 120 μM GSSG compared to the addition of 180 μM GSSG.

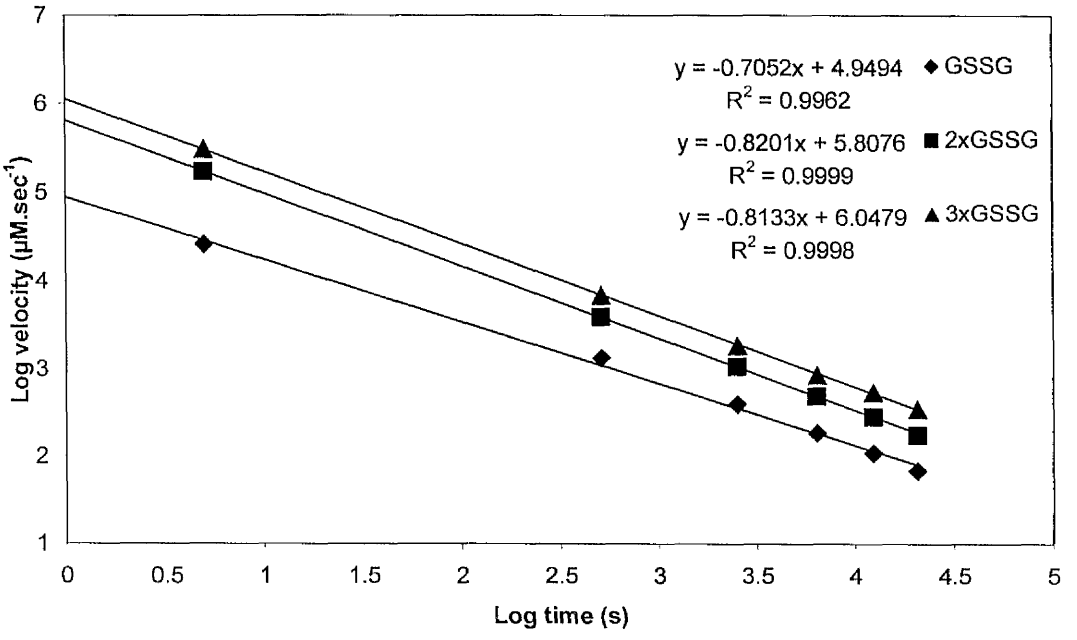

Figure 8.3: The velocity of sulphide oxidation in the presence of 60 μM GSSG, 120 μM GSSG and 180μM GSSG. The regression equations and R^2 values for these relationships are displayed on the figure.

If the oxidation of sulphide in the presence of 60 μM GSH is compared to the oxidation of sulphide in the presence of 60 μM GSSG, the relationship between the log velocities of sulphide oxidation with log time under normoxic conditions is not significantly different ($F_{1, 8}$ = 3.95; p = 0.08). However, the initial log velocities (y-intercepts) increased significantly between the addition of 60 μM GSH and the addition of 60 μM GSSG ($F_{1, 9}$ = 37.09; p < 0.001). Thus GSSG significantly increased the rate of sulphide oxidation when compared to a similar concentration of GSH.

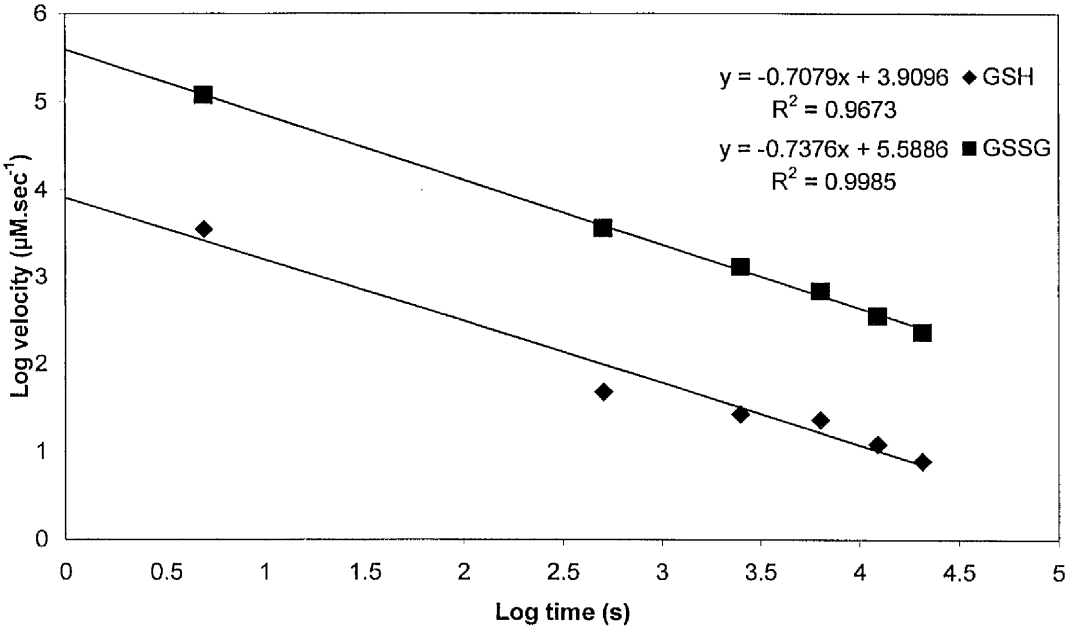

Figure 8.4: The velocity of sulphide oxidation in the presence of 60 µM GSH and 60 µM GSSG. The regression equations and R^2 values for these relationships are displayed on the figure.

The oxidation of sulphide under hypoxic conditions in the presence of 60 µM GSH and 60 µM GSSG was also examined (Fig. 8.4). The log velocities of sulphide oxidation decreased linearly with increasing log time in the presence of 60 µM GSH and subsequently 60 µM GSSG. The log velocities of sulphide oxidation with log time in the presence of 60 µM GSH, compared to 60 µM GSSG were not significantly different ($F_{1, 8} = 0.198$; $p = 0.67$). However, the initial log velocities (y-intercepts) increased significantly between the addition of 60 µM GSH and the addition of 60 µM GSSG ($F_{1, 9} = 415.60$; $p < 0.001$) under hypoxic conditions. Thus GSSG significantly increased the rate of sulphide oxidation when compared to a similar concentration of GSH under hypoxic conditions.

Discussion

Throughout the experiments described above the free acid form of GSH and GSSG were used. The pH of the distilled hypoxic or normoxic water would therefore be acidic upon the addition of glutathione. However, the sulphide solution is a base solution, and although the pH of the sulphide solution was adjusted to pH 8, the resulting pH of the glutathione and sulphide solution would still increase. Small changes in pH can significantly affect the ratio of glutathione between the oxidised and reduced form (Keire *et al.*, 1992). Hence at higher pH values, GSSG formation would be favoured, and at lower pH values GSH formation would occur. As a result, the increase in pH upon the addition of sulphide to the GSH and distilled water solution would cause the formation of GSSG. Upon the addition of sulphide to 60 μM GSH sulphide oxidation occurred. But the initial rate of sulphide oxidation was significantly lower than upon addition of sulphide to 60 μM GSSG. Since there was no significant difference in the subsequent rates of sulphide oxidation with time (slopes) it is probable that the sulphide oxidation displayed upon the addition of sulphide to the GSH solution is via GSSG formed as a consequence of increasing pH. Doubling the GSH concentration had no effect on either the initial rate of sulphide oxidation or the rate of sulphide oxidation with time which again suggests GSH is not able to directly oxidise sulphide.

Upon the addition of sulphide to 60 μM GSH sulphide oxidation occurred, even under hypoxic conditions. But the initial rate of sulphide oxidation was significantly lower than upon addition of sulphide to 60 μM GSSG also under hypoxic conditions. This suggests that GSSG is mediating sulphide oxidation during hypoxia. But the benefit of GSSG mediated sulphide oxidation under hypoxic conditions *in vivo* is suspect. Injecting mice with normoxic GSSG has been shown to protect mice against sulphide

poisoning. But a hypoxic GSSG sulphide solution was still toxic to the mice (Smith and Abbanat, 1966), which led the authors to conclude that, for irreversible sulphide detoxification, oxygen needed to be available.

Addition of sulphide to the 60 μM, 120 μM and 180 μM GSSG solutions led to a significant increase in the sulphide oxidation rates as GSSG concentration increased. Therefore, it is possible to surmise that GSSG directly stimulates sulphide oxidation. The oxidation of sulphide via GSSG and not GSH suggests that it is the disulphide bridge that is cleaved by sulphide as suggested by Smith and Abbanat (1966). It is probable that the GSSG reacts with the HS^- ion species of sulphide as suggested by Bagarinao and Vetter, 1989.

GSSG oxidation in the cell via glutathione reductase monopolises NADPH pools during challenge with reactive oxygen species (ROS) in order to reduce GSSG to GSH, the main scavenger of ROS in the cell. A challenge with sulphide during periods of normoxia would result in the irreversible oxidation and hence detoxification of sulphide by GSSG. However, GSSG would be reduced to GSH as a consequence, hence bypassing the glutathione reductase and NADPH step of the glutathione redox cycle. In the glutathione redox cycle the rate limiting step is the reduction of GSSG to GSH due to the comparatively low enzyme activity rate of glutathione reductase compared to glutathione peroxidase, and the limited availability of the energy source NADPH to drive glutathione reductase activity (Jaeschke, 1990). Therefore, during periods of oxidative stress sulphide exposure has two distinct benefits for the cell. Firstly, the reduction of GSSG to GSH without utilising NADPH confers an energy benefit on the cell, and secondly the rate of reduction from GSSG to GSH is increased, which increases the ability of the cell to deal with an oxidative challenge from ROS.

In Chapter 2, sulphide exposed *A. islandica* and *Mytilus edulis* increased total tissue volume to inner shell volume ratios compared to *A. islandica* and *M. edulis* from the same habitat not exposed to sulphide. The net gain in total tissue volume was attributed to an unknown energy benefit derived from sulphide exposure. It is possible that the energy benefit gained by sulphide reducing GSSG to GSH may be the reason for the higher condition indices found in *A. islandica* and *M. edulis* exposed to sulphide.

Chapter 9

General Discussion

The aims of this thesis were to examine the effects of sulphide on two species of bivalve, *Arctica islandica* and *Mytilus edulis*. The two species were selected as an example of a sulphide tolerant species, and a not-tolerant species, respectively. However, both species are tolerant of hypoxia (Theede *et al.*, 1969; Bayne, 1971; Coleman, 1974; Holwerda and De Zwaan, 1979).

All the experiments were designed to maintain aerobic conditions throughout, in an attempt to separate the effects of sulphide from those of hypoxia. Hence, during each experiment the oxygen concentration was monitored to ensure that it was not affected by either exposure to sulphide or by the respiration of the bivalves. One of the problems faced when examining the effects of sulphide on marine invertebrates is the propensity of sulphide to spontaneously oxidise in the presence of oxygen. Maintaining a constant sulphide concentration during each experimental run is of paramount importance during experimental design, as fluctuations in sulphide concentration have the potential to affect the response of the animal under investigation. Hence, the quality of the results is directly correlated to the level of control that is achieved over the concentrations of sulphide and oxygen in the experimental set-up. The half life of sulphide in the presence of oxygen can vary between 24 minutes to 65 hours, depending on factors such as pH, oxygen concentration, temperature and ionic composition (Millero, 1986). Hence in an ideal situation, constant monitoring and adjustment of these parameters would be desirable. A system has been developed by which electrodes are integrated into a

computer system, via digital/analogue input and output boards, allowing feedback control of oxygen tension, pH and sulphide (Vismann, 1996b). Although this system is ideal for sulphide exposure experiments involving marine invertebrates, the cost of such a system is relatively high. Hence, during the present study a slightly different approach was used, which although adapted to suit each individual experiment, was basically a flow-through system. Thus the exact sulphide concentration could be achieved, without affecting the oxygen concentrations whilst the pH was kept constant. The obvious exception is Chapter 8, where it was the rate of oxidation of sulphide that was measured in a closed system. The reliability of each system was rigorously tested to ensure that a constant sulphide concentration could be maintained without compromising pH or oxygen concentration. Thus, the use of flow-through systems was a reliable method of exposing marine invertebrates to sulphide under tightly controlled conditions.

Initially the LT_{50} values for both *A. islandica* and *M. edulis* were established (Chapter 2). It was apparent from the results of the LT_{50} experiments that the *A. islandica* from the sulphide-rich habitat had longer survival times than those from a low-sulphide habitat. The difference in survival times suggests that it may be possible to acclimatise *A. islandica* to sulphide. There was also a significant difference in the average shell height of the *A. islandica* from both areas, with the mean height of the *A. islandica* from the low-sulphide area being almost double the size of those from the sulphide-rich area. However, the reason behind the size discrepancy is unknown, but is hypothesised to be due to periodic mass mortalities in the area as a result of extended periods of sulphide exposure and anoxic conditions caused by a combination of stratification in the summer months, and the eutrophication of a high organic load in the sediment (Baden *et al.*, 1990; Eriksson and Baden, 1997). It seems possible that the population of *A. islandica* at this location (Laholm Bay) is

derived from subsequent spat settlements, which are sporadic in *A. islandica*, and a number of years may pass between spat settlements (Kennish and Lutz, 1995).

Further analysis of the LT_{50} survivors showed that the condition index (shell meat volume to inner shell volume ratio) increased consistently with sulphide exposure in both *A. islandica* and *M. edulis*. These results were unexpected and may indicate that the bivalves are able to utilise the reducing potential of sulphide as an energy source as suggested by Völkel and Grieshaber (1994, 1996, 1997). However, the mechanism that is responsible for transferring the electrons from sulphide remains elusive.

Experiments were carried out to examine the effect of sulphide on the ventilation rate of *M. edulis* (Chapter 3). During sulphide exposure, the change in ventilation rate should be indicative of modifications in the physiological response of *M. edulis* to sulphide exposure. It was immediately apparent that *M. edulis* remained open and continued to ventilate at all the sulphide concentrations tested, and seemed either unable to detect sulphide in the water column, or simply did not respond to sulphide exposure. Furthermore, the ventilation rates of *M. edulis* appeared to be under direct physiological control, adding to the weight of evidence presented by other researchers on the degree of control exerted by *M. edulis* on physiological adaptations to a changing environmental stimulus (Cranford, 2001; Widdows, 2001). Unexpectedly, the greatest inhibition of ventilation rate was upon exposure to the lowest sulphide concentration. Subsequently, increases in sulphide exposure concentration had less effect on the inhibition of ventilation, to the extent that the mean ventilation rates of the *M. edulis* exposed to the highest concentration of sulphide were only slightly lower than the pre-exposure (control) rates. These results suggested that *M. edulis* was maintaining aerobic respiration in the presence of sulphide.

There was, however, the concern that other effects of sulphide toxicity, such as the inhibition of nerve and muscle tissue (Julian *et al.*, 1998) may have affected the ventilation rates. Thus, experiments were designed to examine the physiological response of isolated hearts from *M. edulis* to sulphide exposure (Chapter 4). The use of isolated hearts would limit the influence of nerve tissue and direct control on the physiological response of the hearts, which would enable changes in heart rate to be correlated to changes in metabolic rate.

The hypothesis raised by the LT_{50} experiments on *A. islandica*, which indicated that pre-exposure to low concentrations of sulphide increased sulphide resistance, could therefore also be examined in *M. edulis*. The results indicate that it is the H_2S ion species of sulphide that elicits a response in the isolated hearts, which adds evidence to that presented by Vismann (1996a), who showed that sulphide toxicity is dependant on the concentration of the H_2S ion species. The results of the isolated heart experiments also indicated that *M. edulis* could be acclimatised to sulphide, as surmised from the LT_{50} results for *A. islandica* (Chapter 2). The isolated hearts from the *M. edulis* acclimatised to sulphide contracted at a significantly higher rate upon exposure to sulphide. The isolated hearts consist of smooth unstriated muscle (Motley, 1933). Hence the effect of sulphide appears to occur at an intracellular level, suggesting that a response to sulphide exposure is induced. Because increased heart rates are frequently indicative of elevated rates of aerobic respiration, the acclimatisation process appears either to protect against the inhibitory effects of sulphide on aerobic respiration, or promote the production of a sulphide insensitive enzyme that replaces the inhibited cytochrome c oxidase.

Thus, the results of the two chapters that examine physiological changes to sulphide exposure, the first in intact *M. edulis* (Chapter 3) and the second using isolated hearts from *M. edulis* (Chapter 4), both present evidence that could be interpreted as

indicating that *M. edulis* maintains elevated metabolic rates in the presence of sulphide. Hence in the presence of sublethal concentrations of sulphide, *M. edulis* appears to maintain aerobic respiration. Aerobic respiration in the presence of sulphide has been demonstrated in the marine echiuran worm, *Urechis caupo* (Eaton and Arp, 1993). However, it is commonly perceived that marine invertebrates only briefly maintain aerobic respiration in the presence of sulphide until cytochrome c oxidase is inhibited, at which point it is energetically more viable to switch to anaerobic metabolism (Vismann, 1991b; Oeschger and Vetter, 1992; Grieshaber *et al.*, 1992; Jahn *et al.*, 1996; Grieshaber and Völkel, 1998).

If aerobic respiration increases in *M. edulis* in the presence of sulphide under normoxic conditions, there should be no evidence of anaerobic respiration. It is only possible to demonstrate anaerobiosis directly if the end products of anaerobiosis are quantified (Grieshaber *et al.*, 1994). In bivalves, although other compounds are known to be produced, succinate is commonly used as an indicator of anaerobiosis (de Zwaan and Wijsman, 1976; Grieshaber *et al.*, 1994). Hence, the end-products of anaerobiosis, including succinate, were quantified from both *A. islandica* and *M. edulis* exposed to sulphide under normoxic conditions. The concentration of succinate in selected tissues of intact *M. edulis* and *A. islandica* showed no significant change upon exposure to increasing concentrations of sulphide. Thus there was no evidence of anaerobiosis. It appears, therefore, that the intact *M. edulis* and *A. islandica* did not switch to anaerobiosis during exposure to sublethal concentrations of sulphide.

Sulphide partially reverses the aerobic direction of succinate dehydrogenase activity from the oxidation of succinate to fumarate, to the reduction of fumarate to succinate. The activity of fumarate reductase is likewise altered; some fumarate will be reduced to succinate instead of *vice versa*, under normoxic conditions (Chapter 6). Hence,

contrary to established opinion, succinate is not a reliable indicator of anaerobiosis in the presence of sulphide, due to the effect of the redox potential of sulphide on succinate dehydrogenase and fumarate reductase. Overall, the Krebs cycle continues in an aerobic direction in the presence of sulphide, with the net oxidation of succinate to fumarate, due to the higher redox potential of oxygen compared with that of sulphide. However, under conditions of increasing hypoxia, it is possible that a critical point will be reached, at which sulphide will actively promote the production of succinate from fumarate.

Succinate concentrations in isolated hearts from sulphide acclimatised *M. edulis* were compared with those from *M. edulis* not acclimatised to sulphide. The isolated hearts from the sulphide acclimatised *M. edulis* contained significantly lower concentrations of sulphide than those from the *M. edulis* that were not acclimatised to sulphide. These results are interesting, as they are somewhat different from those displayed by the intact *M. edulis*. Intact *M. edulis* displayed direct control of ventilation rates (Chapter 3). The advantages of direct control is that there are more sensitive adaptations of ventilation to changing environmental parameters displayed by the animal, which promotes a more efficient response. It is possible, therefore, that the direct control of ventilation, displayed by intact *M. edulis*, but not by isolated tissues (Chapter 3), increases the effectiveness of the response of *M. edulis* to sulphide exposure. Thus, tissues from intact *M. edulis*, unlike those from isolated hearts, show no significant differences in the accumulation of anaerobic end-products, and therefore no evidence of anaerobiosis.

If anaerobiosis does not occur in *M. edulis* in the presence of sulphide (under normoxic conditions), it is possible that aerobic respiration continues. Therefore the sulphide must either be oxidised to prevent the inhibition of cytochrome c oxidase by sulphide, or this part of the respiratory chain must be bypassed. In both instances,

monitoring oxygen consumption would demonstrate whether aerobic respiration continues under these conditions. The oxygen consumption of isolated hearts from *M. edulis* increased significantly as the sulphide exposure concentration was increased. Hence, it would appear that *M. edulis* maintains aerobic respiration, and an aerobic electron flow through the Krebs cycle, in the presence of sublethal concentrations of sulphide. Since succinate does not accumulate in intact *A. islandica*, and they display an even higher resistance to sulphide under aerobic conditions than *M. edulis* (Chapter 2), it is likely that this is also true of *A. islandica*.

There is some evidence that the Complex II enzymes, succinate dehydrogenase and fumarate dehydrogenase are inhibited by sulphide (Chapter 6). Succinate dehydrogenase is not only active in the Krebs cycle, but is the initial electron carrier in the respiratory chain. Thus, inhibition of succinate dehydrogenase by sulphide has the potential to inhibit the respiratory chain at the initial as well as the terminal step. If an alternative terminal oxidase exists and can remain active whilst cytochrome c oxidase is inhibited by sulphide, as argued by Völkel and Grieshaber (1992, 1996, 1997), it is conceivable that there would also need to be an enzymatic step to bypass the possible inhibition of succinate dehydrogenase. However, the alternative is that sulphide is oxidised, and therefore detoxified, prior to reaching and inhibiting cytochrome c oxidase by a "sulphide oxidase". A thorough examination of the cellular oxidative end products of sulphide in both *M. edulis* and *A. islandica* was initiated to examine the existence of a possible sulphide oxidase or other enzyme involved in sulphide detoxification.

Thiosulphate is the main product of sulphide detoxification in marine invertebrates (Gorodezky and Childress, 1994; Johns *et al.*, 1997). Thiosulphate accumulated in the tissue samples from *M. edulis* and *A. islandica* in the presence of sulphide (Chapter 5 and 7), confirming that thiosulphate is also the main product of sulphide

detoxification in these two species of bivalve. The concentrations of sulphite were significantly lower in *A. islandica* than in *M. edulis*. Sulphite is produced as a transient in the two step oxidation of sulphide to thiosulphate (O'Brien and Vetter, 1990). Because sulphide oxidation to thiosulphate is more efficient in invertebrates from sulphidic habitats (Doeller *et al.*, 1999b), lower concentrations of sulphite are expected in the more sulphide tolerant *A. islandica* than in *M. edulis*.

The concentrations of sulphide in the tissues and blood of both *A. islandica* and *M. edulis* are similar (Chapter 5 and 7), and increase as sulphide exposure concentrations increase. These concentrations are also similar to those found by other authors in a range of sulphide tolerant species (Oeschger and Vetter, 1992; Wilmot and Vetter, 1992; Völkel and Grieshaber, 1994). This is significant as it appears that none of these species is able to exclude sulphide from entering the soft tissues (Chapter 5). It is somewhat surprising, therefore, that both *A. islandica* and *M. edulis* do not simply close their valves upon exposure to sublethal concentrations of sulphide. The monitoring of the ventilation rates from *M. edulis* upon exposure to sulphide, seemed to indicate that this bivalve was unable to detect sulphide at concentrations between 0 µM and 1200 µM, as discussed above, which may be the reason that *M. edulis* does not close its valves in the presence of sulphide. It is possible that the same is true for *A. islandica*.

The concentrations of the amino acid, cysteine and the tri-peptide, glutathione were monitored along with those of sulphide, sulphite, and thiosulphate, because it has been previously noted that both are active in cellular protection mechanisms (Tietze, 1969; Jaeschke, 1990; Reed, 1990; Locigno and Castronovo, 2001). The concentrations of cysteine were higher in those *M. edulis* that had not been acclimatised to sulphide, when compared to *A. islandica* and sulphide acclimatised *M. edulis* (Chapter 5). Cysteine is involved with the reactivation of succinate

dehydrogenase (Smith and Abbanat, 1966), and possibly other proteins, after their inhibition by sulphide under aerobic conditions. Hence lower concentrations of cysteine would be expected in sulphide acclimatised *M. edulis* due to the depletion of cysteine after repairing disulphide bridges cleaved by sulphide. In addition, under hypoxic conditions, cysteine cleaves disulphide bridges and would exacerbate the damage to protein structure caused by sulphide exposure.

Glutathione concentrations were significantly higher in the *M.* edulis that had not been acclimatised to sulphide, when compared to *A. islandica* and sulphide acclimatised *M. edulis* (Chapter 5). It is possible that the differences in glutathione concentration are due to glutathione activity in sulphide detoxification. The results of the study presented in Chapter 5 indicated that cysteine and glutathione may be involved in the response of *M. edulis* to sulphide exposure. However, the differences in thiol production between the sulphide acclimatised *M. edulis* and those not acclimatised to sulphide was not clearly visible upon exposure to sulphide. Experiments were subsequently designed using *A. islandica*, in an attempt to define better the effects of the acclimatisation process on thiol production in the presence of sulphide.

The *A. islandica* with the highest sulphide tolerance, collected during the course of this study, were from Laholm Bay in the Kattegat on the west coast of Sweden (Chapter 2). Laholm Bay is a sulphide-rich hypoxic habitat characterised by fine mud. Thiol production in these *A. islandica* was compared to that in *A. islandica* from Kockholmen. Kockholmen is situated north of Laholm Bay in the Skagerrak, and is a sulphide-free normoxic habitat characterised by coarse sand. The concentrations of cysteine in the blood of the *A. islandica* from Kockholmen were significantly higher than those in the *A. islandica* from Laholm Bay. Furthermore, upon exposure to sulphide, the cysteine concentrations in the blood of the *A. islandica* from

Kockholmen displayed a significant linear increase, unlike those in the blood of the *A. islandica* from Laholm Bay, which remained constant. These findings support those reported in Chapter 5 and add credibility to the theory that cysteine is involved in the re-activation of succinate dehydrogenase after its inhibition by sulphide (Smith and Abbanat, 1966). This process is known to occur *in vitro* but has not yet been demonstrated *in vivo*.

It has been hypothesised that 1 mole of oxidised glutathione (GSSG) can detoxify 0.5 moles of sulphide (Smith and Abbanat, 1966). Sulphide-stimulated glutathione production was demonstrated in the *A. islandica* from both Laholm Bay and Kockholmen (Chapter 7). There was sufficient glutathione present in the tissues of the *A. islandica* from both Kockholmen and Laholm Bay to detoxify all the sulphide that accumulated in the tissues upon exposure to 200 µM – 1200 µM sulphide. Subsequent experiments were conducted *in vitro* to ascertain the effect of GSSG on sulphide.

GSSG mediates the oxidation of sulphide, and becomes reduced to the sulphydryl form, GSH, in the process. This process appears to occur under normoxic conditions, and to a lesser extent under hypoxic conditions, perhaps because less oxygen is available (Chapter 8). The protective effect of the addition of GSSG during a sulphide challenge (Smith and Abbanat, 1966) does not occur under hypoxic conditions. Hence, oxygen is required for the irreversible detoxification of sulphide. However, under hypoxic conditions, animals switch to anaerobiosis, thus the detoxification of sulphide, to protect the respiratory chain, would not be necessary for survival.

Sulphide exposure has been shown to stimulate glutathione production (Chapter 7). Secondly, sufficient GSSG is produced in *A. islandica* upon exposure to sulphide to oxidise the sulphide in the tissues (Chapter 7). Thirdly, GSSG oxidises sulphide and

becomes reduced in the process (Chapter 8). And finally, oxidation of sulphide is an effective detoxification mechanism. It therefore appears that *A. islandica* uses GSSG as a "sulphide oxidase" to detoxify sulphide at a cellular level.

The reduction of GSSG to GSH by sulphide has the dual advantage of not only detoxifying the sulphide, but the reduction of the glutathione also occurs without the energy expenditure usually needed to keep the glutathione redox cycle turning. It is possible that this energy saving contributes to the increase in body condition observed in the *A. islandica* during the LT_{50} experiments (Chapter 2). There is an additional benefit. The reduction of GSSG to GSH is the rate-limiting step in the glutathione redox cycle. Sulphide-stimulated GSSG reduction increases the turnover rate of the cycle and thus makes more GSH available to deactivate reactive oxygen species (ROS).

Upon the addition of sulphide, the concentrations of thiosulphate in *A. islandica* from both Kockholmen and Laholm Bay, were significantly higher in the blood of the *A. islandica* from Laholm Bay (sulphide-rich, hypoxic site) than in those from Kockholmen (sulphide-free, normoxic site). Conversely, the concentrations of thiosulphate were significantly higher in the tissues of the *A. islandica* from Kockholmen upon exposure to sulphide, when compared to those from Laholm Bay. Thus it appears that there are different sulphide detoxification mechanisms expressed in the *A. islandica* from the two areas. The first mechanism is active in the blood of the *A. islandica* from Laholm Bay, and the second is active in the tissues of the *A. islandica* from Kockholmen. A possible explanation is that the detoxification mechanism in the blood is induced by long-term sulphide exposure. The second mechanism is a short-term detoxification mechanism active in the tissues of the *A. islandica* not acclimatised to sulphide. Although the reason for this difference remains obscure, an attractive hypothesis is that the mechanism expressed in the blood of the

A. islandica from Laholm Bay (sulphide-rich, hypoxic site) is more energy efficient than that expressed in the tissues of the *A. islandica* from Kockholmen (sulphide-free, normoxic site).

A number of authors have reported on the binding and possible detoxification of sulphide to haem-containing blood compounds (Patel and Spencer, 1963; Smith and Gosselin, 1964, 1966; Bagarinao and Vetter, 1989; Powell and Arp, 1989; Oeschger and Vetter, 1992). Furthermore, different sites of detoxification have been reported in different species (Bagarinao and Vetter, 1989; Völkel and Grieshaber, 1994; Johns *et al.*, 1997). However, there have been no other reports of differences in the site of detoxification in the same species from different habitats.

In summary, *A. islandica* were very resistant to sulphide, with an LT_{50} of 6 – 8 weeks, depending on the concentration of the sulphide, whereas *M. edulis* had an LT_{50} of only 1 – 3 weeks. However, both *A. islandica* and *M. edulis* were found to undergo an increase in body condition upon exposure to sublethal concentrations of sulphide for extended periods (Chapter 2). *A. islandica* and *M. edulis* both appear not to switch to anaerobiosis upon exposure to sublethal concentrations of sulphide (Chapter 6), and maintain an aerobic electron flow through the Krebs cycle during sulphide exposure (Chapter 6). Oxygen consumption of isolated hearts is stimulated by exposure to sulphide (Chapter 6) indicating that aerobic respiration may also be stimulated upon exposure to sulphide in intact *M. edulis* (Chapter 3). Sulphide acclimatisation at a cellular level was demonstrated in isolated hearts from *M. edulis* (Chapter 4), and there is some evidence that intact *A. islandica* can also be acclimatised to sulphide (Chapters 2 and 7).

The key adaptations to sulphide exposure which protect aerobic respiration from inhibition by sulphide and which are displayed by *M. edulis* and *A. islandica* are

sulphide-stimulated cysteine and glutathione production. Sulphide-stimulated cysteine production was demonstrated in both *A. islandica* and *M. edulis* that were not acclimatised to sulphide (Chapters 5 and 7). Cysteine is possibly involved in the repair of partially denatured proteins that were damaged by sulphide exposure. Sulphide-stimulated glutathione production was demonstrated in *A. islandica* and *M. edulis* (Chapters 5 and 7). Furthermore, oxidised glutathione (GSSG) detoxifies sulphide in *A. islandica* at an intracellular level in the gill, foot, mantle and heart tissue (Chapter 7). Glutathione-mediated sulphide oxidation was demonstrated *in vitro* under normoxic conditions, and to a lesser extent under hypoxic conditions (Chapter 8). Another sulphide detoxification mechanism was found to be active in the blood of *A. islandica* from a sulphidic habitat, which is absent or poorly expressed in *A. islandica* from a sulphide-free habitat (Chapter 7).

The results of this thesis have improved the understanding of both the effects of sulphide on *A. islandica* and *M. edulis*, and the long term and short term sulphide detoxification mechanisms expressed by these bivalves. However, there are a number of interesting points that warrant further investigation.

Since succinate dehydrogenase is inhibited by sulphide exposure, and given its close structural and functional similarities with fumarate reductase (Chapter 6), it would be interesting to examine the effect of sulphide on fumarate reductase. Fumarate reductase is structurally and functionally an ideal candidate to replace succinate dehydrogenase as the first electron carrier of the respiratory chain during sulphide exposure.

The use of GSSG to detoxify sulphide is the primary sulphide detoxification strategy in *A. islandica* at an intracellular level (Chapters 7 and 8). An investigation into the concentrations of glutathione in other sulphide-tolerant species would establish

whether this detoxification strategy is limited to *A. islandica*, or occurs in other sulphide tolerant species.

The fate of thiosulphate was not investigated during this series of experiments, but it is generally thought to be excreted (Gorodezky and Childress, 1994; Johns *et al.*, 1997; Hauschild *et al.*, 1999). However, as thiosulphate oxidation to sulphate is glutathione dependant in sulphur bacteria and in mammals (Sörbo, 1964), further investigation of the fate of thiosulphate in invertebrates in warranted.

Although sulphide-stimulated cysteine synthesis was demonstrated in the blood of the *A. islandica* from Kockholmen (sulphide-free, normoxic site), the possible role of cysteine, in the repair of proteins denatured by sulphide, needs to be defined. The sulphide detoxification mechanism present in the blood of the *A. islandica* from Laholm Bay (sulphide-rich, hypoxic site) also needs to be investigated.

The results of the studies discussed above have given new directions to follow in the attempt by researchers to monitor and characterise the effects of sulphide on the physiology and biochemistry of both *M. edulis* and *A. islandica.* By pursuing the additional experiments proposed above, it should be possible to gain a better understanding of how invertebrates survive and, in some cases, appear to thrive in potentially lethal sulphidic habitats.

Appendix 1

The sublethal effects of sulphide on *Nephrops norvegicus* (Linnaeus)

Introduction

The Norway Lobster, *Nephrops norvegicus* is a marine decapod crustacean that constructs simple burrows in soft sediments (Rice and Chapman, 1971; Field *et al.*, 1998). *N. norvegicus* forms the basis of many commercial fisheries in the North Atlantic, Irish Sea and Kattegat (Chapman, 1980; Bailey *et al.*, 1983; Hagerman and Uglow, 1985; Hosie *et al.*, 1991). The distribution of *N. norvegicus* is affected by fishing effort and by environmental conditions (Chapman, 1980; Chapman and Bailey, 1987; Tully and Hillis, 1995; Marrs *et al.*, 1996). In some areas, such as the Kattegat, *N. norvegicus* is subjected to moderate to severe hypoxia for extended periods which is caused by eutrophication processes combined with seasonal stratification (Baden *et al.*, 1990; Hagerman *et al.*, 1990; Hosie *et al.*, 1991). During these hypoxic episodes, *N. norvegicus* emerge from the protection of their burrows and are more susceptible to predation, and capture by bottom trawls (Eriksson and Baden, 1997). Even if not captured in the trawl, 50% of escapees from the trawl suffer visible damage (Harris *et al.*, 1997). In addition, even if the lobsters avoid predation or capture by fishing, extended periods of low oxygen availability can affect feeding activity, which can lead to starvation (Hagerman and Baden, 1988). During periods of severe hypoxia, *N. norvegicus* become inactive (Eriksson and Baden, 1997), which is presumably to keep energy expenditure at a minimum (Helm and

Trueman, 1967; De Fur and Magnum, 1979). During these periodic hypoxic events, dead and dying *N. norvegicus* have been caught by fishing vessels (S. Baden *pers. comm.*) Hence hypoxic events, caused by eutrophication, can have a dramatic effect on the survivability and distribution of *N. norvegicus*.

Another problem faced by marine invertebrates in some habitats is the accumulation of hydrogen sulphide, which is commonly produced during eutrophication processes where it is formed as a by-product during the reduction of sulphates by sulphur-reducing bacteria as a by-product of carbon fixation (Theede *et al.*, 1969; Jørgensen, 1982a, b). Sulphide has been found to be present in muddy sediments below the oxic/anoxic interface (Fenchel and Reidl, 1970; Grieshaber *et al.*, 1992; Thiermann *et al.*, 1996; Johns *et al.*, 1997). The burrowing of *N. norvegicus* into the sediment could bring them into contact with sulphide contained in the interstitial water of the sediment. However, there are other factors that can combine to expose *N. norvegicus* to sulphide on the surface of the sediment. In the presence of oxygen, sulphide is quickly oxidised to less toxic substances. Thus sulphide accumulation occurs only under hypoxic conditions, primarily in the sediment (Hagerman *et al.*, 1996).

Hence it is primarily during hypoxic events that *N. norvegicus* may be exposed to sulphide. Sulphide appears to have an additive effect to that of hypoxia on crustaceans, and exposure to sulphide decreases their survival time when compared with hypoxia alone (Hagerman, 1998). Thus hydrogen sulphide is an important environmental parameter that can further limit the survival and distribution of *N. norvegicus*.

Preliminary research has indicated that *N. norvegicus* was able to oxidise sulphide to thiosuphate in the hepatopancreas, gill, muscle tissue and blood (Astall, 1993). Subsequently it was shown that the haemocyanin from *N. norvegicus* was able to rapidly oxidise sulphide *in vitro* (Taylor *et al.*, 1999). Several decapod crustaceans including *B. thermydron* have been used in whole animal studies to show the effect of sulphide on heart rate and scaphognathite beat frequency. Vetter *et al.* (1987) found that sulphide concentrations, up to 1.4mM, had no effect on *B. thermydron*, but other Crustacea, from non-vent habitats, showed a 40-90% decrease in heart rate and scaphognathite beat frequency, with increasing sulphide exposure concentration. This was thought to be indicative of sulphide poisoning. Thus it is tempting to speculate that while hydrothermal vent decapods have the highest sulphide tolerance, it is an ability shared by decapod crustaceans from some other habitats.

The aims of this study were to examine the effects of sulphide exposure, under normoxic conditions, on not only some aspects of the physiology of *N. norvegicus*, but also on behaviour, anaerobiosis, and the production of sulphide oxidation products. By examining all these parameters, it was hoped that a better understanding would be gained of how *N. norvegicus* integrates these different responses to deal with sulphide exposure.

Materials and Methods

Animal collection and Maintenance

N. norvegicus were collected by beam trawl from the Clyde Sea south of Little Cumbrae (55° 43` N; 4° 56` W) from a depth of approximately 80m. The lobsters were subsequently transported to the University Glasgow in seawater, where they

were maintained in a re-circulating seawater aquarium at 10 °C ± 1 °C under a 12:12 hour light: dark regime (salinity = 35 ± 1 $^{o}/_{oo}$). All the lobsters were male, as only male lobsters were present in the trawl (see Discussion) and they were not fed whilst under observation. The *N. norvegicus* were selected to be of similar size (CL = 30 - 40 mm), measured from the tip of the rostrum to the back of the carapace.

Stock sulphide solution

The sulphide stock solution (50mM) was prepared from $Na_2S.\ 9H_2O$ (Sigma, S-4766 Assay 100%) every day prior to use. Due to the highly volatile nature of sulphide in the presence of oxygen, the stock solution was maintained under a nitrogen atmosphere to prevent spontaneous oxidation. The concentration of the stock solution was determined using iodometric back titration (Parsons *et al.*, 1984). However, colorimetric determination of sulphide modified from Parsons *et al.* (1984) was used to monitor sulphide concentration during the experiments. Both methods of determining sulphide concentration are described in detail in Chapter 2. The pH of the sulphide solution was initially adjusted to pH 8 using 6N HCl, and then maintained at pH 8 by the buffering properties of the seawater.

Behavioral studies

The ability of *N. norvegicus* to detect sulphide, and the subsequent behavioural response were examined. The *N. norvegicus* were selected to be of similar size (CL = 37.2 ± 4.3 mm). The *N. norvegicus* were subsequently divided into two groups, and the antennules removed from one group. Individual lobsters were placed in a glass aquarium (1m x 1m) that had been divided into two equal parts, and filled to a depth of 15 cm with normoxic seawater (10 ± 1°C). The aquarium was constantly aerated. The lobsters were left undisturbed over night to acclimatize to the experimental conditions. Sulphide solution was very carefully introduced to the

animals in the area between the rostrum and the mouth parts using a hypodermic needle and a 5 ml syringe. Care was taken to cause minimal mechanical disturbance to the seawater in the aquarium. The sulphide concentrations used were 0 μM, 50 μM, 500 μM and 5000 μM. Individual *N. norvegicus*, with (n = 10) and without antennules (n = 10) were exposed to each of the sulphide concentrations, starting with the lowest and progressing to the highest. The initial response of the lobsters to each of the sulphide concentrations was subsequently observed and recorded. As soon as the lobsters had settled after exposure to sulphide, a higher concentration was added.

Monitoring of heart and scaphognathite rates

The effect of sulphide on the physiology of *N. norvegicus* was examined by exposing lobsters to differing concentrations of sulphide, in a flow-through apparatus maintained at a constant temperature (10 ± 1°C). Four medium sized (CL = 30 – 40 mm) male *N. norvegicus* were placed in separate Perspex tubes (length = 280 mm; internal diameter = 45 mm), and aerated seawater was pumped continuously through each tube at a slow rate (74 – 240 $ml.min^{-1}$) using a peristaltic pump (Fig. 10.1). Care was taken to ensure that all air bubbles were removed from the system. A second peristaltic pump was used to pump a stock sulphide solution into the mixing chamber that was located upstream of the tubes in which the animals were kept. By adjusting the relative flow rates of the two pumps it was possible to obtain the required sulphide concentrations within the tubes whilst still maintaining normoxic conditions. The Po_2 of the water was initially monitored in the outflow water as described above.

The scaphognathite beat frequency and heart rate of all four animals was monitored using the impedance technique (Helm and Trueman, 1967; Trueman *et al.*, 1973;

Aagaard *et al.*, 1995). Fine silver electrodes were inserted through small holes in the carapace, situated on both sides of the heart and on both sides of one of the scaphognathites, and held in place with cyanoacrylate adhesive (Taylor and Brand, 1975; Taylor, 1977; DeFur and Magnum, 1979; Styrishave and Depledge, 1996). The change in impedance between the two electrodes caused by both the heart beat and scaphognathite beat of the lobsters was monitored using an impedance monitor (Strathkelvin Instruments, Glasgow), connected to a Maclab interfaced to a Mac computer running Chart 3.4 software.

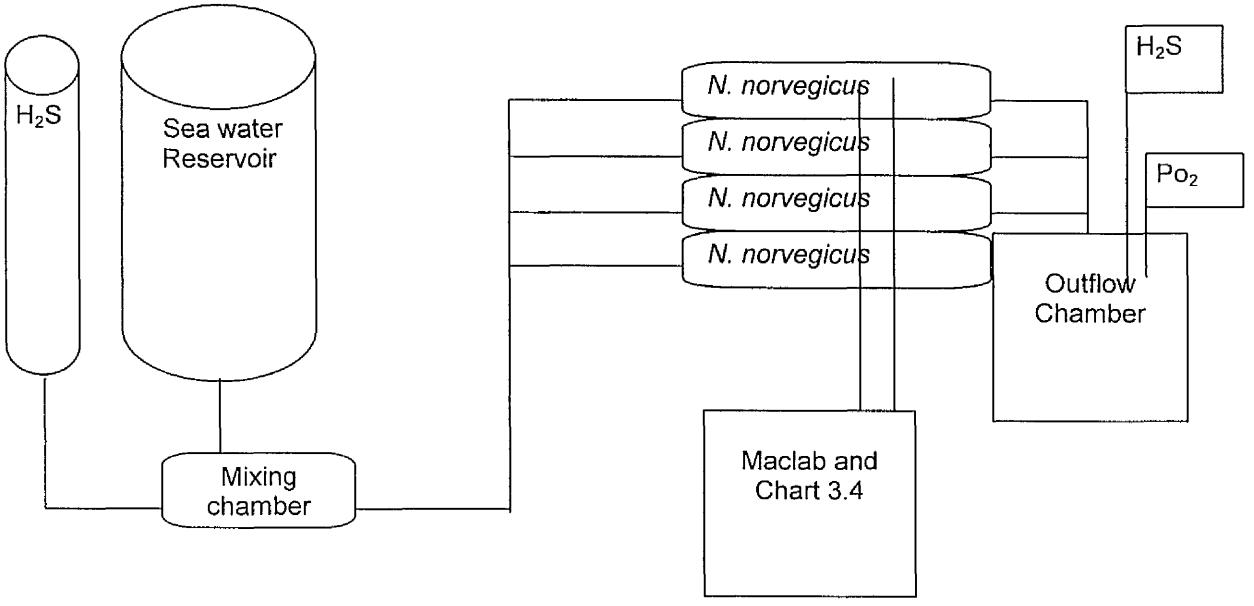

Figure 10.1: Diagrammatic representation of the closed system used to monitor scaphognathite beat frequency and heart rates of Nephrops norvegicus *exposed to selected concentrations of sulphide.*

The lobsters were left undisturbed overnight to acclimatize to the experimental conditions. Recordings of both the heart rate and scaphognathite rate of lobsters in water containing no sulphide were made for a period of 30 min. The lobsters where then exposed to increasing concentrations of sulphide (actual sulphide

concentrations measured in the outflow water were 150 μM, 609 μM, 830 μM and 1056 μM ± 10 μM) for periods of 30 min during which continuous recordings of hear rate and scaphognathite rate were made. Following exposure to the highest sulphide concentration, the second pump was turned off and the tubes were flushed with sea water containing no sulphide at the same flow rate used during the exposure period. During this recovery period, lasting a further 30 minutes, further recordings of heart and scaphognathite rate were made.

Sulphide tolerance experiments

N. norvegicus were placed separately in an exposure tank (n = 10) and a lid fitted. The lid was larger than the exposure tank with an overhanging lip, so that seawater pumped into the tank could overflow, displacing any air below the lid and preventing any interaction between the air and the seawater in the exposure tank as illustrated in Fig. 2.1.

Aerated seawater (10 ± 1 °C) was pumped continuously through the exposure tank at a slow rate (74 – 240 $ml.min^{-1}$) using a peristaltic pump. A second peristaltic pump was used to pump a stock sulphide solution into the mixing chamber located upstream of the exposure tank in which the animals were kept. By adjusting the relative flow rates of the two pumps it was possible to obtain the required sulphide concentration within the exposure tank, whilst still maintaining normoxic conditions. The Po_2 of the water was initially monitored in the exposure tank, using a microcathode oxygen electrode (Strathkelvin Instruments, Oxygen Meter 781), that had been calibrated prior to use against aerated sea water (at 10 °C), and against a solution having a Po_2 of zero (sodium sulphite in 0.01M sodium tetraborate). The actual sulphide concentration in the exposure tank water was measured using the colorimetric method described by Parsons *et al.* (1984) (Chapter 2).

The lobsters were allowed to acclimatise overnight to the experimental conditions, before a known concentration of sulphide was introduced into the tank. A steady sulphide concentration of 500 μM was maintained until 50% mortality occurred. Mortality was assumed when the lobsters no longer moved their antennules, and no longer responded to mechanical stimulation. After 50 % mortality was achieved, the survivors were sacrificed, and samples of the tail muscle and hepatopancreas were analysed for thiols as described in Chapter 5, and for anaerobic end products as described in Chapter 6.

Anaerobic end products

N. norvegicus were exposed to sulphide at different concentrations and the accumulation of the end products of anaerobiosis in the presence of sulphide was subsequently determined. During experimentation, *N. norvegicus* (n = 5) were placed in an aquarium of sulphide-free seawater (10 °C) as described in Chapter 5. A lid was fitted, and the lobsters allowed to acclimatise to the experimental apparatus for 12 hours prior to exposure to a single concentration of sulphide for 30 minutes (Fig. 2.1). The *N. norvegicus* were exposed to one of 5 concentrations of sulphide. The concentrations used were 200 μM, 500 μM, 800 μM, 1000 μM and 1200 μM. The exposure period was 30 minutes after which samples of haemolymph were rapidly removed and frozen in liquid nitrogen. The frozen haemolymph samples were prepared for HPLC and subsequently analysed for lactate, using the method developed by Wormersley *et al.* (1985) which is discussed in detail in Chapter 6.

Thiol concentrations

N. norvegicus were exposed to sulphide at different concentrations and the accumulation of thiols in the presence of sulphide, subsequently determined. *N. norvegicus* (n = 5), were placed in the same apparatus described above (Fig. 2.1.)

and exposed to one of 5 concentrations of sulphide. The concentrations used were 200 µM, 500 µM, 800 µM, 1000 µM and 1200 µM. The lobsters were exposed to sulphide for 30 minutes after which haemolymph samples were removed and immediately frozen in liquid nitrogen. To measure the concentrations of thiols (sulphite, thiosulphate, sulphide, glutathione and L-cysteine) the frozen blood samples were analysed using the bromobimane method (Völkel and Grieshaber, 1992). Subsequently, the concentrations of the thiols in the samples were determined using HPLC. The method is described in detail in Chapter 5.

Statistical analysis

Data were analysed using Minitab version 11 statistical package. Results were compared using a one-way ANOVA. The data showed a normal distribution (NS values 0,869 – 0,994), and there was no significant heterogeneity of variance (Bartlett's value < 9.49; $p < 0.05$). Tukey's pairwise comparisons were used to show significant differences ($p < 0.05$) between the different measured parameters. Since 95% confidence intervals (CI = 95%) are used as the error bars some diagrams, error bars that do not overlap indicate significant differences between the means. Other diagrams use standard errors as error bars. Unless otherwise stated, statistical significance is quoted at a 95% confidence level ($p < 0.05$).

Results

Behavioral experiments

To examine the ability of *N. norvegicus* to sense sulphide, initially, sulphide-free seawater at a similar temperature to that in the aquarium was used to establish if the lobsters would react to the small current formed by injecting the solution so close to the antennules. None of the lobsters from either group reacted to the injection of the

sulphide-free seawater if the injection process was carried out slowly. However, there was a marked difference in the responses of the two groups of lobsters to injections of sulphide. However, these results were subjective and hence difficult to quantify (Table 10.1).

Table 10.1: Behavioural observations of two groups of Nephrops norvegicus *(n = 10) exposed to sulphide, the first group with antennules, and the second with the antennules removed.*

Sulphide concentration (µM)	***N. norvegicus* with antennules**	***N. norvegicus* without antennules**
0	Reared slightly, or stepped backwards slightly	No reaction
50	Moved backwards quickly, sometimes with one tail-flip	No reaction
500	Tail-flipped away between 2 – 4 times	No reaction
5000	Frantic tail-flipping around the aquarium	No reaction

Monitoring of heart and scaphognathite rates

Changes in the heart rate and scaphognathite rate were monitored in *N. norvegicus* exposed to sulphide (0 µM – 1200 µM) for 30 minutes and subsequently to sulphide-free seawater for an additional 30 minutes. During the sulphide exposure period, the individual animals ceased all locomotory activity. Activity was resumed early on in the recovery period. The average heart or scaphognathite rates recorded at the specified time in the experiment were collated for 10 lobsters and a mean heart or

scaphognathite rate calculated (Fig. 10.6 and Fig. 10.7). The heart or scaphognathite rates displayed at time zero were the pre-exposure or control rates recorded prior to the addition of sulphide. The heart or scaphognathite rates displayed after 30 minutes reflect the mean heart or scaphognathite rates recorded during the first minute of the recovery period when sulphide was no longer pumped through the exposure tubes.

The lobsters exposed to 150μM of sulphide showed very little variation in heart rate during the exposure period, but there was an increase in heart rate during the first 15 minutes of the recovery period (Fig. 10.6), followed by a decrease in the heart rates to rates similar to the pre-exposure (control) rates. The heart rates of the lobsters exposed to 609 μM, 830 μM and 1056 μM sulphide all showed a decrease in heart rate during sulphide exposure. Subsequently, there was an increase in heart rate, after 30 minutes, at the beginning of the recovery period, to levels only slightly higher than those recorded prior to the addition of sulphide (controls). Finally, the heart rates stabilised after 15 minutes of the recovery period at similar rates to those recorded prior to exposure to sulphide (control) rates (Fig. 10.6).

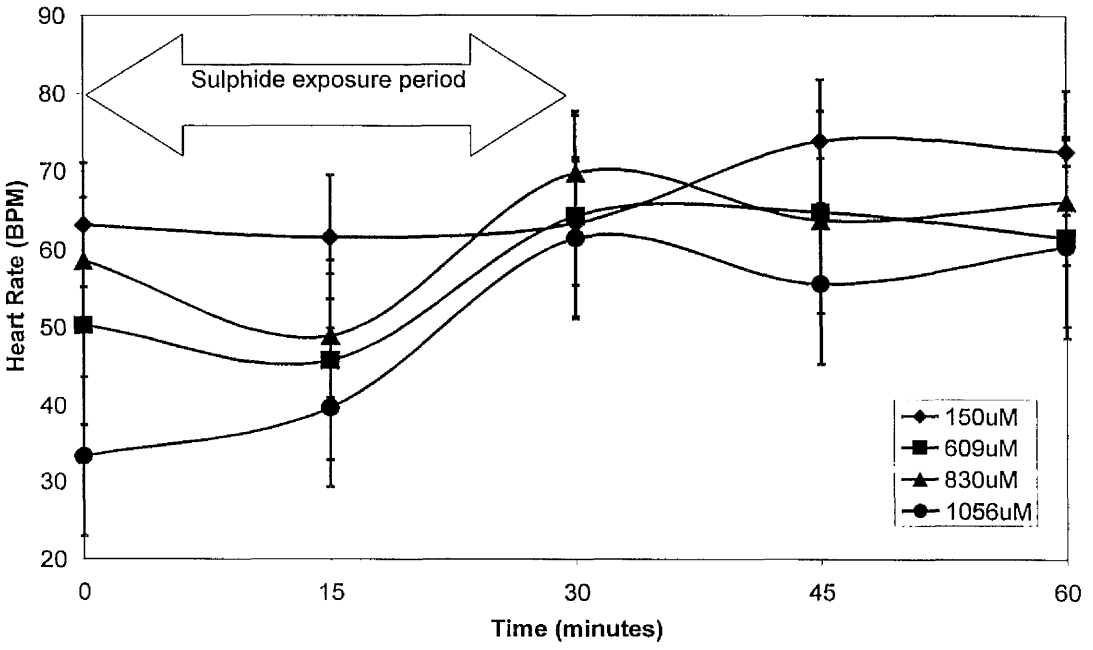

Figure 10.6: The mean heart rates of Nephrops norvegicus *exposed to increasing concentrations of sulphide for 30 minutes and subsequently allowed to recover in sulphide-free seawater for an additional 30 minutes (n = 10). Values are means ± standard error.*

The scaphognathite beat rates of the animals exposed to 150µM sulphide showed no change when exposed to sulphide, but there was a slight increase in the scaphognathite beat rate during the first 15 minutes of the recovery period. The scaphognathite beat rate then stabilised at approximately 20 BPM higher than the resting rate (Fig. 10.7). The scaphognathite beat rates of the animals exposed to 609 µM, 830 µM and 1056 µM sulphide decreased during sulphide exposure. However, at the end of the sulphide exposure period when sulphide-free water was added, the scaphognathite beat rates increased to rates slightly higher than those recorded prior to the addition of sulphide. Finally, the scaphognathite beat rates stabilised at similar rates to the pre-exposure (control) rates (Fig. 10.7).

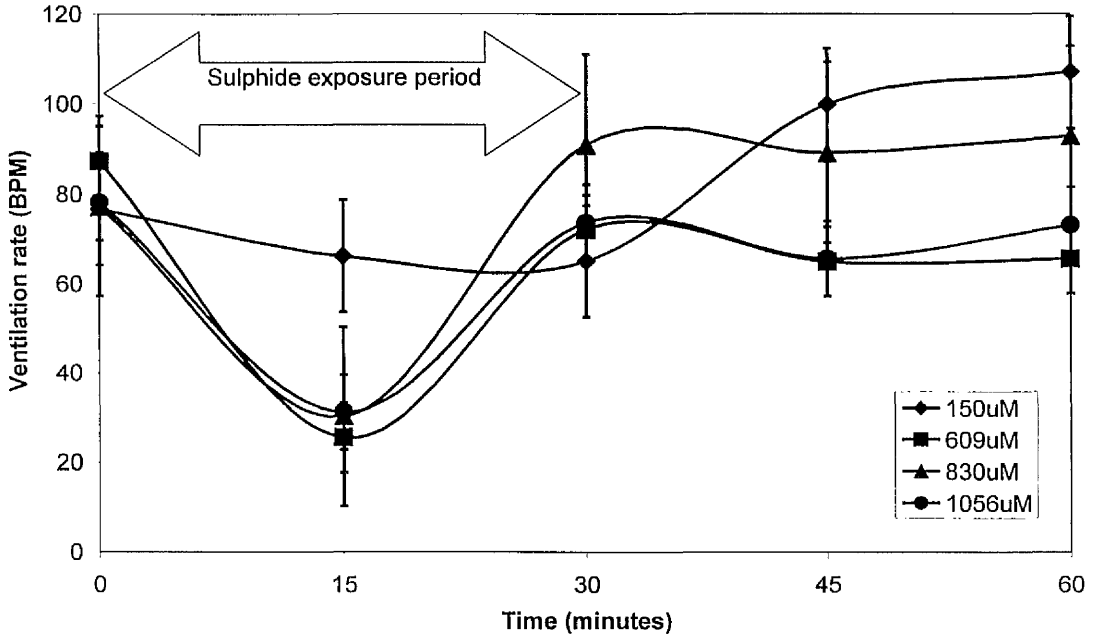

Figure 10.7: The mean scaphognathite beat rates of Nephrops norvegicus *exposed to increasing concentrations of sulphide for 30 minutes and subsequently allowed to recover in sulphide-free seawater for an additional 30 minutes. Values are means ± standard error (n = 10).*

The values of the mean heart rates (Fig. 10.6) recorded prior to the addition of sulphide (controls) were rather variable. Hence it is difficult to gauge objectively the effect of the different concentrations of sulphide on the heart or scaphognathite rates. Therefore, the mean difference in heart and scaphognathite rates between the pre-exposure (control) rates and those after 15 and 30 minutes were calculated to determine the effect of each sulphide concentration on the heart and scaphognathite rates (Fig. 10.8 and Fig 10.9).

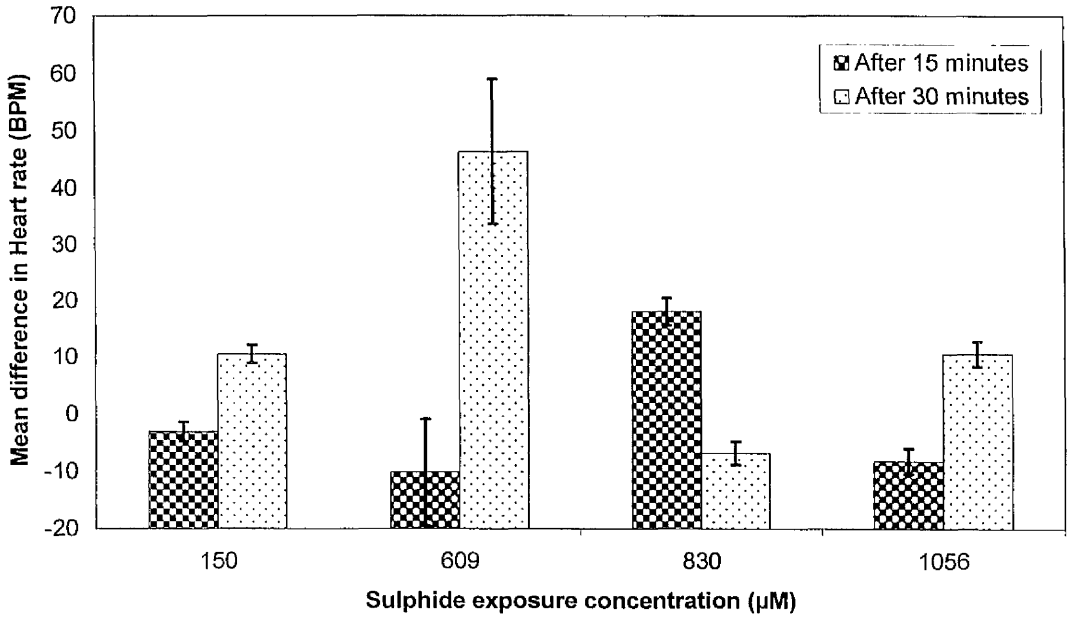

Figure 10.8: The mean difference in the heart rates of Nephrops norvegicus *after 15 minutes of sulphide exposure, and after 30 minutes (beginning of the sulphide-free, recovery period). Values are means ± standard error (n = 10).*

The mean pre-exposure (control) heart rates are represented by zero in Fig. 10.8. During sulphide exposure (after 15 minutes), and at the beginning of the sulphide-free recovery period (after 30 minutes), there was no discernable or significant trend in the effect of sulphide exposure on the differences between the heart rates after 15 minutes and 30 minutes, from the control heart rates.

The mean scaphognathite rates recorded prior to sulphide exposure (controls) are represented by zero in Fig. 10.9. Hence positive differences between the control rates and the scaphognathite rates during sulphide exposure (after 15 minutes) and during the sulphide-free recovery period (after 30 minutes) are represented by positive values, and negative differences are represented by negative values (Fig. 10.9).

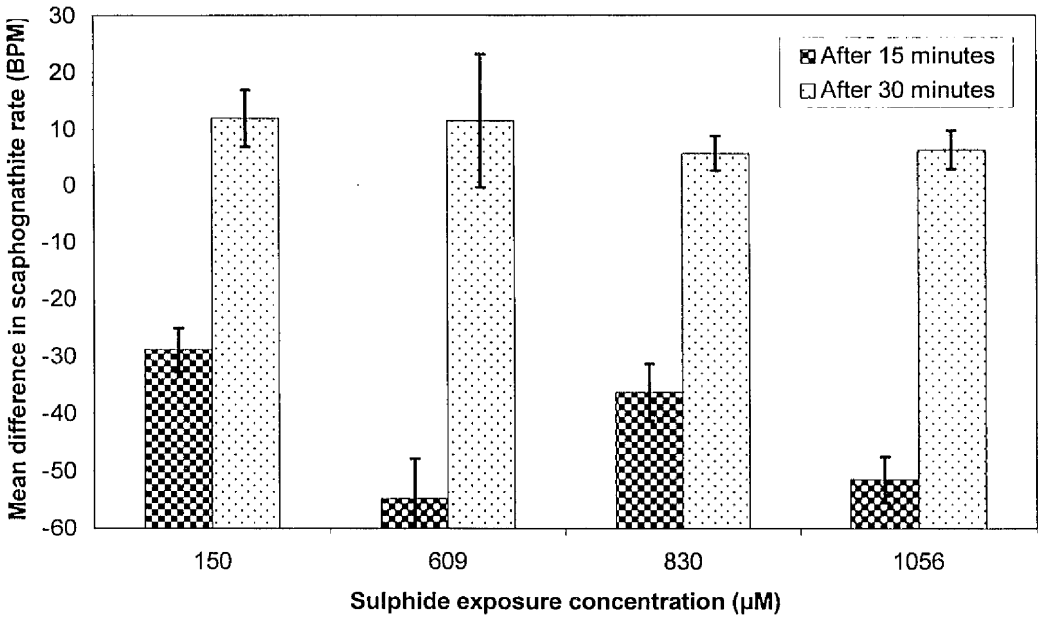

Figure 10.9: The mean difference in the scaphognathite rates of Nephrops norvegicus *after 15 minutes of sulphide exposure, and after 30 minutes (beginning of the sulphide-free recovery period). Values are means ± standard error (n = 10).*

The mean differences in scaphognathite rate during sulphide exposure (after 15 minutes) were consistently significantly different from the pre-exposure (control) scaphognathite rates (Fig. 10.9). As these were negative values, the mean scaphognathite rates (Fig. 10.7) during sulphide exposure were lower than the control rates. However, at the beginning of the recovery period, when sulphide-free seawater was pumped through the tubes containing the *N. norvegicus* (after 30 minutes), the mean differences in scaphognathite rates were not significantly different from the pre-exposure (control) scaphognathite rates.

Sulphide tolerance experiments

N. norvegicus were exposed to 500 µM sulphide under normoxic conditions in a flow-through system to ascertain the LT_{50} of this species. It took 22.5 hours for LT_{50} to be achieved. Samples of tail muscle and hepatopancreas were removed from the

survivors of the LT_{50} experiment and analysed for anaerobic end products (Fig. 10.2) and thiol content (Fig. 10.3).

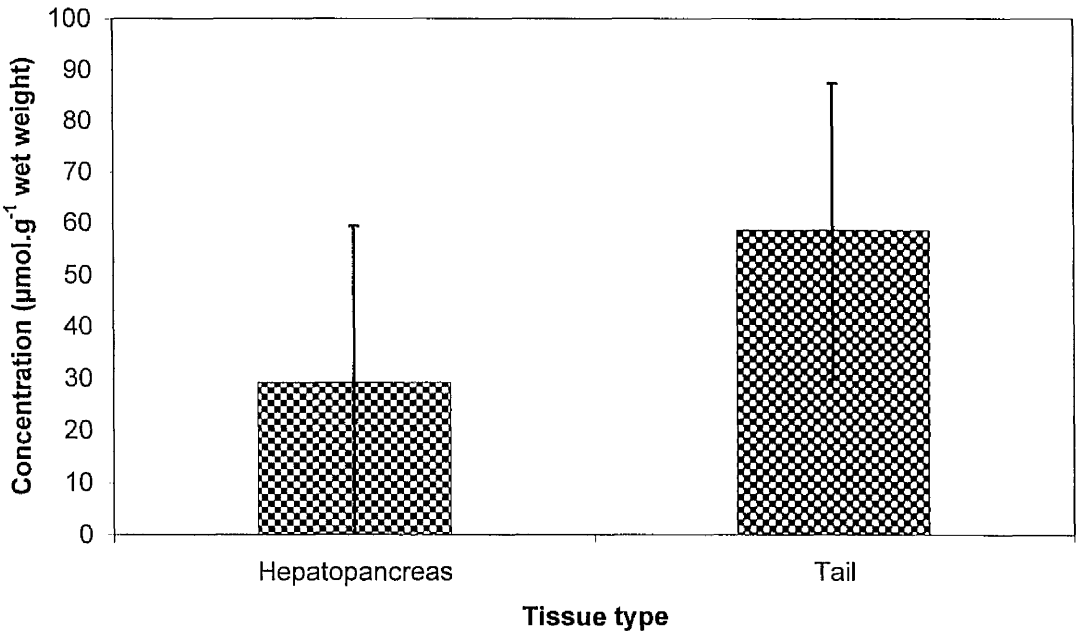

Figure 10.2: The concentrations of lactate in the hepatopancreas and tail muscle of Nephrops norvegicus *after 22.5 hours of exposure to 500 µM sulphide under normoxic conditions (n = 5). The values are means ± 95% CI.*

The concentrations of lactate in the tail muscle were double those in the hepatopancreas after 22.5 hours of exposure to 500 µM sulphide under normoxic conditions but, due to considerable variation in lactate concentration between individuals, this difference was not significant.

Although, with the exception of thiosulphate, the concentrations of the all the thiols in the hepatopancreas appear to be higher than those in the tail muscle, these differences were not significant.

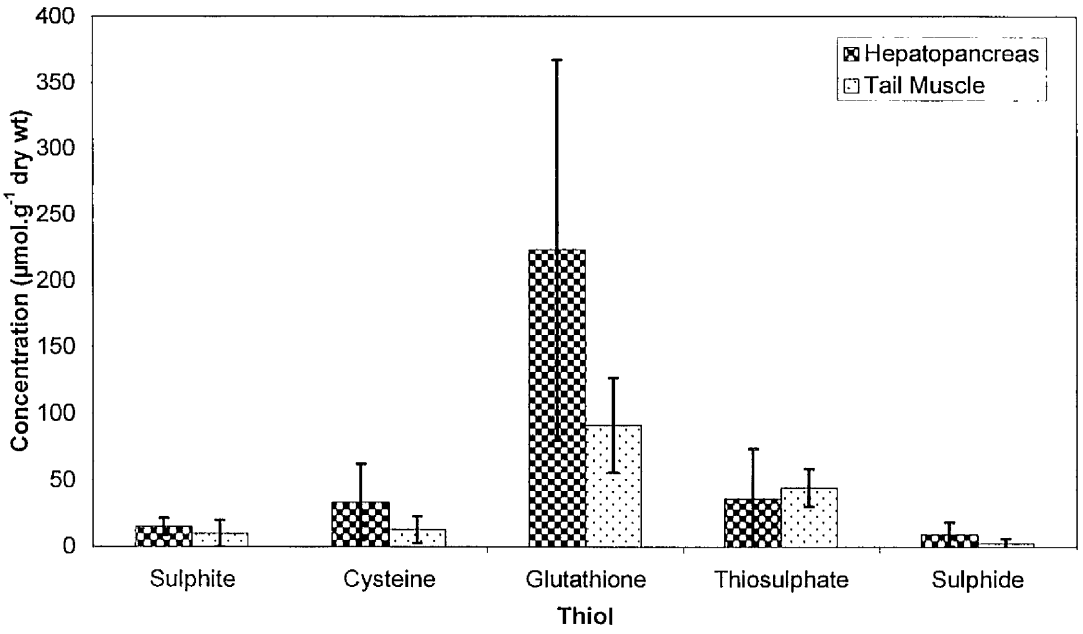

Figure 10.3: The concentrations of thiols in the hepatopancreas and tail muscle of Nephrops norvegicus *after 22.5 hours of exposure to 500 µM sulphide under normoxic conditions (n = 5). The values are means ± 95% CI.*

Anaerobic end products

The concentrations of lactate in the haemolymph of *N. norvegicus* were quantified after the lobsters had been exposed to sulphide (0 µM – 1200 µM) for 30 minutes (Fig. 10.4). Each *N. norvegicus* was exposed to only one concentration of sulphide. No lactate was found in the haemolymph of the lobsters upon exposure to 0 µM, 200 µM and 500 µM sulphide. However, there was a stepwise increase in lactate concentration with increasing sulphide concentrations between 800 µM – 1200 µM.

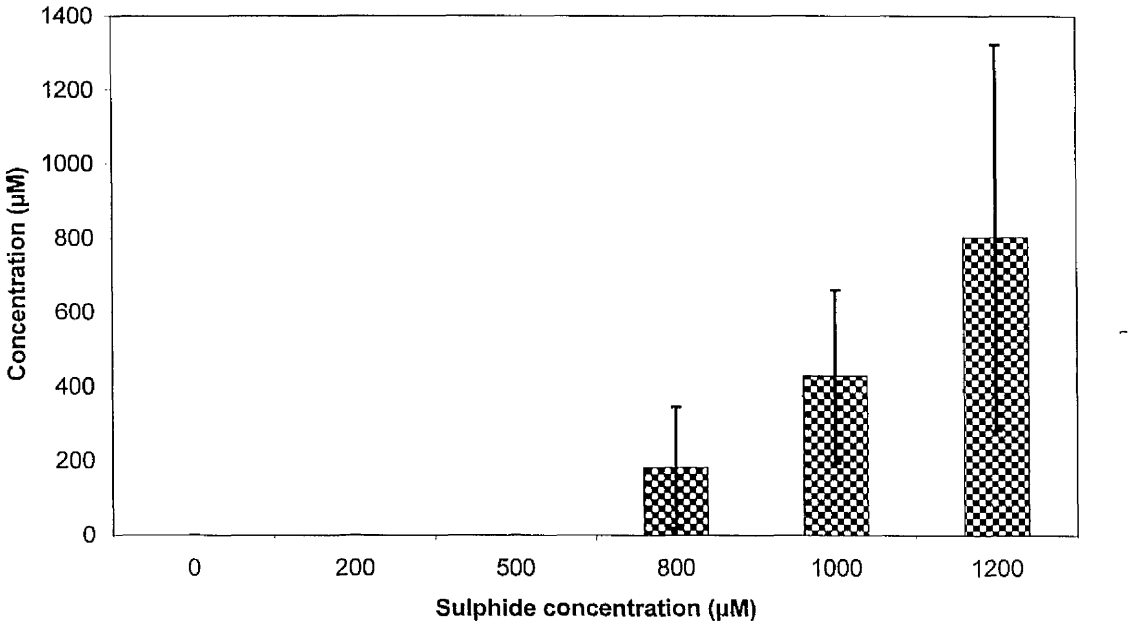

Figure 10.4: The concentrations of lactate in the haemolymph of Nephrops norvegicus *exposed to increasing concentrations of sulphide for 30 minutes (n = 5). Values are means ± 95% CI.*

Thiol concentrations

The concentrations of thiols in the haemolymph of *N. norvegicus* were quantified after the lobsters had been exposed to sulphide (0 µM – 1200 µM) for 30 minutes. Each *N. norvegicus* was exposed to only one concentration of sulphide. Although sulphite was detected in the haemolymph, the concentrations were not significantly different from zero at any of the sulphide exposure concentrations. The concentrations of cysteine ranged between 0.01 – 0.9 µM, and the concentrations of glutathione ranged between 0.01 – 0.18 µM upon exposure to sulphide. There were, however, no significant differences in the concentrations of either cysteine or glutathione in the haemolymph of the *N. norvegicus* kept in sulphide-free seawater (controls), and those exposed to sulphide for 30 minutes. The concentrations of thiosulphate in the haemolymph (Fig. 10.5) increased significantly with increasing sulphide exposure concentration when compared to those in the haemolymph of the

N. norvegicus kept in sulphide-free seawater (controls). The concentrations of sulphide in the haemolymph, however, did not differ significantly between the *N. norvegicus* kept in sulphide-free seawater (controls), and those exposed to sulphide for 30 minutes (Fig. 10.5).

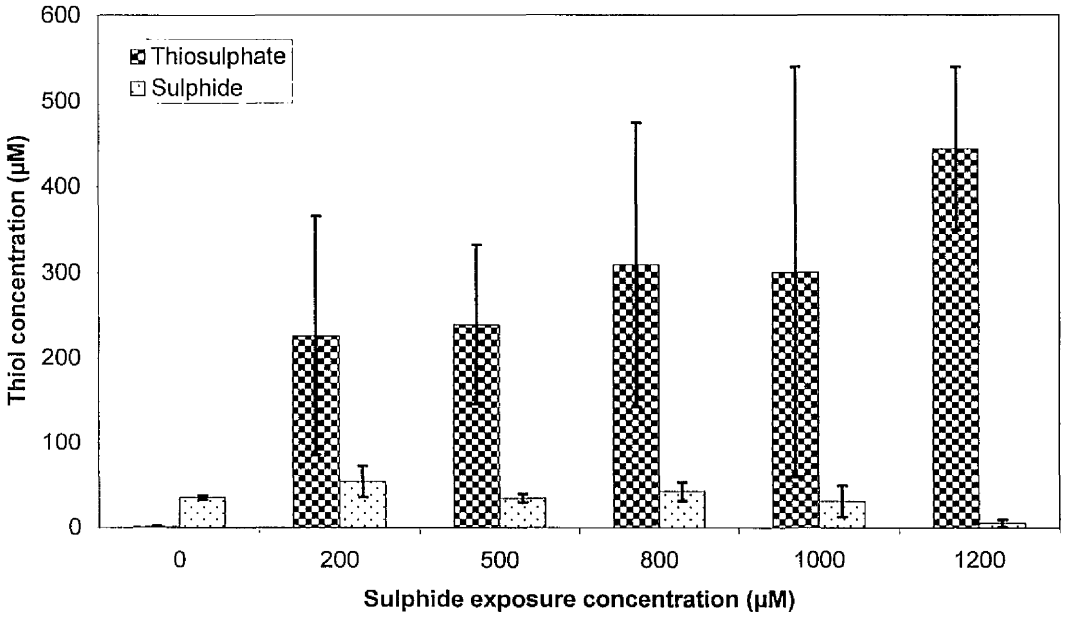

Figure 10.5: The concentrations of thiosulphate and sulphide in the haemolymph of Nephrops norvegicus *exposed to sulphide for 30 minutes (n = 5). Values are means ± 95% CI.*

Discussion

Behavioral experiments

Experiments were designed to examine the ability of *N. norvegicus* to sense sulphide in the water column. Previous experiments have shown that the antennules of the hydrothermal vent shrimp *Rimicaris exoculata* can detect sulphide (Renninger *et al.*, 1995). Hence the effect of sulphide on *N. norvegicus* was examined in animals with antennules, and in those with the antennules removed. There was a marked

difference in the responses of the two groups of lobsters. Those without antennules did not react to sulphide at any of the concentrations tested. However, the lobsters with intact antennules immediately displayed avoidance behaviour even at the lowest sulphide concentration (50 µM). The avoidance behaviour increased as sulphide exposure concentrations increased, until, at the highest exposure concentration, the lobsters frantically tried to escape the sulphide by rapid tail flipping. The brown shrimp, *Crangon crangon* was also reported to display such a "panic reaction" to sulphide, but during exposure to only 20 µM (Vismann, 1996a). The results of these experiments confirm those of other studies and indicate that it is the antennules of *N. norvegicus* that alert the animal to the presence of sulphide. Furthermore, the *N. norvegicus* immediately attempted to evade the sulphide. It is therefore possible that *N. norvegicus* uses avoidance behaviour as the first defence against exposure to sulphide. A similar behavioural adaptation was shown to exist in the amphipod *Corophium volutator*, which burrows in sediments that could potentially contain lethal concentrations of sulphide (Meadows *et al.*, 1981).

Monitoring of heart and scaphognathite rates

Changes in the heart rate and scaphognathite rate were monitored in *N. norvegicus* exposed to sulphide (0 µM – 1200 µM) for 30 minutes and subsequently to sulphide-free seawater for an additional 30 minutes. During the addition of sulphide at each concentration, there was a significant reduction in ventilation rate in all the *N. norvegicus* (Fig. 10.6 and Fig. 10.7). There are two possible explanations for the observed reduction in ventilation rate: either the lobsters were attempting to reduce the amount of sulphide to which the gills were exposed, or they were already suffering from the toxic effects of sulphide which may have affected the muscles controlling the scaphognathites. Sulphide is a neuromodulator in mammals (Abe and Kimura, 1996), and subsequent research has shown that sulphide is also a smooth

muscle relaxant (Hosoki *et al.*, 1997). Hence, the reasons for the reduction in ventilation rates remain unclear.

Vetter *et al.* (1987) exposed a number of crustaceans to sulphide, and reported that those species that were intolerant of sulphide showed a reduction in heart rate with increasing sulphide concentration. Although the heart rates of *N. norvegicus* decreased upon exposure to sulphide, these decreases were not significant. Laboratory studies have shown that when confronted with declining ambient oxygen levels, *N. norvegicus* showed no modification of its heart rate (Hagerman and Uglow, 1985). Similarly, the sulphide tolerant *B. thermydron* showed no modification of heart rate with increasing sulphide concentration (Vetter *et al.*, 1987). *N. norvegicus* therefore appear to be resistant to 0 µM – 1056 µM sulphide exposure over short periods.

After 30 minutes, at the start of the recovery (sulphide-free) period, the scaphognathite rates increased to rates higher than those recorded prior to sulphide exposure. During the recovery of crustaceans from sustained hypoxia, there is usually an elevated period of oxygen consumption at the beginning of the recovery period (Helm and Trueman, 1967; Vismann and Hagerman, 1996; Bourgeois and Felder, 2001). This is accompanied by increased ventilatory rates (Bayne, 1971; Taylor, 1977; Vetter *et al.*, 1987), possibly in an attempt to pass more oxygen over the gills to repay the oxygen debt incurred during the hypoxic event. Sulphide inhibits the terminal step of the respiratory chain, which inhibits aerobic respiration (National Research Council, 1979). Although the lobsters were exposed to sulphide under normoxic conditions, the inhibition of the respiratory chain may induce a similar reaction to that described above when crustaceans recover from sustained hypoxia. *N. norvegicus* becomes inactive whilst exposed to hypoxia (Eriksson and Baden, 1997), and this was also observed in the lobsters exposed to sulphide under

normoxia. However, it is also possible that the elevated scaphognathite rates at the start of the sulphide-free recovery period were indicative of an attempt by the lobsters to flush the remaining sulphide from the gills.

Sulphide tolerance experiments

The LT_{50} value of *N. norvegicus* exposed to 500 µM sulphide was 22.5 hours. It is difficult to compare this value to those reported by other authors, as the sulphide concentrations used in different experiments, vary somewhat Table 10.2.

Table 10.2: Reported sulphide tolerance values for some selected species of Crustacea.

Species	**Sulphide (µM)**	**LT_{50} (hours)**	**Author**
Metapenaeus monoceros	0.5	48	Kang and Matsuda, 1994
Penaeus indicus	0.4 – 0.6	96	Gopakumar
Metapenaeus dobsoni	0.5 – 1	96	and Kuttyamma, 1996
Crangon crangon	20	1	Vismann, 1996a
Monoporeia affinis	25	14,2	Sandberg *et al.*, 1999
Rhepoxynius abronius	138	48	Knezovich *et al.*, 1996
Eohaustorius estuarius	138	48	Knezovich *et al.*, 1996
Saduria entomon	150	75	Hagerman and Vismann, 1993
Calocaris macandreae	1000	29	Johns *et al.*, 1997
Callianassa subterranea	1000	24	Johns *et al.*, 1997

N. norvegicus appears to be more tolerant of sulphide than some non-burrowing crustaceans, but less tolerant than the thalassinideans which construct complex, deep burrows often in muddy substrata containing sulphide (Atkinson and Taylor, 1988). However, the female *N. norvegicus* could have a higher sulphide tolerance

than the males which spend more time on the sediment surface. But female *N. norvegicus* spend most of the year in simple burrows, and were not present in the trawl catch; therefore only male *N. norvegicus* were used.

Although the concentrations of lactate in the tail muscle were approximately double those in the hepatopancreas of the *N. norvegicus* exposed to 500 µM for 22.5 hours, these differences were not significant. The concentrations of thiols in the tail muscle were not significantly different from those in the hepatopancreas of the *N. norvegicus* exposed to 500 µM for 22.5 hours. However, the concentrations of glutathione were approximately a factor of 30 higher than the concentrations of sulphide in the respective tissues. Given the role of glutathione in the tissues of *A. islandica* and *M. edulis* in sulphide detoxification (Chapters 5, 7 and 8), the data suggest that glutathione may also be involved in sulphide detoxification in *N. norvegicus*.

Anaerobic end products

During exposure to sulphide, lactate was found to accumulate only in the lobsters exposed to 800 – 1200 µM sulphide. Hence there is no evidence of anaerobiosis in *N. norvegicus* exposed to 0 – 500 µM sulphide. A similar pattern was reported in *Calocaris macandreae* exposed to different sulphide concentrations for 24 hours, the lactate concentrations did not differ significantly upon exposure to lower concentrations of sulphide (Johns *et al.*, 1997). However, a critical concentration of sulphide exposure was reached where lactate accumulated in significant concentrations. Hence it appears that *N. norvegicus* does not switch to anaerobiosis at sulphide exposure concentrations below 500 µM over a 30 minute period. It is unfortunate that the rates of oxygen consumption were not measured upon exposure to sulphide, as it is possible that sulphide stimulated oxygen consumption occurs at these lower sulphide exposure concentrations, as demonstrated in *M. edulis* (Chapter 6).

Thiol concentrations

No significant concentrations of sulphite were detected in the haemolymph at any of the sulphide concentrations (0 µM – 1200 µM). The concentrations of cysteine and glutathione in the haemolymph were in the nanomolar range, and did not vary significantly as sulphide exposure concentrations increased.

The concentrations of thiosulphate, however, increased significantly between the pre-exposure concentrations, when the lobsters were not exposed to sulphide, and upon exposure to sulphide. It appears, therefore, that thiosulphate is the main product of sulphide detoxification in *N. norvegicus*, as it is in some other crustaceans (Gorodezky and Childress, 1994; Johns *et al.*, 1997) and some other marine invertebrates (Powell and Somero, 1986a; O'Brien and Vetter, 1990; Vismann, 1991b; Jahn *et al.*, 1996; Hauschild *et al.*, 1999).

Following the addition of sulphide, the sulphide concentrations in the haemolymph of *N. norvegicus* did not increase significantly from the pre-exposure (control) concentrations. It is therefore possible that these lobsters were able to oxidise sulphide to thiosulphate in the blood as swiftly as it was diffusing into the haemolymph. These results support those found by previous authors on the sulphide oxidising activity of haemolymph from *N. norvegicus* (Astall, 1993). Sulphide was subsequently demonstrated to have no effect on the binding properties of haemocyanin to oxygen *in vitro* (Taylor *et al.*, 1999). However, sulphide binding by haemoglobin has been demonstrated in the hydrothermal vent tubeworm, *Riftia pachyptila* (Zal *et al.*, 1997; Zal *et al.*, 1998), and in the symbiont-containing clam *Lucina pectinata* (Kraus, 1995; Frenkiel *et al.*, 1996; Cerda-colon *et al.*, 1998; Silfa *et al.*, 1998; Rosado-Ruiz *et al.*, 2002). Hence it is possible that there is a haem-containing molecule in the haemolymph of *N. norvegicus* that is involved in the sulphide detoxification.

In summary, the LT_{50} of *N. norvegicus* at 500 µM was 22.5 hours. *N. norvegicus* uses the antennules to sense sulphide, and appears to avoid sulphide when possible. However, if the lobsters cannot escape the sulphide, they cease all locomotory activity, and decrease scaphognathite rates perhaps in an attempt to limit the amounts of sulphide that is absorbed by the gills.

The sulphide that enters the haemolymph of the lobsters is oxidised to thiosulphate whilst still in the haemolymph. This process appeared to be efficient enough to keep the concentrations of sulphide in the haemolymph constant throughout a 30 min exposure period at sulphide concentrations between 0 µM – 1200 µM. There was no significant effect of sulphide on the heart rates of *N. norvegicus* at sulphide concentrations between 0 µM – 1056 µM which suggests that *N. norvegicus* is able to tolerate these concentrations of sulphide. However, these heart rates were also recorded during a 30 minutes sulphide exposure period. The concentrations of lactate start to accumulate after only 30 minutes of 800 µM sulphide in the haemolymph. All of which suggest that *N. norvegicus* is tolerant of high concentrations of sulphide, but only for very short periods. Further experiments are necessary to examine the effects of differing concentrations of sulphide on anaerobiosis, and heart and scaphognathite rates of *N. norvegicus* over longer periods.

A tentative overall strategy for *N. norvegicus* to cope with sulphide exposure may be to first sense sulphide and move to sulphide-free areas. In cases where evasion is not possible the lobsters may reduce gill ventilation rates either to limit sulphide uptake via the gills or due to inhibition of muscular activity by the sulphide. Simultaneously, sulphide is oxidised to thiosulphate in the haemolymph, as a detoxification mechanism. During short-term (30 minutes) sulphide exposure, at concentrations in excess of 500 µM the lobsters switch to anaerobiosis. However,

the success of this strategy is directly correlated to the sulphide exposure concentration, and the duration of exposure.

Further experiments to examine oxygen consumption of *N. norvegicus* in the presence of sulphide, and experiments to determine the concentrations of thiols, and lactate in the tissues and blood of *N. norvegicus* upon exposure to sulphide concentrations below 500 μM, and over varying time scales are necessary to prove this hypothesis.

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Index