Sulphide metabolism in burrowing marine Crustacea

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To my wife and family,
Bridget, Ellie and Peter
and our parents.

The conquerors of the shelf will have to get wet,
(Cousteau & Dumas, 1953).

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Abstract

Sulphide occurs widely in marine sediments and is highly toxic to most organisms. Its principal poisoning effect occurs at extremely low concentrations and is the result of inhibition of mitochondrial cytochrome c oxidase. Mud-shrimps, (Crustacea: Decapoda: Thalassinidea), construct burrows in sublittoral muddy sediments. The sediment in which they burrow may often be markedly reduced and conditions within the burrow are usually hypoxic and hypercapnic. Field measurements taken during this study also indicate significant exposure to sulphide in two species of mud-shrimp. Direct evidence has demonstrated that *Callianassa subterranea* may be exposed to potentially toxic levels of sulphide in the burrow water (37 ± 44 μM, range 0 - 206 μM). Sulphide, thiosulphate and sulphite were also found in the haemolymph and tissues of freshly caught animals (*Calocaris macandreae* and *Callianassa subterranea*) indirectly indicating that they are exposed to ambient sulphide. The concentrations of sulphide found in the burrow water and thiol concentrations within the mud-shrimps also showed seasonal variations which may be correlated with the input of organic material.

Mud-shrimps appear to be physiologically adapted to tolerate elevated levels of sulphide that they may encounter in their natural habitat. Laboratory experiments carried out on three species, *Calocaris macandreae*, *Callianassa subterranea* and *Jaxea nocturna*, have shown that they have a high tolerance of sulphide. High micromolar concentrations of sulphide (360 ± 320 μM) were also found not to affect the irrigation behaviour of the mud-shrimp *Calocaris macandreae* suggesting tolerance of sulphide exposure.

The mud-shrimp *Calocaris macandreae* can tolerate substantial exposure to sulphide before showing signs of anaerobic metabolism. An oxygen-dependent 'oxidation' mechanism appears to exist which defends mitochondrial cytochrome c oxidase from sulphide poisoning. Sulphide diffusing into the body tissues is oxidized primarily to thiosulphate, in the hepatopancreas and muscle tissues, and

accumulates rapidly even during brief exposure to low micromolar concentrations of sulphide. Sulphite also appears as a secondary 'oxidation' product. No evidence of chemoautotrophic bacterial symbionts could be found and it is therefore proposed that the 'oxidation' of sulphide is facilitated by the animal's tissue.

The 'oxidation' mechanism allows aerobic metabolism to be maintained despite the presence of sulphide although the mud-shrimp's sensitivity to sulphide is apparently greater at lower oxygen partial pressures. Sulphide also has a concentration-dependent capacity to either stimulate or inhibit aerobic metabolism in mud-shrimps. Aerobic metabolism appears to increase during exposure to sulphide, when oxygen is available, and can be maintained even under severe hypoxic and sulphidic conditions. The mud-shrimps resort to anaerobiosis (which may operate concurrently with aerobic metabolic pathways) when the 'oxidation' mechanism is saturated, when oxygen supply becomes limited and when intracellular sulphide concentrations become toxic. The possibility of metabolic depression to reduce energy expenditure may also occur in response to sulphide exposure and is discussed.

Haemocyanin function was also found to be unaffected by sulphide and oxygen affinity increased in the presence of thiosulphate. This may be a physiological adaptation to increase the amount of oxygen available for sulphide 'oxidation' to produce the metabolites thiosulphate and sulphite during severely hypoxic and sulphidic conditions. Mudshrimps are therefore physiologically adapted to tolerate elevated levels of sulphide which they may encounter in their natural habitat. The 'oxidation' of sulphide in the mitochondria in a small number of other marine invertebrates has been established and it therefore seems likely that this may be the case in mud-shrimps. Indirect evidence suggests that the underlying biochemical mechanism of mitochondrial sulphide 'oxidation' is enzymatic ('sulphide oxidase'), although direct evidence of this, the isolation of such an enzyme and its characterization remain to be elucidated.

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1. Introduction.

1.1. Sulphide in the aquatic environment

Sulphide is a naturally occurring toxin which commonly occurs in aquatic environments. The role of sulphide as an environmental factor for aquatic animals, however, has received relatively little attention, although the risk to sediment-dwelling invertebrates of being exposed to sulphide was recognised during the late 1960s and early 1970s (Theede *et al.*, 1969; Fenchel & Reidl, 1970; Boaden & Plat, 1971). More recently, a rapidly growing interest in sulphide within marine and limnic environments has developed, stimulated by the discovery of complex communities of animals around geothermal (hydrothermal) vents on the ocean floor (Corless & Ballard, 1977; Felbeck, 1981; Grassle, 1986). Many of these animals may be exposed to high concentrations of sulphide and are apparently able to overcome its potentially toxic effects. Some of these animals form endo-symbiotic associations with chemoautotrophic bacteria to meet their energy requirements from geothermally produced hydrogen sulphide. Over 230 invertebrate species associated with hydrothermal vents have been described, which include molluscs, annelids, crustaceans and members of the new phylum Vestimentifera (Childress & Fisher, 1992). Biomass around these hydrothermal vent sites is highly localized and may be up to 300 times that of the surrounding abyssal regions.

Extensive work has taken place over the last few years on hydrothermal vent organisms such as the vestimentiferan *Riftia pachyptila* (Felbeck, 1981), the bivalves *Bathymodiolus thermophilus* and *Calyptogena magnifica* (Powell & Somero, 1986b) and the decapod crustacean *Bythograea thermydron* (which lacks symbionts) (Vetter *et al.*, 1987). This work has also led to a renewed interest in animals which inhabit other sulphide-rich environments in shallower waters. Many infaunal marine organisms survive in habitats which contain potentially toxic concentrations of sulphide such as in marine sediments, and

may possess mechanisms to detoxify and derive energy from sulphide. A small and growing number of these animals have been investigated and include the polychaete *Arenicola marina* (Völkel & Grieshaber, 1992, 1995, 1996), the echiuran *Urechis caupo* (Arp *et al.*, 1992, Eaton & Arp, 1993), the bivalve *Solemya reidi* (Anderson *et al.*, 1987) and the Baltic brackish water isopod *Saduria entomon* (Hagerman & Vismann, 1993). This project has investigated another group of animals, the thalassinideans or mud-shrimps, which form burrows in marine sediments and may be exposed to significant ambient concentrations of sulphide.

1.2. Sulphur and sulphide in the biosphere

Sulphur (atomic number = 16, relative atomic mass = 32.06) is an element in the oxygen group (VI) of the Period Table and exists in a wide range of oxidation states in the biosphere. These range from sulphide (2-) in its most reduced form through oxidation states 0, 2+ and 4+ to sulphate (6+) in its most oxidized form. It behaves principally as a non-metal forming covalent species, shows a great tendency to catenation and reacts combining with most other elements to form a wide range of inorganic and organic compounds (Atkins, 1989).

The reduced and oxidized forms of sulphur can be inter-converted by various organisms in the biosphere during the sulphur cycle (Figure 1.1). The assimilatory reduction of sulphate by micro-organisms and plants leads to the formation of cysteine which may subsequently be incorporated into higher organisms as proteins via methionine (Schiff & Saida, 1987; Andreae, 1990). Cysteine and methionine may also undergo oxidation in micro-organisms, plants and animals to produce sulphate which may be used to form polysaccharide and steroidal sulphates. Chemosynthetic bacteria also mediate the inter-conversion of sulphide to form sulphate, whilst bacterial dissimilatory sulphate reduction may occur to form sulphide (Schiff & Saida, 1987; Andreae, 1990).

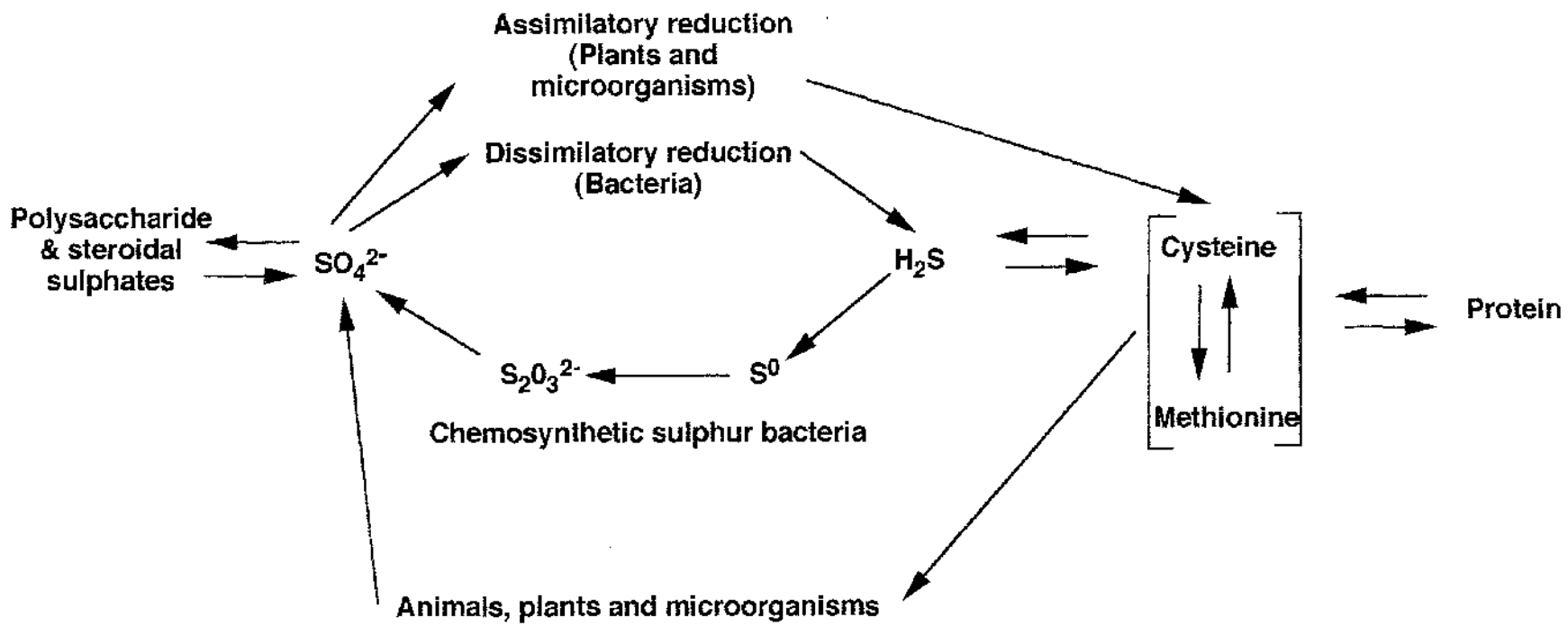

Figure 1.1 Reactions of sulphur in the biosphere (after Schiff & Saida, 1987).

In the aquatic environment sulphur commonly occurs in its reduced form as sulphide as a result of a variety of processes and mechanisms. These include geothermally produced sulphide at hydrothermal vent sites (Edmond *et al.*, 1982), bacterial production of sulphide during periods of eutrophication and stratification of the water column (Kitching *et al.*, 1976; Aure & Stigebrand, 1989; Mann & Lazier, 1991) and production of dimethylsulphide by marine plankton (Andreae, 1990). In marine sediments, sulphide is produced under anoxic conditions beneath the redox potential discontinuity layer due mainly to the activity of sulphate-reducing bacteria, and may reach concentrations in the micromolar to millimolar range. Sulphide is oxidized rapidly in the presence of oxygen (Theede *et al.*, 1969; Fenchel & Reidl, 1970; Theede, 1973; Jørgensen & Fenchel, 1974; Jørgensen, 1988). It can therefore accumulate to significant levels only in anoxic or severely hypoxic environments (Cline & Richards, 1969; Chen & Morris, 1972; Invorgensen & Jørgensen, 1979; Jørgensen, 1982). Thus, high sulphide levels are a correlate of ambient oxygen deficiency. Many infaunal marine organisms survive in habitats which contain potentially toxic levels of sulphide in interstitial water. Animals that inhabit marine sediments may therefore be subjected to sulphide and its potentially toxic effects.

1.3. Sulphide chemistry and toxicity

In aqueous solution sulphide exists in three different chemical species; hydrogen sulphide (H_2S), the hydrosulphide anion (HS^-) and the bisulphide anion (S^{2-}). Sulphide therefore is conventionally expressed as total sulphide: [Sulphide] = $[S^{2-}]$ + $[HS^-]$ + $[H_2S]$ (Vismann, 1991a; Bagarinao, 1992). The proportion of each species is highly dependent on pH (see section 2.1.1.) (Goldhaber & Kaplin, 1975). At physiological and environmental pHs (7 - 8), however, hydrogen sulphide and the hydrosulphide anion predominate. This has a considerable effect on sulphide toxicity because H_2S is the form which freely diffuses across membranes (see section 2.4.2.) (Bagarinao, 1992).

Hydrogen sulphide is extremely toxic to aerobic organisms (Evans, 1967; Nicholls, 1975; National Research Council, 1979; Beauchamp *et al.*, 1984; Vetter *et al.*, 1991). Its principal poisoning effect, which is reversible, is the result of the inhibition of mitochondrial cytochrome c oxidase by binding to cytochrome aa_3. This results in the disruption of the electron transport chain and inhibition of aerobic metabolism (Nicholls, 1975). The isolated enzyme, taken from various organisms as homogenates, has been shown to be very sensitive to sulphide and is inhibited at nanomolar to low micromolar concentrations of sulphide (Hand & Somero, 1983; Bagarinao, 1992). Aerobic mitochondrial metabolism, however, is inhibited at higher sulphide concentrations (2 - 38 μM); approximately 1 - 2 orders of magnitude greater (Bagarinao, 1992). Since the enzyme is located at the inner mitochondrial membrane, sulphide may be oxidized by the mitochondria before cytochrome c oxidase is inhibited.

To a lesser extent sulphide may also effect other enzymes (approximately 20), metabolites and cofactors. These include ATPase, carbonic anhydrase and succinate dehydrogenase (Bagarinao, 1992). Sulphide is a very strong nucleophile and may therefore reduce disulphide bridges and bind to haem-type enzymes which results in the inhibitory effects described above (National Research Council, 1979). Respiratory pigments may also be affected. In the presence of sulphide, haemoglobin may undergo a number of chemical reactions (Kraus *et al.*, 1996) either binding to the iron-containing haem centre (Somero *et al.*, 1989; Kraus *et al.*, 1996) or to the tetrapyrrole ring of vertebrate haemoglobins. Haemoglobins may react with sulphide to form haematins which may also catalyse the 'oxidation' of sulphide. These reactions may significantly reduce oxygen affinity causing the molecule to become dysfunctional. The effect of hydrogen sulphide on haemocyanin is unclear, although, in contrast to haemoglobin, it may not be poisoned by sulphide (Vetter *et al.*, 1987; Hagerman & Vismann, 1993). In addition, sulphide may generate reactive radicals and alter membrane permeability (Bagarinao, 1992). Finally, it has also been established

that the survival of animals which resort to anaerobiosis to overcome the poisoning of cytochrome c by sulphide may be reduced, due to the inhibition of anaerobic metabolic pathways (Theede, 1969; Vismann, 1990, Vismann, 1991b).

1.4. Animals in sulphide rich environments

Animals inhabiting sulphide-rich environments, such as marine sediments, hydrothermal vents, methane seeps, and those exposed to sulphide during eutrophication events and the stratification of the water column, tend to have an increased tolerance of sulphide in comparison with other species which are less likely to be exposed to it. The sulphur present in most living organisms (sulphide tolerant and non-tolerant) occurs in the form of sulphur-containing amino acids (cysteine and methionine) and as sulphate (SO_4^{2-}). In sulphide-tolerant animals, however, additional forms of sulphur are often abundant in haemolymph and tissues: sulphide (S^{2-}), thiosulphate ($S_2O_3^{2-}$), sulphite (SO_3^{2-}) and elemental sulphur (S^0) (Childress & Fisher, 1992). These sulphur groups are 'oxidation' products of sulphide and their presence in sulphide-tolerant animals has been widely interpreted as sulphide 'detoxification' metabolites. A range of adaptive physiological and biochemical 'oxidation' mechanisms to counter the toxic effects of sulphide and in some cases derive energy have been proposed in a variety of animal groups (Vetter *et al.*, 1991; Vismann, 1991b; Bagarinao, 1992; Völkel & Grieshaber, 1995). These adaptations are now believed to be fairly common in animals inhabiting the marine and limnic environments and include meiofauna (Fenchel & Reidl, 1970; Powell *et al.*, 1979, 1980; Ott *et al.*, 1991, 1996) and various macroinvertebrates (Theede *et al.*, 1969, 1973) such as annelids (Degn & Kristensen, 1981), polychaetes (Vismann, 1990; Llanso, 1991; Völkel & Grieshaber, 1994, 1995), oligochaetes (Felbeck *et al.*, 1983), sipunculan worms, (Völkel & Grieshaber, 1992), echiuran worms (Eaton & Arp, 1993), vestimentiferan and pognophoran worms

(Powell & Somero, 1983; Childress *et al.*, 1984), priapulidan worms (Oeschger & Vetter, 1992), bivalve molluscs (Felbeck, 1983, Powell & Somero, 1986a; Levitt & Arp, 1991; Herry *et al.*, 1989; O'Brien & Vetter, 1990), amphipods (Vargo & Sastry, 1977), isopods (Vismann, 1991a) and decapod crustaceans (Vetter *et al.*, 1987; Astall 1993), as well as some shallow-water marine fish (Bagarinao & Vetter, 1989, 1990). These physiological and biochemical 'detoxification' strategies for protection against sulphide and perhaps for the derivation of energy are summarized below and include specific examples :-

1. Evolution of an alternative sulphide insensitive cytochrome c oxidase, as has been proposed to exist within the mitochondria of the intertidal polychaete *Arenicola marina* (Völkel & Grieshaber, 1996).

2. Exclusion of sulphide at the body wall because of impermeability (not described to date) or by the presence of a layer of sulphide oxidizing bacteria covering the cuticle as has been reported to occur in some marine nematodes (Ott *et al.*, 1991)

3. The use of anaerobic metabolism during exposure to sulphidic conditions when aerobic metabolism is inhibited. This has been established in a small number of marine invertebrates including the bivalve *Solemya reidi* (Anderson *et al.*, 1990) and the Baltic brackish water isopod *Saduria entomon* (Hagerman & Vismann, 1993).

4. The use of sulphide-binding proteins with subsequent unloading at sites of 'oxidation', or upon return to low external sulphide concentrations, as in the haemoglobin of the hydrothermal vent vestimentiferan *Riftia pachyptila* (Arp *et al.*, 1984, 1987).

5. Symbiotic association with chemoautotrophic bacteria capable of sulphide 'oxidation'. The symbionts, using energy from sulphide 'oxidation', fix carbon dioxide into organic compounds for translocation to the host, e.g. the bivalve *Solemya reidi* (Felbeck, 1983; Anderson *et al.*, 1987) and the hydrothermal vent vestimentiferan *Riftia pachyptila* (Felbeck, 1981, 1983).

6. Eukaryotic sulphide 'oxidation' with the potential utilization of energy released in sulphide 'oxidation' to drive the generation of ATP, e.g. the fish *Fundulus parvipinnis* (Bagarinao & Vetter, 1990), the priapulid worm *Halicryptus spinulosus* (Oeschger & Vetter, 1992) and the polychaete worm *Heteromastus filiformis* (Oeschger & Vismann, 1994).

7. Behavioural adaptations to actively avoid sulphide as described in *Corophium volutator* (Meadows *et al.*, 1981).

Most studies of these mechanisms have been carried out in various 'worms' and some bivalves. Only very limited work, however, has been conducted on the sulphide metabolism of Crustacea (Vetter *et al.*, 1987; Vismann, 1991b; Astall, 1993; Hagerman & Vismann, 1993; Gorodezky & Childress, 1994). In comparison with other benthic invertebrates, it is generally believed that crustaceans have a low physiological tolerance of sulphide and may use behavioural adaptations to overcome its toxic effects (Theede *et al.*, 1969; Vargo & Sastry, 1978; Meadows *et al.*, 1981; Vismann, 1991b). Much work has been conducted on the biology and ecology of the hydrothermal vent-crab *Bythograea thermydron* (Vetter *et al.*, 1987; Sanders & Childress, 1992; Gorodezky & Childress, 1994). Very little, however, has been carried out on other crustaceans from different sulphidic environments. Mud-shrimps (Crustacea: Decapoda: Thalassinidea) construct burrows in sediments in eulittoral and sublittoral marine environments in which they may be exposed to sulphide.

They occur in a substantially different biotope than other animals previously reported from sulphidic environments. Given the abundance and dominance of mud-shrimps in many intertidal and sublittoral environments (Buchanan, 1963; Buchanan & Warwick, 1974; Calderon-Perez, 1981; Nash *et al.*, 1984; Atkinson, 1986; Anderson *et al.*, 1991), the ecophysiological effects of sulphide on these animals may be of considerable importance and interest.

1.5. Thalassinidean biology and ecology

Mud-shrimps, (Crustacea: Decapoda: Thalassinidea), construct complex burrows of species-specific architecture, which usually penetrate deep into intertidal and sublittoral sediments which may often be anoxic and reduced (Dworschak, 1983; Nash *et al.*, 1984; Atkinson & Taylor, 1988; Nickell & Atkinson, 1995; Ziebis *et al.*, 1996). These animals have attracted increasing attention in many recent ecophysiological studies on marine soft-sediment benthos. This is because mud-shrimps are often abundant and may form a large component of the infaunal macrofauna (Buchanan, 1963; Buchanan & Warwick, 1974; Calderon-Perez, 1981; Rowden & Jones, 1994). In addition, their bioturbatory activities strongly influence the benthic community structure and the chemico-physical structure of the sediment water interface (Aller, 1983; Nickell, 1992, Ziebis *et al.*, 1996).

The Infra-order Thalassinidea (Superfamily Thalassinoidea) Latreille comprises of approximately 500 species (Saint-Laurent & Lelouef, 1979). There are eight thalassinidean species in U.K. waters: *Upogebia stellata* (Montagu), *Upogebia deltaura* (Leach), *Upogebia pusilla* (Petagna), *Calocaris macandreae* Bell (Plate 1.1), *Callianassa subterranea* (Montagu) (Plate 1.2), *Callianassa tyrrhena* (Petagna), *Jaxea nocturna* Nardo and *Axius stirhynchus* Leach. A number of these infaunal species inhabit the muddy eulittoral and sublittoral sediments around the west coast of Scotland and may be locally abundant. During the course of this study the biology and ecology of two different species

were investigated in relation to sulphide: *C. macandreae* (Plate 1.1) and *C. subterranea.* (Plate 1.2).

Within the burrow environment, mud-shrimps lead a fairly sedentary and solitary lifestyle. Apart from a brief pelagic larval stage which may be abbreviated in some species, such as in *Calocaris macandreae*, they rarely if ever, leave their burrows, although brief excursions in the immediate vicinity of the openings for purposes of excavation and feeding may occur (Nickell & Atkinson, 1995). The burrows themselves are some of the deepest (> 50 cm; sometimes several metres deep) and most complex formed by decapod Crustacea (Dworschak, 1983; Atkinson & Taylor, 1988; Atkinson & Nash, 1990; Ziebis *et al.*, 1996). Burrow architecture has been shown to be species-specific and varies considerably from simple 'Y' or 'U' morphs to more complex and intricate designs (Dworschak, 1983; Suchanek, 1985; de Vaugelas, 1990). The burrow of *C. macandreae* (Plate 1.3) typically consists of groups of interconnecting tunnels (usually with three openings at the sediment surface), penetrating the sediment to around 10 cm, below which a secondary development of circular tunnels at around 20 cm typically occurs (Nash *et al.*, 1984; Atkinson, 1986). In the case of *Callianassa subterranea*, the burrow (Plate 1.4) consists of a lattice of tunnels and chambers connected to the surface by one or more inhalant and exhalant shafts and may extend to depths of greater than 86 cm into the sediment (Atkinson & Nash, 1990; Nickell & Atkinson, 1995). Feeding behaviour may vary considerably amongst the thalassinideans but is believed to have a strong influence on burrow morphology (Nickell & Atkinson, 1995; Ziebis *et al.*, 1996). Three major feeding types have been proposed; suspension feeders, deposit feeders and seagrass harvesters which are associated with different types of burrow architecture (Suchanek, 1985). The mud-shrimps *C. macandreae* and *C. subterranea* are primarily sub-surface deposit feeders (Buchanan, 1963; Calderon-Perez, 1981; Nickell & Atkinson, 1995). *C. subterranea*, however, may supplement its diet by suspension feeding (Nickell &

Atkinson, 1995). Due to these activities burrow shape may be continuously modified (Nickell & Atkinson, 1995). This plasticity in feeding and interaction of different trophic modes may be used to exploit the most beneficial food resource.

In contrast to the burrowing activities of mud-shrimps, rather little is known about the reproductive behaviour of the animals. The timing, frequency and location of mating is virtually unknown, although it has been suggested that mating occurs in intersecting burrows (Rodriques, 1976; Felder & Lovett, 1989). The mud-shrimp *Calocaris macandreae*, however, is a protrandous hermaphrodite, the testes and ovaries develop simultaneously for approximately three years before the testes degenerate leaving the *vas deferenia* filled with spermatophores (Buchanan, 1963). Cross fertilization however, may not be precluded. The ovaries continue to develop before the first eggs are laid at a latter stage (during year 5 in Northumbrian specimens) (Buchanan, 1963). The breeding season in most species of mud-shrimp is known to be divided into two distinct peaks (minor and major episodes) which occur in the spring and summer (Hailstone & Stephensen, 1961; Buchanan, 1963; Forbes, 1979; Dworschak, 1988). Single breeding seasons have, however, been reported in some thalassinidean species (Tunberg, 1986; Felder & Lovett, 1989; Hanekom, 1989). The life-spans of mud-shrimps, including callianassids, have been reported to range between 2- 5 years (Devine, 1961; Hailstone & Stephenson, 1961; Forbes, 1977; Dworschak, 1988) although the mud-shrimp *Calocaris macandreae* apparently survives for 5 - 10 years (Buchanan. 1963; Calderon-Perez, 1981). Moulting appears to occur annually in *C. macandreae* from 4 years old onwards, although several moults may take place throughout the year in younger individuals (Buchanan, 1963).

The bioturbatory effects of mud-shrimps, which also include the recycling of organic material, nutrients and trace elements, may be considerable in terms of sediment and also in water movement through the burrow (Pemberton *et al.*,

1976; Aller *et al.*, 1983; Suchanek, 1983; Waslenchuck *et al.*, 1983; Abu-Hilal, 1988; de Vaugelas, 1990; de Vaugelas & Buscail, 1990; Nickell, 1992; Ziebis *et al.*, 1996). The composition and structure of the macro and microbiotic assemblages may also be strongly influenced by these activities (Suchanek, 1983; Posey, 1986; Branch & Pringle, 1987; Griffis & Chavez, 1988; Posey *et al.*, 1991).

Conditions within the burrows are very different from those at the sediment surface. Irrigation may, however, be infrequent and large areas of the burrow lumen may not be in contact with oxygenated water (Forster & Graf, 1992; 1995). The burrow water is usually hypoxic and hypercapnic (Anderson, 1989; Anderson *et al.*, 1991; Nickell, 1992; Astall *et al.*, 1997; Ziebis *et al.*, 1996). Oxygen availability within the burrows of *Calocaris macandreae* has been shown to be low, with minimum Po_2 values in the region of 15 Torr (Anderson *et al.*, 1991). As a consequence of permanent hypoxia and short periods of anoxia in mud-shrimp burrows, sulphide levels may be elevated to some degree in the burrow water. Additionally, during burrowing, these animals may be subjected to toxic levels of sulphide that are present in the interstitial water. Interestingly, mud-shrimps are among the few species to survive the low oxygen partial pressures and high sulphide levels in the vicinity of fish cages in sea lochs (Atkinson, 1987).

Thalassinidean mud-shrimps are known to be able to survive exposure to hypoxia (Anderson *et al.*, 1991) and show a number of adaptations to these conditions. These include behavioural responses to declining oxygen tensions such as irrigating the burrow to draw in oxygenated sea water (Anderson 1989; Astall, 1993; Astall *et al.*, 1997). In addition, in an attempt to ensure oxygen delivery is not compromised, physiological adaptations have been shown, such as increasing ventilation, maintaining a constant heart rate, reducing metabolism to reduce energy expenditure and the possession of a high affinity respiratory pigment (Anderson, 1989; Astall, 1993; Nickell, 1992, Anderson *et al.*, 1994).

Species such as *Calocaris macandreae* are also able to maintain their rates of oxygen consumption constant over a wide range of oxygen partial pressure down to a critical Po_2 (Pc) of approximately 20 Torr (Anderson *et al.*, 1991). Similar or even lower Pc values have been recorded in several other species of mud-shrimp (Atkinson & Taylor, 1988; Paterson & Thorne, 1995; Astall *et al.*, 1997). Mud-shrimps also appear to have a greater tolerance of anoxia than many other decapods. For example, *Neotrypaea* (as *Callianassa*) *californiensis* and *Lepidophthalmus louisianensis* (as *Callianassa jamaicense)* can survive anoxia for 3 - 4 days (Thompson & Pritchard, 1969; Felder, 1979). Although their ability to survive long periods of reduced oxygen availability is now well established, there have been very few studies of their ability to cope with exposure to sulphide.

1.6. Project aim and specific objectives

The aim of the study was to investigate the possible ecophysiological mechanisms that allow thalassinidean mud-shrimps to overcome the potentially toxic effects of sulphide that may be encountered in their burrows. The following specific objectives have been addressed during the course of the work. A field programme was established to estimate the concentrations of sulphide in burrow and sediment interstitial water to determine whether these animals are likely to be exposed to sulphide in the burrow environment. In addition, sediment characteristics (Eh & pH) were also determined. In the laboratory, the tolerance of sulphide of a range of thalassinidean species was investigated and the distribution of sulphide and its 'oxidation' products in tissues and haemolymph following exposure to sulphide determined. In addition, the effects of sulphide on aerobic and anaerobic metabolism were also investigated and the effect of sulphide on whole animal respiration and metabolism was established. The effects of sulphide on haemocyanin function and the effect of thiosulphate (a sulphide 'oxidation' product) on haemocyanin oxygen transport

properties from a small number of decapod crustaceans was also determined. Finally, the behavioural responses of mud-shrimps during exposure to sulphide were investigated .

Plate 1.1 *Calocaris macandreae* Scale bar = 2 cm.

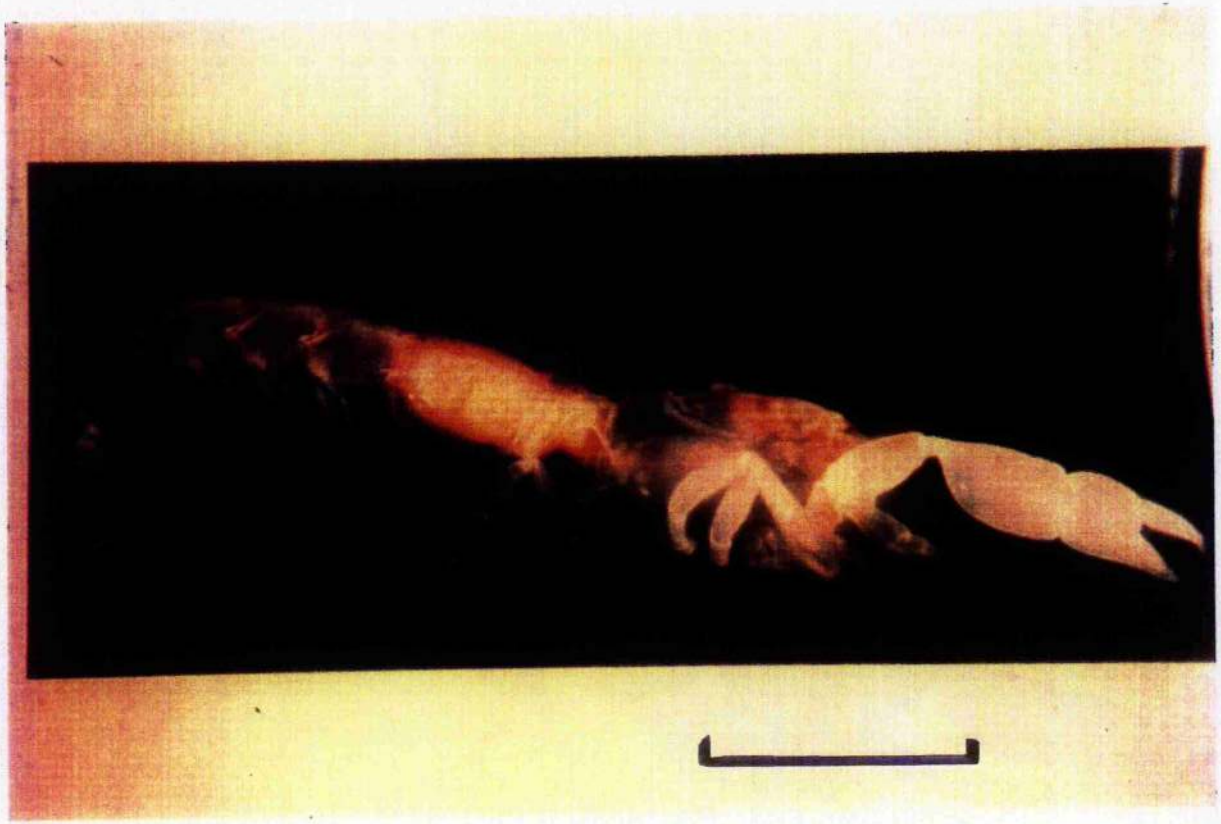

Plate 1.2 *Callianassa subterranea.* Scale bar = 2 cm.

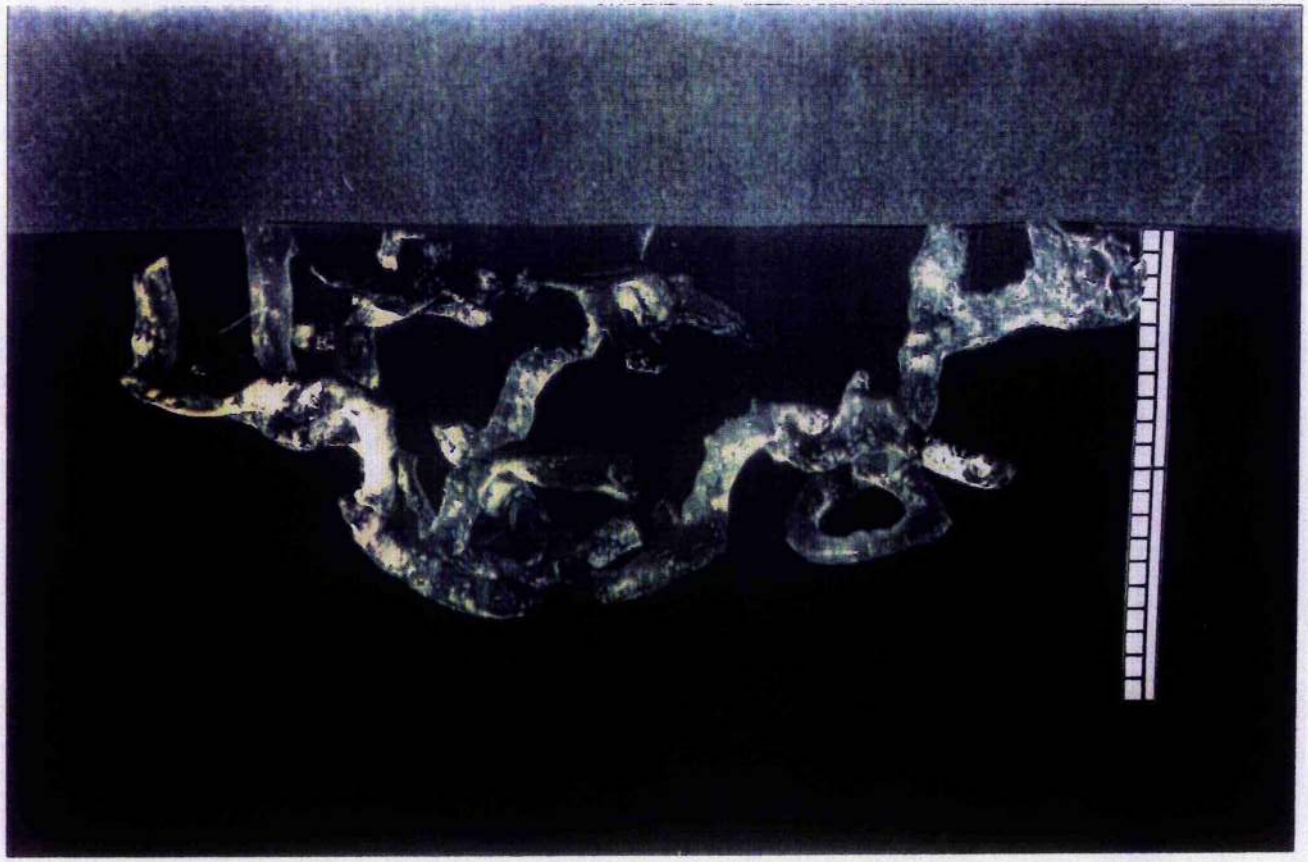

Plate 1.3 Resin cast of the burrow of *Calocaris macandreae*. Lateral view. Scale bar = 25cm (photograph by R.J.A. Atkinson).

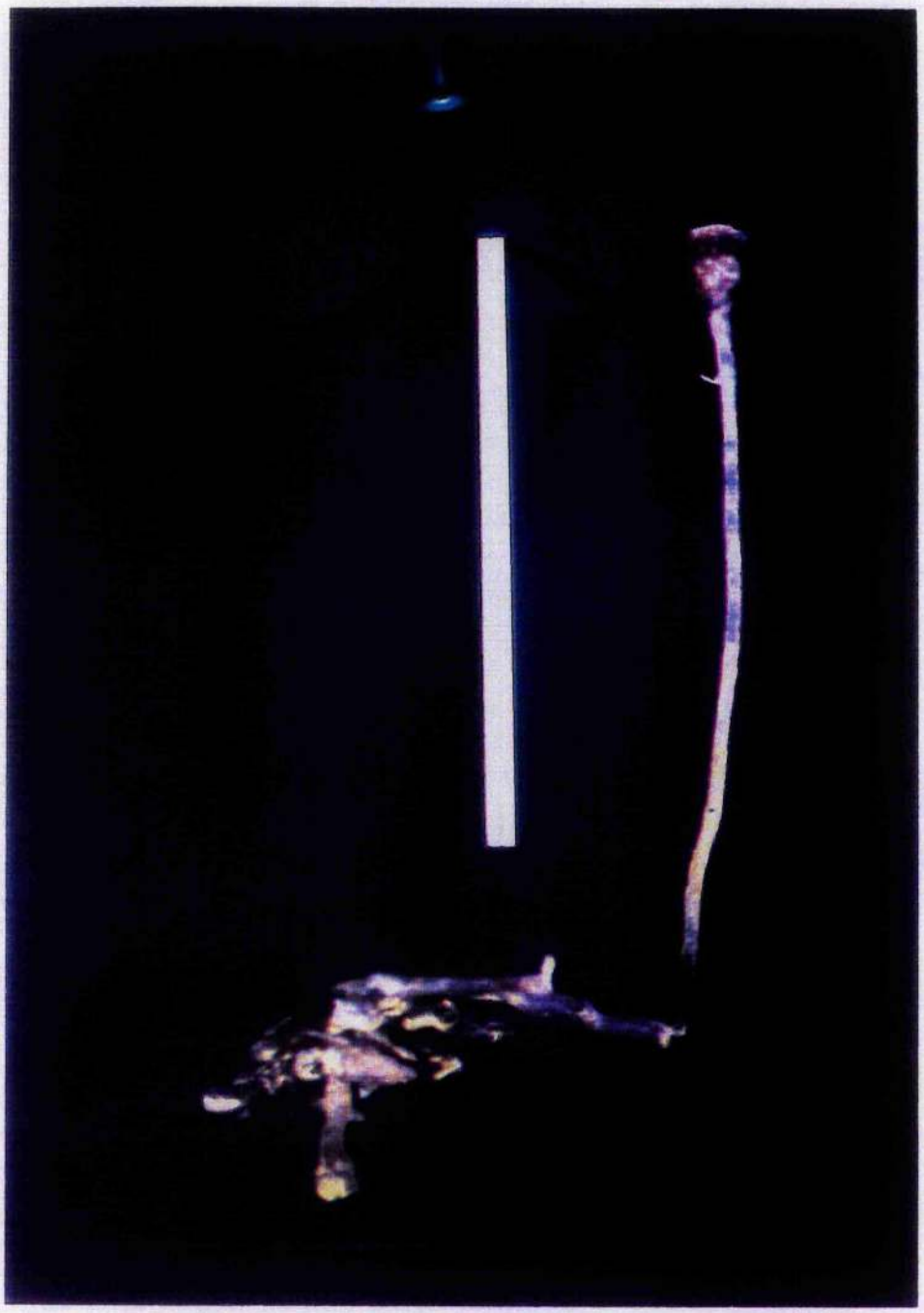

Plate 1.4 Resin cast of the burrow of *Callianassa subterranea*. Lateral view. Scale bar = 50 cm (photograph provided by R.J.A. Atkinson).

2. Sulphide in marine sediments

2.1. Introduction

2.1.1. Sulphide chemistry in water

The chemistry of sulphide in water is complex. In solution, hydrogen sulphide (H_2S) dissociates into the hydrosulphide anion (HS^-) and the bisulphide anion (S^{2-}). This speciation is highly dependent on pH (Goldhaber & Kaplin, 1975; National Research Council, 1979). Sulphide is conventionally expressed as total sulphide: [Sulphide] = $[S^{2-}]$ + $[HS^-]$ + $[H_2S]$ (Vismann, 1991a) and the proportion of H_2S and of the S^{2-} and HS^- ions in any given solution of known pH (Figure 2.1) can be calculated from the total sulphide concentration using the dissociation constants for each ion species (Goldhaber & Kaplan, 1975). These values vary with temperature and salinity but are approximately pK_1 = 6.87 and pK_2 = 13.6 in sea water (salinity = 36 ‰) at 10°C. (Goldhaber & Kaplan, 1975).

In the presence of oxygen, sulphide reacts spontaneously and may form elemental sulphur, sulphite, thiosulphate, sulphate and may also polymerise (Bagarinao, 1992). Metals, anions and organic molecules, even at trace concentrations, can also act as catalysts and significantly increase the rate of sulphide oxidation (Stumm & Morgan, 1981). Sulphide also precipitates with metals such as iron, manganese, lead, copper, cadmium, mercury and zinc. Oxidation may also occur through microbial activity (Jørgensen, 1982). Despite the susceptibility of sulphide to oxidize it can co-exist with oxygen in solution, under certain conditions, for relatively long periods of time. The half-life of sulphide in oxygen-saturated sea water is typically in the order of a few hours (Chen & Morris, 1972) and may be considerably longer for solutions made up with distilled water. A number of attempts to elucidate the kinetics and mechanisms of the auto-oxidation of sulphide have failed to establish any unified results (Stumm & Morgan, 1981).

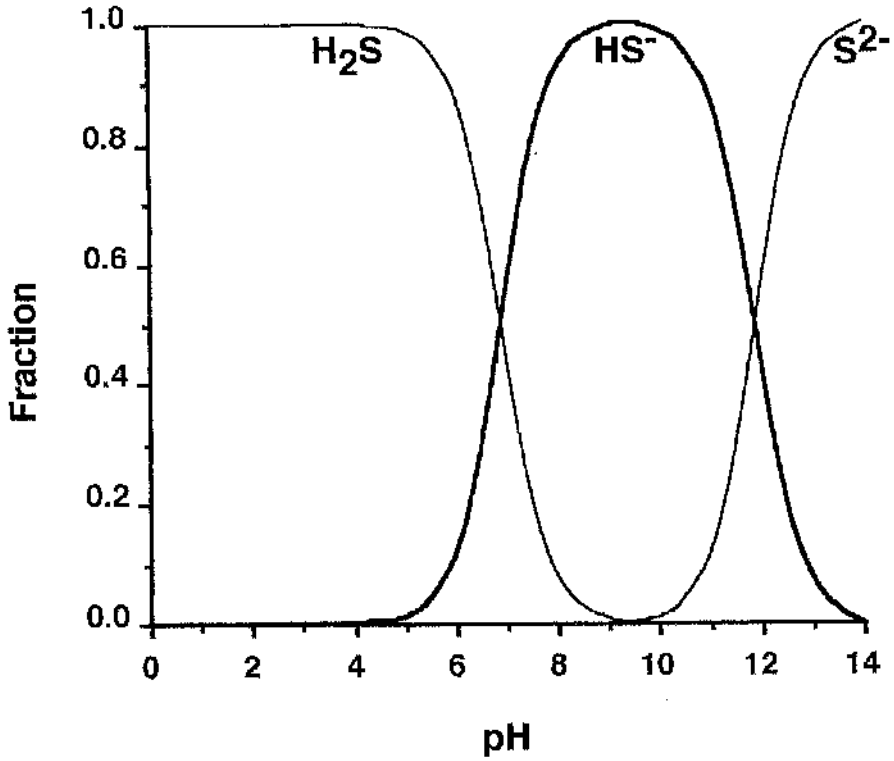

Figure 2.1 The approximate fraction of total sulphide concentration present as H_2S, HS- and S^{2-} over the pH range 0 - 14 in sea water at room temperature (National Research Council, 1979). The exact distribution varies with temperature and salinity (Goldhaber & Kaplan, 1975; Millero, 1986)

In sea water, however, the kinetics of sulphide oxidation are approximately first order, with reaction rates that decrease exponentially with increasing sulphide concentration and half-lives that decrease exponentially with increasing oxygen : sulphide ratios (Almgren & Hagstrom, 1974) and which are affected by pH and temperature (Millero, 1986). In addition, the solubility of sulphide is inversely proportional to salinity and temperature. The largely unpredictable reactive properties of sulphide so far described therefore mean that there is considerable difficulty in establishing controlled experimental conditions. This will be addressed in more detail in Chapter 3.

2.1.2. Sulphide in marine sediments

Marine sediments are characterized by vertical zonation of their physical and chemical properties. This is often generalized as an upper aerobic layer occurring above an anoxic, reduced layer (Fenchel & Reidl, 1970) which results primarily from microbial activity. The oxic and anoxic layers are characteristically separated by a transition zone where oxidized conditions give way to reduced conditions (Redox Potential Discontinuity Layer, RPDL). There may, however, be considerable variation in the scale of this zonation (Meadows & Campbell, 1980) ranging from just a few millimetres or centimetres in muddy intertidal sediments to up to 50 cm in sediments on the outer continental shelf. This may be due to a number of factors which include the sedimentation rate of organic matter, the oxygen content of the overlying water as well as the temperature, grain size and organic content of the sediment. The bioturbatory effects of meiofaunal and macrofaunal activity may also have significant effects on the concentrations of sulphide occurring in the sediment (Boaden & Platt, 1971; Nickell, 1992; Wetzel *et al.*, 1995).

Specific conditions exist within the vertical zonation of the sediment that result primarily from the activities of different bacteria. In the upper layer, heterotrophic bacteria require oxygen to break down organic matter but this is

limited by the diffusion rate of oxygen from the surface. Beneath this oxidized layer reducing and anoxic conditions exist which results from anaerobic microbial activity (Vismann, 1991a). Initially, organic material is degraded by fermenting bacteria producing substrates such as acetate, butyrate and propionate. Alternative electron acceptors, such as sulphate, nitrate or carbon dioxide are then used by bacteria to decompose the remaining organic matter (Bagarinao, 1992). This results in the production of reduced end products such as sulphide, ammonia, methane, and hydrogen (Fenchel & Blackburn, 1979a, 1979b).

The presence of sulphide is therefore a common feature of marine sediments. It is produced under anoxic conditions mainly by the activity of sulphate-reducing bacteria which are important in the breakdown of organic matter (Sorensen *et al.*, 1979). Sulphate is abundant in sea water, occurring at millimolar concentrations, which, under anoxic conditions, allow high rates of sulphate reduction. Concentrations of sulphide in marine sediments, however, may vary enormously from micromolar to millimolar concentrations (Table 2.1).

Microbial activity is also important in the oxidation of sulphide. In light, sulphide is oxidised to sulphur and sulphate by green and purple anaerobic bacteria. At the interface between anoxic and aerobic conditions sulphide is oxidized chemically and by aerobic chemolithotrophic bacteria (Nelson *et al.*, 1986; Meadows & Campbell, 1988). Sulphide is also reversibly precipitated in anoxic conditions by metals. Due to the rapid oxidation of sulphide by the chemical and biological processes described above it can only accumulate in hypoxic and anoxic environments (Cline & Richards, 1969; Chen & Morris, 1972; Invorgensen & Jørgensen, 1979; Jørgensen, 1982; Jørgensen, 1988). Animals that inhabit marine sediments may therefore be subjected to sulphide and its potentially toxic effects.

Table 2.1 Values (Range) for concentrations of sulphide in the interstitial water of marine sediments from different locations.

Location	Sulphide	Source
Mission Bay (USA) salt marshes	1 - 3.5 mM	Vetter *et al.*, 1989
St. Pol de Léon (France) intertidal flats	3.1 - 340 μM	Völkel & Grieshaber, 1992
Weser estuary (Germany) intertidal flats	70 μM	Oeschger & Vismann, 1994
Arhus Bight (Denmark) intertidal beach	26 - 1189 μM	Oeschger & Pedersen, 1994
Outer Königshaven, Waden Sea	5 μM - 1 mM	Thiermann *et al.*, 1996
North Sea (NW Europe)	190 μM	Theede *et al.*, 1969
Lower Medway estuary, Kent (England)	2.4 - 3.0 mM	Wharfe, 1977
Thames estuary (England)	80 - 400 μM	Ingold & Havill, 1984
Loch Sween (Scotland)	198 - 793 μM	Pers. comm. Clyde River purification board
Loch Sween (Scotland) near fish cages	2.6 - 3.5 mM	Pers. comm. Clyde River purification board
Clyde estuary (Scotland) inshore	12 μM	Astall, 1993
Clyde estuary (Scotland) offshore	2.7 mM	Astall, 1993

2.1.3. Sulphide exposure in the field

The specific objectives of the field work were to establish whether mud-shrimps are exposed to sulphide in the sediments that they inhabit. The concentrations of sulphide in the burrow water were therefore determined together with those in the interstitial water. The redox potential of the sediment was also measured and used as an indicator of the conditions within the

sediment. Experiments were also carried out in the laboratory to study the behavioural responses of these animals to sulphide exposure.

2.2. Materials and methods

2.2.1. Sulphide measurement

The general methods for the determination of sulphide have been established for a number of years (Vetter *et al.*, 1989). These techniques can be grouped into four categories: a) colorimetric, b) chromatographic, c) electrochemical and d) iodometric techniques. There are a number of different variants in each category. In this study four such techniques, one from each category, were compared and are described in detail below. Their accuracy, precision and practicality were investigated to determine their suitability in any given application.

2.2.1a. Colorimetric determination of sulphide

The spectrophotometric methylene blue technique for the determination of sulphide in natural waters has been established for many years (Cline, 1969; Gilboa-Garber, 1971). The determination of sulphide by this principle was originally described by Fisher in 1883 (Bagarinao, 1992). The technique has been extensively modified for specific applications since that time (Cline, 1969). The determination of sulphide in natural waters involves initial fixation and stabilization of the sulphide by addition of the sample to a solution of alkaline zinc acetate. The principle of the assay involves the formation of methylene blue from the reaction of sulphide with a strongly acidic solution of N,N-dimethyl-*p*-phenylenediamine and ferric chloride. The absorbance of methylene blue is then measured spectrophotometrically at a wavelength of 670 nm.

The technique was adapted to allow the routine determination of sulphide concentrations in small volumes (μl) of water. The fixation stage involved the addition of a 250 μl water sample to an Eppendorf tube (1.5 ml) containing 500 μl of 0.12 M zinc acetate and 125 μl of NaOH. The solution was then mixed thoroughly for a few seconds (Miximatic, Jencons Scientific Limited). The samples could then be stored at room temperature for a number of days prior to

analysis (Völkel & Grieshaber, 1992). The concentration of sulphide in the sample was determined by the addition of 125 μl of 0.3 % N,N-dimethyl-*p*-phenylenediamine in 5.5 N HCl and 125 μl of 11.5 mM $FeCl_3$ in 0.6 N HCl to the sample tube. After thorough mixing, the Eppendorf tubes were then incubated in darkness for 15 minutes at 20° C before the absorbance (at 670 nm) of the solution was determined using a spectrophotometer (Philips PU 8700 or Shimazdu UV - 1201). The colour is stable for a number of hours (Gilboa-Garber, 1971). The concentration of sulphide in the solution was then determined using a previously-prepared calibration curve constructed using solutions containing 10 - 100 μM sulphide prepared by serial dilution of a 100μM standard. This calibration conforms to Beer's law over the absorbance range of 0 - 0.8. To enable the assay to be used with a greater range of sulphide concentrations (0 - 1 mM sulphide), smaller volumes of the samples were added to the assay and the final volume of the assay mixture maintained by the addition of the appropriate quantity of distilled water. This allowed the absorbance values to be maintained within the range of the calibration curve.

The 100 μM standard solution was prepared from $Na_2S.9H_20$ crystals that had been pre-washed and then dried. The standard solutions were made up using distilled water through which nitrogen had been bubbled for at least 60 minutes to remove the oxygen from solution. The exact concentration of the 100 μM standard solution was established by iodometric back titration, as described below, and then used to correct the calibration. Distilled water blanks (normally 3 per assay) were also run and their absorbance values subtracted from those obtained for the sulphide solutions.

2.2.1b. Chromatographic determination of sulphide

The concentrations of reduced thiols, including sulphide, were also determined by High Performance Liquid Chromatography (HPLC) (Fahey *et al.*, 1981; Newton *et al.*, 1981; Vetter *et al.*, 1989; Völkel & Grieshaber, 1992).

The principle of this technique is based on the derivatizing reaction between monobromobimane and reduced inorganic and organic thiols. During this reaction a fluorescent marker is added to the reduced sulphur compounds present. These derivatized sulphur compounds can then be separated by their charge : size ratio during reverse phase HPLC and quantified by fluorimetry.

Samples (50 μl) of water and haemolymph were pipetted into 1.5 ml Eppendorf tubes containing 10μl of 46 mM monobromobimane, 50 μl 160 mM HEPES and 16 mM EDTA (pH 8.00) and 50 μl of HPLC grade acetonitrile. The samples were stored in the dark at room temperature for 30 minutes to allow the fluorescent adduct to derivatize. 100 μl of 65 mM methansulphonic acid were then added to stop the reaction. After centrifugation for 10 minutes at 11500 g, 200 μl of the supernatant were removed and stored at -70 °C. The samples remain stable for many months under these conditions (Vetter *et al.*, 1989). The preparation and fixation of tissue samples is described in Chapter 3.

The thiols in water and haemolymph samples were subsequently separated by HPLC using a Merck/Hitachi L-6200 intelligent pump combined with a Merck LiChroSpher 60 RP-select B 125-4 reversed phase column (particle size 5 μM) with a pre-column filter. Thiols were detected by a Shimazdu Fluorescence detector (RFC-530) using an excitation wavelength of 380 nm and an emission wavelength of 480 nm. After thawing, the samples were mixed thoroughly (Miximatic Jencons [Scientific] Limited) and re-centrifuged at 17000g for 20 minutes before being placed in darkness into a 36 cell Merck Hitachi AS-2000 autosampler cooled to 4 °C by a RM6 Lauda cooling unit. The total elution time for each sample was 35 minutes. The HPLC system was controlled by a Merck Hitachi D-6000 manager system which also calculated the areas of each peak from the chromatograms.

The mobile phase consisted of 0.25 % acetic acid (made up in Millipore water and adjusted to pH 4.0 using NaOH) and methanol, the proportions of which were adjusted during the run to provide an increasingly hydrophobic

gradient (Table 2.2). The acetic acid solution was filtered (Cellulose acetate, pore size 0.45 μM, Sartorius) and both the acetic acid solution and the methanol were degassed for several minutes using a water pump prior to use. A flow rate of 1 $ml.min^{-1}$ was used to elute the samples.

The concentrations of thiols in each sample were then calculated from a previously-prepared calibration based on a mixture of thiol standards (500 μM) which included sulphite, cysteine, glutathione, thiosulphate and sulphide. The sulphide standard was made up in oxygen-free distilled water and the exact concentration was determined by iodometric back titration (see below) prior to use. The standards were serially diluted with a 1:5 mix of 0.25 % acetic acid, (pH 4.0) and 12 % methanol to construct a linear calibration over the concentration range 0 - 100 μM. Völkel & Grieshaber (1992) found the relationship between peak area and concentration to be linear over the concentration range of 0 - 2.5 mM. All the samples were also initially diluted by the addition of 40 μl of the sample to 160 μl of the acetic acid/methanol mix. The concentrations of thiols in most of these diluted sub-samples fell within the concentration range covered by the calibration. A small number of samples which contained higher concentrations of thiols, however, required further dilution with the acetic acid/methanol mix to bring them within the linear part of the calibration curve.

The total concentrations of the thiols in the original samples were then calculated. In addition, during every 36 sample run of the HPLC using the autosampler, a calibration sample was run after every fifth sample to confirm the reproducibility of system. In the event of a > 5 % departure from the original calibration values for the 100 μM standards solution the system was checked and recalibrated.

The individual thiols were identified from their retention times on the column (Figure 2.2). In addition, to confirm that the peaks were thiols a small number of samples were pre-treated with 10 μl 2,2'-dithiopyridine (PDS)

immediately prior to the derivatization process. This compound has a greater affinity for reduced thiols than the monobromobimane fluorescing agent. Any sample pre-treated in this manner, therefore, has a significantly reduced peak area on the chromatogram providing confirmation that the peaks were thiol compounds.

Table 2.2 The HPLC mobile phase gradient 0.25 % acetic acid / methanol gradient in terms of % methanol.

Time (min)	% Methanol
0	12
5	12
7	20
15	30
18	30
22	50
26.1	100
29	100
29.1	12
35	12

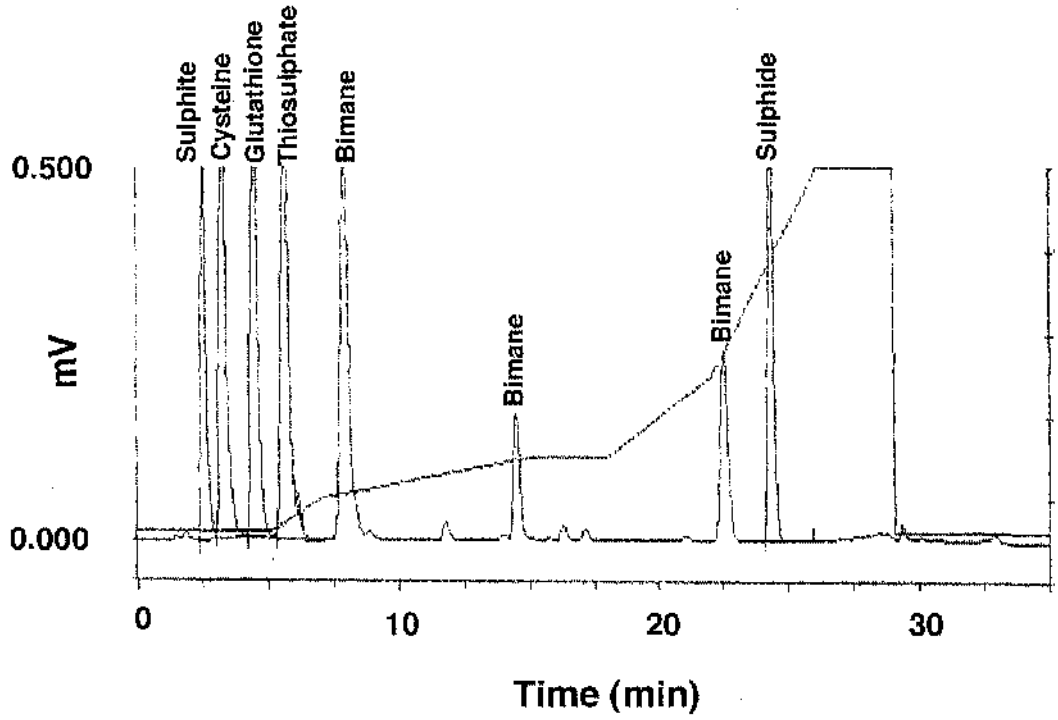

Figure 2.2 An HPLC chromatogram of a 100 μM thiol calibration. Full details of the HPLC protocol are given in 2.2.2.

2.2.1c. Potentiometric determination of sulphide

The direct determination of sulphide by an electrode has a number of distinct advantages over the two previously-described chemical techniques. An ion-selective electrode, such as a sulphide electrode, is an electrochemical half cell responding to a specific ion and obeying the Nernst equation. Rapid determinations of the concentration of sulphide can be achieved provided the pH, temperature and salinity of the sample are known. The conventional operation of sulphide ion-selective electrodes requires their calibration directly before use. This process is extremely time consuming, requires laboratory facilities and, as a result, is impractical for field studies. A technique has recently been described, however, whereby an electrode constant can be determined for any particular sulphide electrode (pers.comm. B. Vismann & L. Hagerman). This remains stable for a number of months and allows the calculation of total sulphide from the voltage generated by the electrode. This technique was adopted throughout the present study. A silver/sulphide ion selective electrode (Russell, ISE94-4169/11, Auctermuchty, Fife) in combination with a calomel reference electrode (Radiometer, Copenhagen, Denmark) was used. The principle and methodology of the technique is described below.

The tip of the sulphide electrode is constructed of silver. This detects the activity of the S^{2-} ion and measures a voltage dependent on the silver ion activity of the half cell reaction:

$$Ag_2S \Leftrightarrow AgS + Ag^{+} + e^{-}$$

This reaction can be expressed in terms of the Nernst equation, equation 1, (pers.comm. B. Vismann & L. Hagerman)

$$1).\ E = k - 2.3\ [RT / 2F] \log A_{Ag^{+}}$$

where E = the Ag - Ag_2S electrode potential in mV, k = an electrode constant specific to the Ag - Ag_2S and reference electrodes, 2.3 [RT/2F] = Nernst factor where R is the gas constant ($J.mol^{-1}.k^{\circ -1}$), F is the Faraday constant ($c.mol^{-1}$), T is temperature (°K) and $\log[A_{Ag^+}]$ is the activity coefficient of the silver cation. Defining A_{Ag^+} in terms of solubility product allows this equation to relate E to the S^{2-} activity ($A_{S^{2-}}$):

$$2).\ E = k - 2.3\ [RT / 2F] \log A_{S^{2-}}$$

The sulphide ion activity coefficient is directly related to the sulphide concentration by the ionic strength of the solution, see equation 3.

$$3).\ [S^{2-}] = A_{S^{2-}} / \gamma_{S^{2-}}$$

where $\gamma_{S^{2-}}$ = ionic strength. It was therefore possible to calculate the electrode constant k (equation 4) by the method described below in a solution of known sulphide concentration.

$$4).\ E = k - 2.3\ [RT / 2F] \log [S^{2-}]$$

The specific constant of the electrode was determined by titration. 20 ml of 30 mM sulphide solution in 1 M NaOH was placed in a beaker on a magnetic stirrer. The use of NaOH maintained the solution at pH 14 which results in all the sulphide being present as the S^{2-} species. The sulphide and reference electrodes were placed into the solution and the initial potential (E) and temperature were recorded. The solution was titrated with 0.2 M $AgNo_3$ until the end point was reached. This was determined by a rapid increase in electrode voltage when all the sulphide was consumed to form AgS^{2-}. The exact initial

concentration of sulphide in the beaker was calculated from the volume of silver nitrate consumed in the reaction and used to determine the electrode constant.

The electrode was then used to determine the S^{2-} ion activity coefficient from which the $[S^{2-}]$ concentration in any given solution of known ionic strength can be calculated. This total sulphide concentration was determined by retrospectively calculating the concentrations of the HS^- hydrosulphide anion and of hydrogen sulphide H_2S. The activity of these species is dependent on pH (Figure 2.1). This calculation was considerably simplified using tables which describe the relationship between sulphide ion activity and total sulphide concentration (see Radiometer Instructions for F1212S & F1712S sulphide selectrodes) at any given pH and ionic strength. These tables were used in conjunction with a computer spreadsheet to calculate the total sulphide concentration from the electrode voltage, pH, temperature and salinity of the solution.

2.2.1d. Iodometric determination of sulphide

Iodometric titration was used to determine the exact concentration of sulphide in the solution prior to its use in the calibration of the three techniques described previously (2.2.1a,b & c). Throughout this study sulphide solutions were always made up in oxygen-free distilled water to minimize sulphide oxidation. The water was deoxygenated by bubbling a stream of nitrogen through the solution for at least 30 minutes to achieve this. In addition, the sodium sulphide crystals ($Na_2S.\ 9H_20$) from which the solutions were made may be contaminated with thiosulphate (Vetter *et al.*, 1989) and are difficult to weigh accurately. As a result, it is often difficult to prepare accurately a solution of known sulphide concentration. Iodometric titration was therefore carried to determine the exact concentration of any prepared sulphide solution.

The technique is based on the principle that sulphide can be accurately determined by the titration of thiosulphate against iodine following the addition

of sulphide to the iodine solution (Parsons *et al.*, 1984). Two separate stages were involved. Initially, the normality of thiosulphate (nominally 0.1 N) was accurately determined. This was achieved by titrating the thiosulphate against an accurately prepared solution of iodine (0.1 N) using starch as an indicator. This was repeated three times to confirm the precision of the standardization. The exact concentration of thiosulphate was then calculated. The reaction is described below

$$I_2 + 2\,Na_2S_2O_3 \Leftrightarrow 2\,I^- + Na_2S_4O_6$$

In the second stage, 25 ml of approximately 1 mM sulphide was added to 5 ml iodine. Iodine is consumed in this reaction (see below).

$$S^{2-} + I_2 \Leftrightarrow 2I^- + S$$

The remaining iodine was then titrated with the thiosulphate. Four replicates were carried out. The difference in the volume of thiosulphate used in the two titrations was used to calculate the exact initial concentration of the sulphide solution.

2.2.1e. Comparison of the methods for the measurement of sulphide.

The reproducibility of each technique was assessed and a direct comparison was conducted between the colorimetric, chromatographic and potentiometric techniques as described in 2.2.1. A solution of approximately 1 mM sulphide was prepared and its exact concentration determined by iodometric back titration. This was than then accurately diluted to 100 μM and assayed (10 replicates) by

the colorimetric and chromatographic methods as described above and the results compared. In addition, comparisons of the colorimetric and the sulphide electrode methods were carried out by using both procedures to determine the concentrations of sulphide in prepared solutions (sulphide range 0 - 200 μM).

2.2.2. Burrow water sampling at Loch Sween, the Clyde Sea and in laboratory mesocosms

Measurements of the concentration of sulphide in the water in the burrows of *Callianassa subterranea* and *Jaxea nocturna* were carried out at Caol Scotnish, Loch Sween (56° 02.6'N, 005° 35.5'W). This site was chosen since mud-shrimps (*C. subterranea* and *J. nocturna*) were common in moderately shallow water (< 10 m depth) enabling a large number of samples to be collected by diving and because the sediments at this site were the most reduced in the Loch (Nickell, 1992). The majority of samples were taken from the burrows of *C. subterranea* which could be identified by their characteristic burrow openings, (Plate 2.1) (Nickell & Atkinson, 1995). Water samples (approximately 10 ml) were collected by inserting a length of narrow bore catheter tubing supported by a piece of stiff wire (75 cm in length) into the burrow. With practice it was possible to insert the end of the catheter tube deep into the burrow (up to 50 cm) down the vertical inhalant shaft. Water was slowly extracted from the burrow lumen using a hypodermic syringe attached to the catheter tubing. The syringe was then sealed and quickly transferred to a support boat where a sub-sample (125 μl) was pipetted into a solution of 500 μl of 0.12 M zinc acetate and 125 μl of 1.5 M NaOH. Any sediment particles drawn into the syringe during sampling were allowed to settle out for 1 - 2 minutes, in the sealed syringe, before fixation in the zinc acetate solution. Samples were subsequently analysed in the laboratory using the method of Cline (1969) as modified by Gilboa-Garber (1971), (see section 2.2.1.).

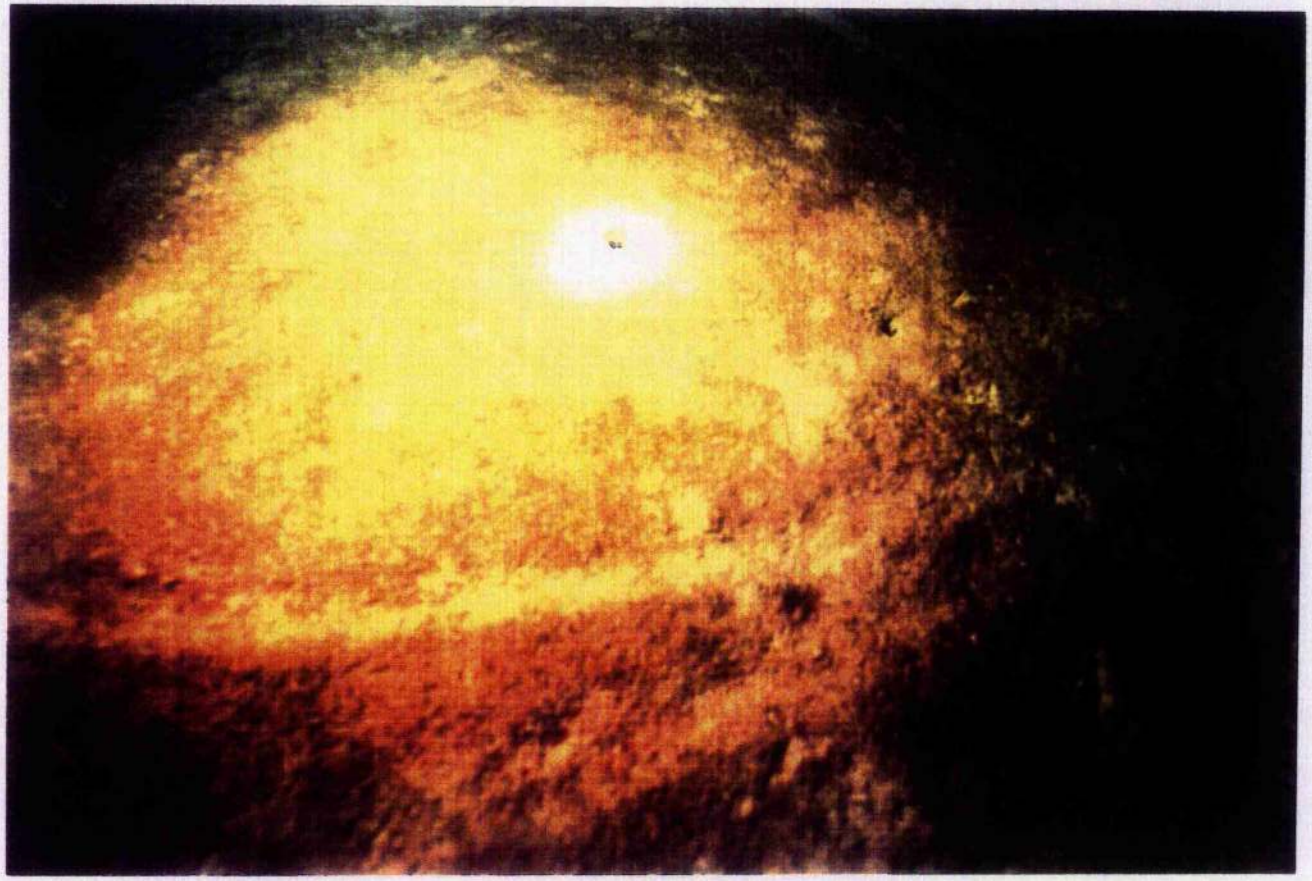

Plate 2.1 An example of the characteristic burrow openings formed by *Callianassa subterranea* photographed at Coal Scotnish Loch Sween using a Nikonos V underwater camera fitted with a wide angle 15 mm Nikonos lens set to infinity and f 5.6. A 35 mm ASA 200 film was used.

Water samples were also taken from the burrows of *Upogebia deltaura*, at Farland Point (55° 46.4' N 004° 53.2') and White Bay (55° 47.8' N 004° 54.4' W), Isle of Cumbrae, from depths of approximately 20 m. Additionally, the concentration of sulphide and the pH of the water in the burrows of a number of thalassinidean species (*Upogebia deltaura, C. macandreae, C. subterranea,* and *Jaxea nocturna*) that had been constructed in laboratory mesocosms were also determined.

Each laboratory mesocosm consisted of a tank (50 cm x 15 cm x 75 cm) filled to a depth of > 40 cm with mud sediment taken from the Firth of Clyde or from Loch Sween. The tanks were placed in a temperature-controlled aquarium (10 ± 1 °C) and the sediment allowed to settle for several weeks before individual animals were placed in each of the aquaria. Burrows were normally established within a few days but water samples were not taken until after the burrows had been established for more than 2 months to ensure that conditions within the burrows had stabilized.

Water samples were subsequently taken from the burrows using a similar procedure to that used in the field (see section 2.2.1a.) except that an in-line filter (5 μm) was used to prevent any suspended sediment entering the syringe. The transparent Perspex walls of the aquaria allowed the position of the sampling tube within the burrow to be seen. The concentrations of sulphide in the water samples were assayed using the colorimetric method described above (see section 2.2.1a.). The pH of the water samples were also determined using a Russell pH electrode (Auctermuchty, Fife) and pH meter (Jenway 3020).

2.2.3. Concentrations of sulphide in interstitial water in Loch Sween and Clyde sediments

A number of attempts were made to use a suction interstitial water sampler to determine the concentration of sulphide in the interstitial water of the sediment in the vicinity of the mud-shrimp burrows throughout the Loch. The sampler

was constructed from a small plastic collector densely packed with glass wool, attached to a 10 cm length of 0.8 mm i.d. glass tubing which was inserted into the sediment (Völkel & Grieshaber, 1992). A 2 ml syringe was then used in an attempt to withdraw the interstitial water by suction. The sampler, however, very quickly became blocked by the very fine sediment particles present; median size in the order of 4 μm (Anderson, 1989). An alternative approach was therefore adopted. A small number of sediment cores (9) were taken, by diving, at the Caol Scotnish site during May 1995 and July 1996. In addition, a small number of Craib cores (3) were also taken in different parts of the loch using the Research Vessel Aora at Sailean Mhòr (56° 01.4'N 005° 35.7'W) Sròn Bheith (56° 00.8' N 005° 36.5'W) and Achnamara (56° 00.7'N 005° 35.6'W) during May 1995. The corer (58 cm by 10 cm diameter) used during diving sampling was constructed from a length of brass tubing which could be split longitudinally. This was pushed into the sediment during a dive, sealed with two large bungs and brought to the surface. It was then placed in a nitrogen-filled glove bag which was continuously flushed from a plastic tube connected to 10 l high pressure nitrogen cylinder (BOC). The flow rate of the gas was controlled using a two-stage Nitrogen gas regulator (Apo Flame Equipment). The core was subsequently transferred to the shore and then split inside the bag. The effectiveness of this method for removing oxygen from the glove bag was tested in the laboratory prior to the field work by placing an oxygen electrode inside it (see section 4.2.1). Samples of sediment (approximately 5 g) were taken at 5 - 10 cm intervals along the core's length and placed in 10 ml plastic tubes that had been filled with nitrogen. The tubes were then centrifuged for 5 minutes at 6500g to separate the interstitial water from the sediment (Vetter *et al.*, 1989). A subsample of the water was then immediately fixed in alkaline zinc acetate (see section 2.2.1a.). The total time taken to process the sediment core was < 30 minutes and the samples were maintained under nitrogen for this period in an attempt to reduce the possibility of sulphide oxidation. The fixed

samples were subsequently returned to the laboratory where their sulphide content was determined using the colorimetric method as described above (see section 2.2.1a). Craib cores (15 x 8 cm) taken using the RV Aora were handled and analysed as described above.

2.2.4. Sediment Eh

Additional sediment cores were also taken using the same technique from the Caol Scotnish site to enable determinations of the redox potential of the sediment to be carried out. Immediately after the core had been split lengthways the Eh of the sediment was determined by inserting a combination Eh electrode (CMPTRL, Russell, Auctermuchty, Fife) into the sediment at 5 - 10 cm intervals along the length of the exposed core. The electrode was inserted into the middle of the core and held in a retort stand to support it. The redox voltage normally became stable within 2 - 3 minutes although, occasionally, this took considerably longer. Box cores were also taken using the RV Aora from sites in the Firth of Clyde in the vicinity of the *Calocaris macandreae* trawl area (55° 46.7' N, 004° 58.8' W). The Eh of these sediment cores was also determined as described above.

2.2.5. Thiol concentrations in freshly caught animals

Attempts to obtain samples of water from the burrows of *C. macandreae* in the field were unsuccessful. This was due in part to the shape of the burrows which prevented the insertion of the catheter deep into the burrow (see Plate 1.3) and to the fact that, even in Loch Sween, most *C. macandreae* occur at depths > 20 m making access by diving difficult . An alternative, indirect technique to determine whether *C. macandreae* is exposed to sulphide in its environment was therefore adopted (Gorodezky & Childress, 1994). Samples of haemolymph were taken from mud-shrimps immediately after capture by trawling and 'bait pump' (see section 3.2.1. Animal collection and maintenance). A sample of

haemolymph (50 μl) was taken from each animal by inserting a narrow gauge hypodermic needle (25G) into the pericardium and withdrawing the haemolymph with a 1 ml syringe. The concentrations of accumulated thiol metabolites in the haemolymph were determined by high performance liquid chromatography following derivatization with monobromobimane as described above (2.2.1b.). The concentration of thiols in the haemolymph of a single specimen of *Callianassa subterranea* captured using the 'bait pump' at the Caol Scotnish site was also determined (see Chapter 3.2.1. Animal collection and maintenance).

2.2.6. Behaviour of mud-shrimps during sulphide exposure

The effect of sulphide on the burrow irrigation behaviour of the mud-shrimp *Calocaris macandreae* was investigated. A glass burrow having three openings (approximately 200 ml in volume) was manufactured in the Department of Chemistry at the University of Glasgow. Its shape and size were similar to burrow casts of this species taken from the field (Atkinson, 1986; Atkinson & Taylor, 1988). Three small sampling apertures were positioned along the length of the burrow and sealed using a small plastic taps. A large glass tank (volume = 6 l) was placed above the glass burrow with the three openings sealed into the base of the tank. The tank and the burrow were then filled with previously UV-sterilized sea water (salinity = 35 ± 1 ‰). Prior to any addition of sulphide an air stone and air line were used to oxygenate the overlying sea water reservoir in the tank. The experiment was conducted in a thermostatically controlled cool room at 10 ± 1 °C.

Animals used during the experiment were collected from the Firth of Clyde and maintained in the sea water aquarium at in the University of Glasgow at 10 ± 1 °C and a salinity of 35 ± 1‰ (see Chapter 3 for details). An individual animal (0.9 - 2.0 g) was placed in the glass burrow and allowed to settle overnight. The overlying sea water in the reservoir tank allowed the animal to draw water into its burrow. The mud-shrimp's general behaviour was then

observed and the frequency and duration of pleopod beating of the mud-shrimp were determined over 20 - 30 minute periods. During each observation period two stop-watches were used to cumulatively time the duration the animal remained quiescent and the durations of pleopod beating. The frequency of pleopod beating was estimated by counting during the irrigation activity. Initially, control measurements were determined for 8 individual animals in the glass burrow filled with sea water. The individual mud-shrimps were then exposed to two different concentrations of sulphide. This was achieved by slowly injecting of 2 ml of nominally 10 mM sulphide and 100 mM sulphide, for the low and high sulphide exposures respectively, into the glass burrow through the sampling apertures near the animal. The pH of both sulphide solutions was adjusted to 8.0 prior to the experiment. The concentration of sulphide in the burrow water throughout the period of the experiment was determined at 4 - 5 minute intervals (see section 2.2.1a.). Control injections of distilled water and sea water were also carried out.

2.2.7. Statistics

The following statistical procedures were used throughout this study (Zar, 1984). Individual data sets were initially tested for normality (Anderson-Darling test) and homogeneity (Bartlett's test or Levene's test). The pairwise analysis of parametric data was conducted by a pooled *t*-test. Any non-parametric data were tested separately by individual pairwise comparison using the following tests: *t*-test not pooled (data normal but with heterogeneous variance), Mann-Whitney (data not normal and homogeneous variance) or Mood test (data not normal and heterogeneous variance). Multiple comparisons of data sets with homogenous variance were performed by one way analysis of variance and subsequent Tukey pairwise comparisons. In some cases these data sets were transformed by square root or log functions to increase homogeneity. Analysis of co-variance was used to compare regression lines. The level of significant difference adopted

was $p < 0.05$. The letters a/b/c/ were used to indicate significant differences graphically (i.e. $a \neq b \neq c$). Minitab version 10 was used to conduct these analyses. Data values cited in the text throughout this thesis are means ± standard deviation (unless otherwise stated) and also include sample sizes. The number of samples in any treatment occasionally varied. During the experiments a small number of animals occasionally died and were therefore ignored. In addition, it was not always possible to maintain large numbers mud-shrimps in the aquarium at the University of Glasgow due to collecting difficulties.

2.3. Results

2.3.1. Sulphide measurement

Both the colorimetric and chromatographic techniques were highly reproducible. In a comparative experiment to determine the concentration of sulphide in a nominally prepared 100 μM solution values of sulphide were found to be 108.6 ± 8.5 μM (colorimetric) and 111.0 ± 6.1 μM (chromatographic) and were not significantly different (*t* - test, $p > 0.05$). Only very small differences were observed between replicate samples and the accuracy and precision of the colorimetric and chromatographic methods were similar (Figure 2.3). Both techniques were also found to be in agreement with the iodometric titration (*t* - test, $p > 0.05$) used to standardize the sulphide concentration. A significant correlation was also found between the determination of sulphide by the potentiometric and colorimetric techniques. (Figure 2.4), (ANOVA, $p < 0.05$, $r^2 = 0.969$).

2.3.2. Burrow water sampling at Loch Sween, the Clyde Sea and in laboratory mesocosms

The concentrations of sulphide in the burrows of *C. subterranea* at the Caol Scotnish site (Loch Sween) were quite variable with an overall mean concentration of 36.6 ± 44.3 μM (range 0 to 206 μM, n = 37). A seasonal trend in the sulphide concentration of the burrow water was seen with the concentrations being significantly lower during the summer (Figure 2.5). No sulphide was detected, however, in the burrows of *Upogebia deltaura* at the two sites in the vicinity of the Isle of Cumbrae, although low concentrations of sulphide in the interstitial water were detected in cores (see section 2.2.3.) taken from the surrounding sediment (Farland Point 7.1 ± 4.0 μM, n = 16; White Bay 7.0 ± 5.1 μM, n = 15).

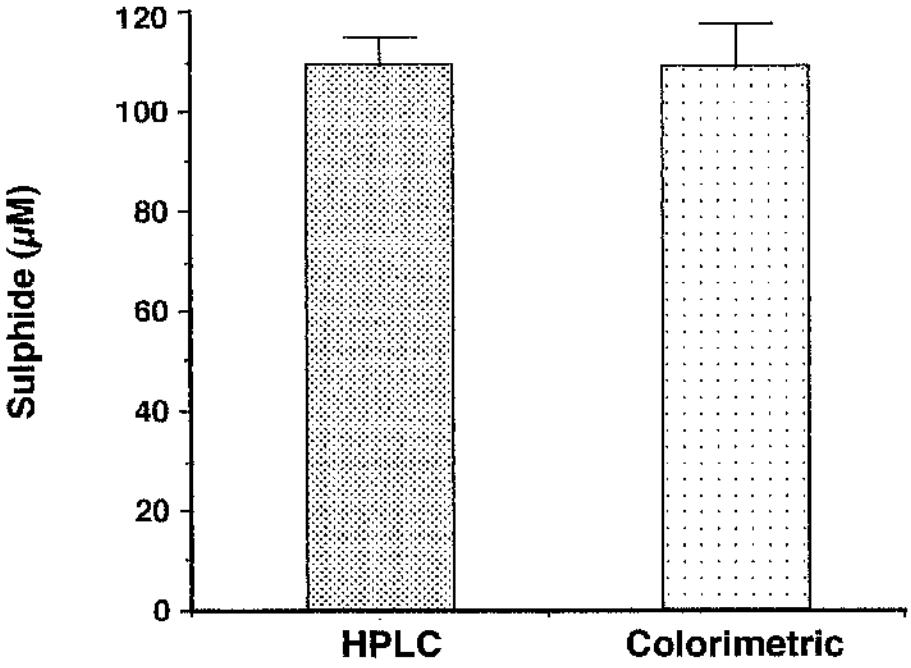

Figure 2.3 Comparison between Colorimetric and HPLC sulphide determination techniques. The concentration of the sulphide solution measured by iodometric back titration was 116 μM. Values are means ± SD, n = 10. Pooled t - test, $p > 0.05$.

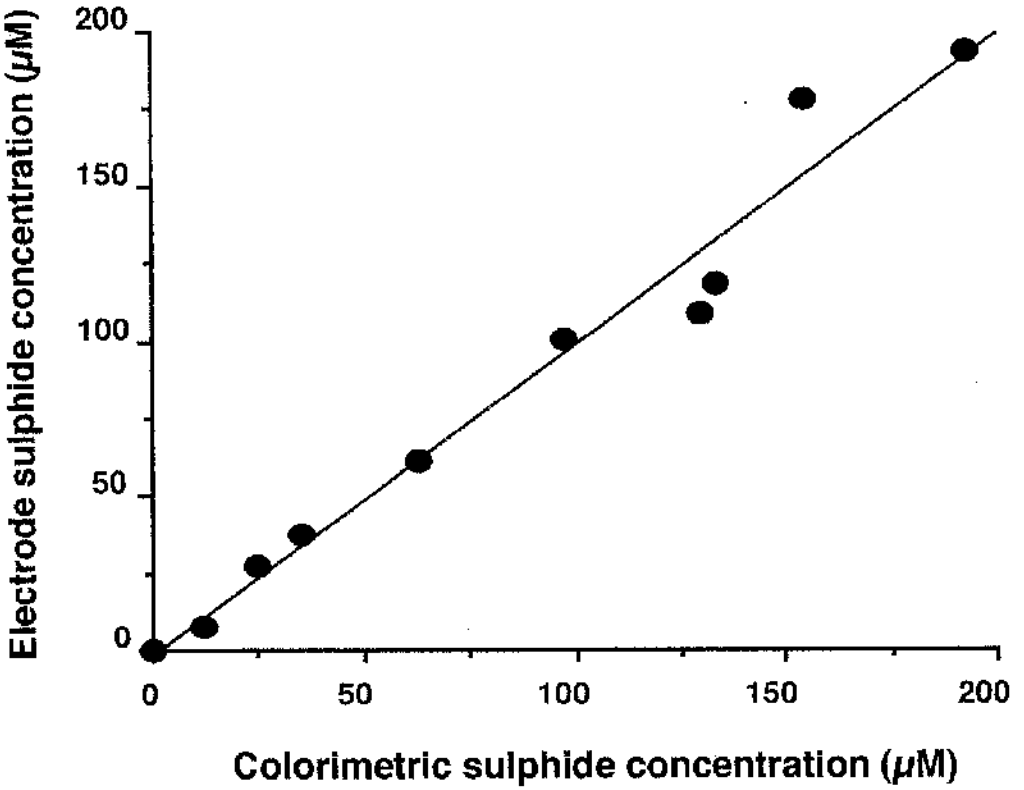

Figure 2.4 The correlation between the values for sulphide concentrations recorded using the potentiometric and colorimetric techniques. Values are means, n = 20 for the colorimetric method and n = 10 for the potentiometric method. ANOVA, $p < 0.05$, $r^2 = 0.969$.

The concentrations of sulphide in the water from burrows constructed in the laboratory were found to be low but detectable, (1.9 ± 1.4 μM, n = 117, Range = 0 - 6.9 μM, MOOD $p < 0.001$). A significant difference in pH was also seen between the burrow water (7.5 ± 0.2) and the overlying water (7.9 ± 0.2) (pooled t - test, $p < 0.001$, n = 15).

2.3.3. Concentrations of sulphide in interstitial water in Loch Sween sediments

The concentration of sulphide found between 1 - 50 cm in the interstitial water of sediment from Loch Sween were found to be extremely variable using the technique described above (34.1 ± 189.5 μM, range = 0 - 1591.9 μM, n = 86). Although sulphide was detected in the majority of the samples, it generally occurred at low micromolar concentrations. In addition, sulphide was not present at detectable concentrations in a number of the samples.

2.3.4. Sediment Eh

The sediment at the Caol Scotnish site was found to be very reduced. A seasonal trend was observed in the Eh of the sediment with the values being significantly more negative during the winter (Figure 2.6). This trend was strongly correlated with the concentrations of sulphide measured in mud-shrimp burrows (Figure 2.5. & 2.6). Only the upper layers (< 5cm) of the sediment were found to have positive Eh values during the spring and summer. It was not possible, however, to determine the Eh value of the initial 0 - 1 cm of sediment because of disturbance during the coring procedure. This uppermost layer of sediment was, however, a much lighter colour which would appear to indicate that it was oxidized. In contrast to the observations made at Loch Sween, the sediment cores taken from the Firth of Clyde at the *Calocaris macandreae* trawl area were seen to be reduced during the summer but oxidized during the winter (Figure 2.7).

2.3.5. Thiol concentrations in freshly caught animals

Although water samples could not be obtained from the burrows of *Calocaris macandreae*, measurements of the concentrations of thiols in the haemolymph of *C. macandreae* revealed the presence of significant concentrations of sulphite and thiosulphate (Figure 2.8). Sulphide was also detected in the haemolymph but at very low micromolar concentrations. In addition, the concentration of thiosulphate in the haemolymph of a single specimen of *Callianassa subterranea* captured using a 'bait pump' at Caol Scotnish during August was 32.8 μM. Sulphite and sulphide were also present at lower concentrations (12.6 μM and 8.4 μM respectively).

Thiosulphate was found to be the predominant product of sulphide 'oxidation' in mud-shrimps with sulphite occurring as a secondary metabolite when exposed to sulphide (see Chapter 3). The concentrations of thiosulphate and sulphite in the haemolymph of *Calocaris macandreae* showed a significant increase during the summer months suggesting that the concentration of sulphide in the sediment may change seasonally. This trend was correlated with the seasonal variation in the redox potential of the sediment which showed a significant decrease during the summer (Figure 2.8).

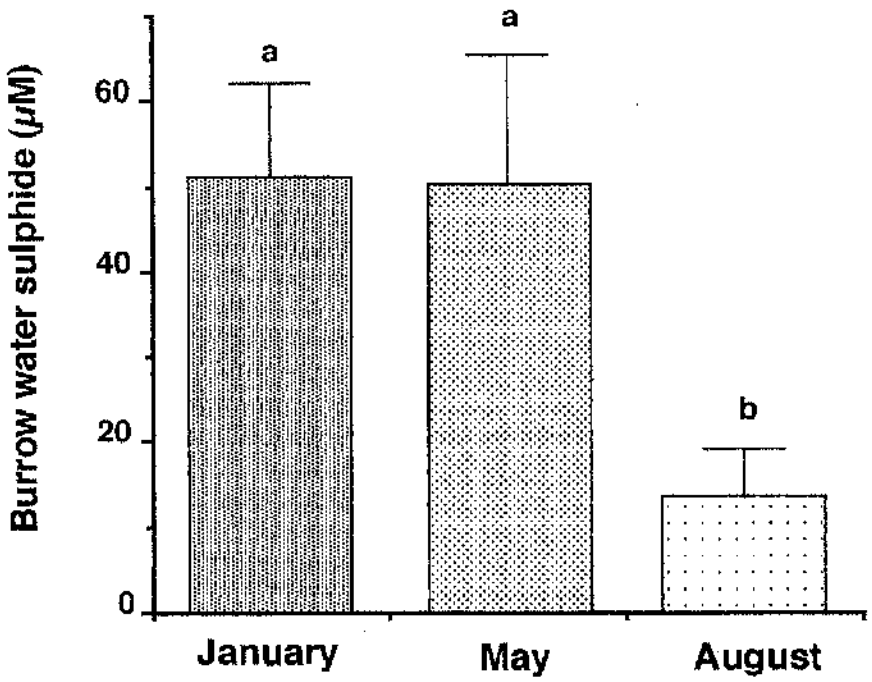

Figure 2.5 The concentration of sulphide in the burrow water of *Callianassa subterranea* in Caol Scotnish during January, May and August. Values are means ± SD. ANOVA & Tukey, $p < 0.05$, a/b = significantly different.

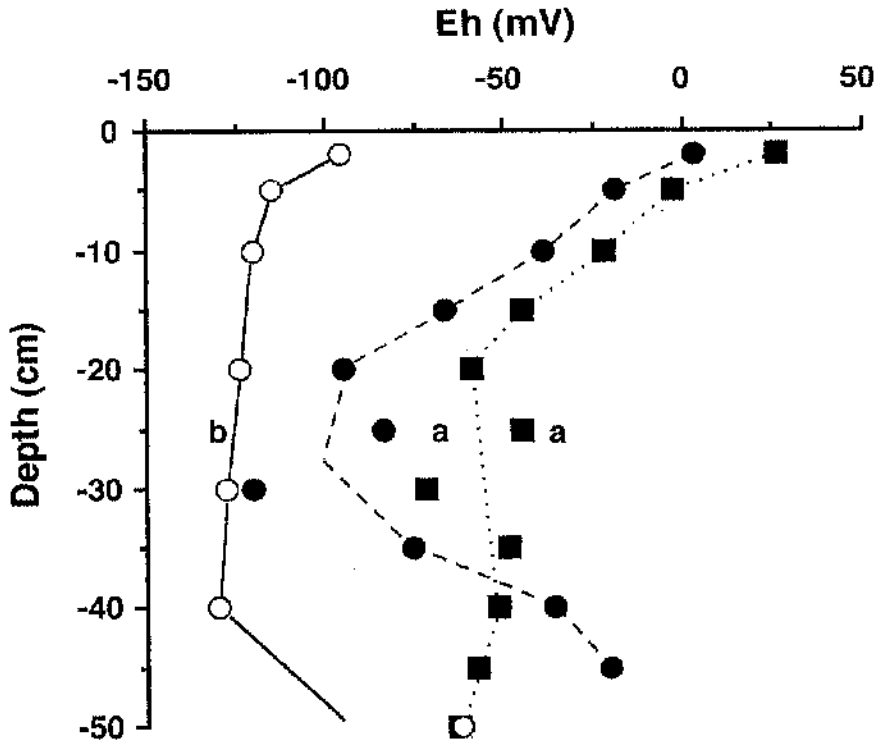

Figure 2.6 Eh values at depths of 5 - 50 cm in cores taken from the surrounding sediment during January (open circles), May (closed circles) and August (closed squares). ANOVA & Tukey, $p < 0.05$, a/b = significantly different.

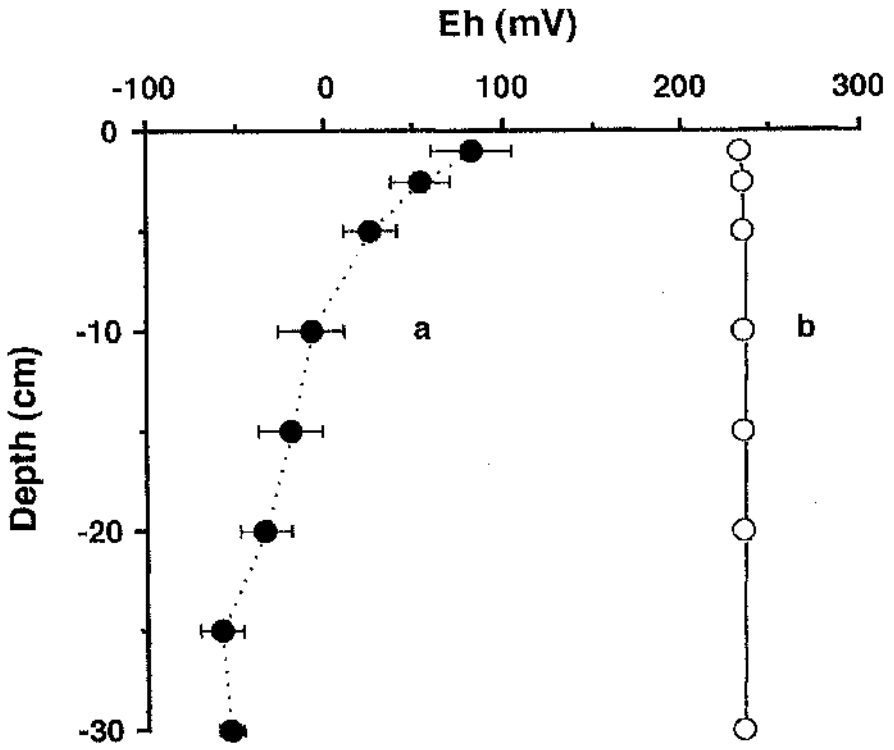

Figure 2.7 Eh values at depths of 5 - 50 cm in sediment cores taken from the *Calocaris macandreae* trawl area during January, n = 1, (open circles) and June, n = 5, (closed circles). *t* - test $p < 0.0001$, a/b = significant difference.

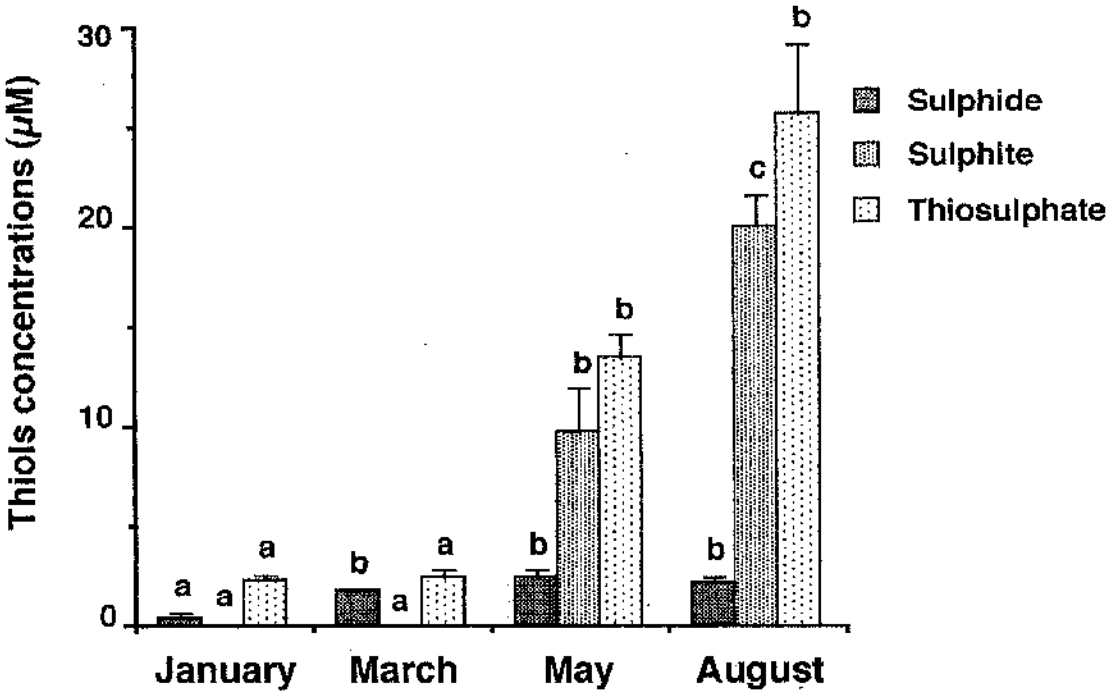

Figure 2.8 The concentration of thiosulphate in the haemolymph of freshly-caught *Calocaris macandreae* taken from the Firth of Clyde during January, March, May and August. Values are means ± SE, n = 10 - 30. ANOVA + Tukey and Mann-Whitney $p < 0.05$, a/b/c = significant difference.

2.3.6. Behaviour of mud-shrimps during sulphide exposure

The irrigation activity of the mud-shrimp *Calocaris macandreae* was observed to be very low under the control conditions of the experiment (Figure 2.9 & 2.10). During these control observations the basic pattern was found to be long periods of no activity interspersed with short periods of intermittent irrigation. The initial control observations and the control injections of distilled water or sea water were found not to differ significantly (ANOVA + Tukey, $p < 0.05$). The mud-shrimps, however, were observed to occasionally walk in the burrow although this was not correlated with pleopod beating. In addition, during the injection of the different solutions into the burrow, the mud-shrimps showed an initial tendency to move away from the source of injection. During exposure to 0.36 ± 0.32 mM sulphide the irrigation activity did not alter significantly (ANOVA + Tukey, $p > 0.05$) from that of the control animals. During exposure to higher sulphide concentrations (6.25 ± 4.82 mM) a significant (ANOVA + Tukey, $p < 0.05$) increase in the frequency of pleopod beating and the duration of bouts of irrigation was observed (Figure 2.9 & 2.10). In addition, the mud-shrimps were observed to tail-flip in an escape-type response away from the sulphide source to a different part of the burrow and also to swim to one of the burrow openings using its pleopods. Even during the exposure to the high sulphide concentrations, however, the mud-shrimps did not abandon their burrows.

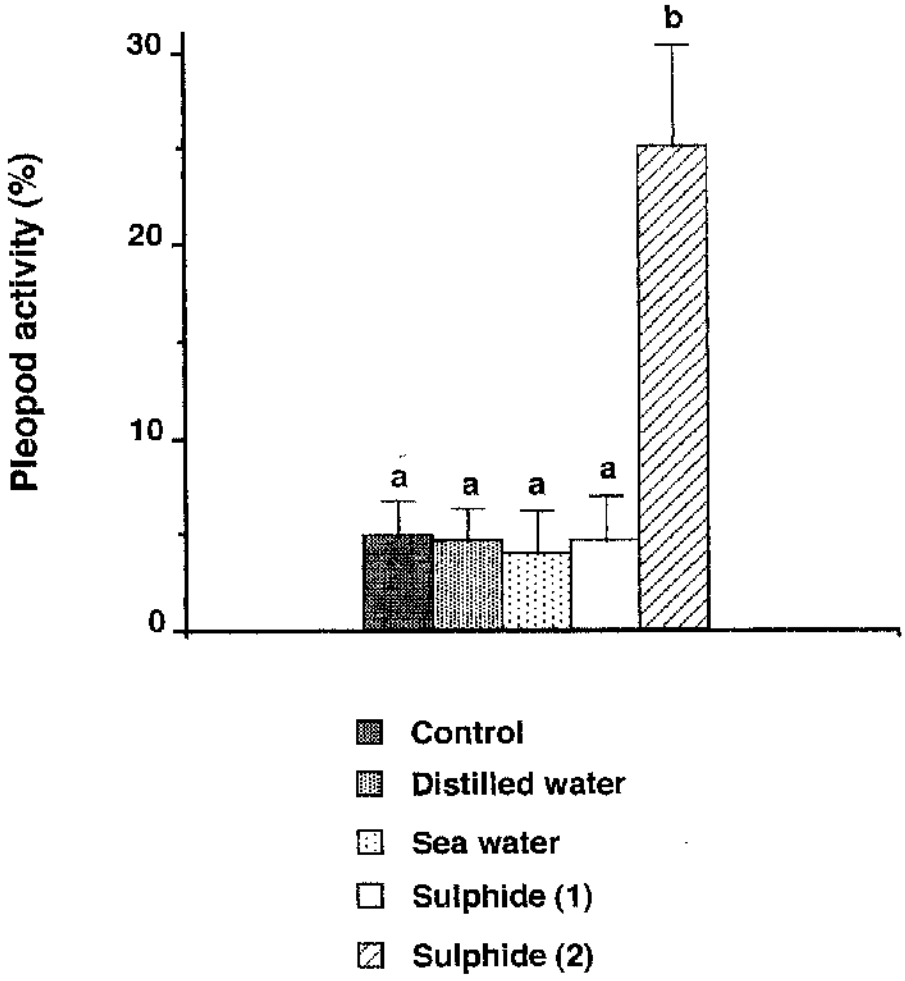

Figure 2.9 The effect of sulphide on the pleopod activity (% duration) of *Calocaris macandreae* maintained in a laboratory glass burrow Sulphide 1 = 0.36 ± 0.32 mM, Sulphide 2 = 6.25 ± 4.82 mM. Values are means ± SE, n = 7 - 8. ANOVA + Tukey $p < 0.05$, a/b = significant difference.

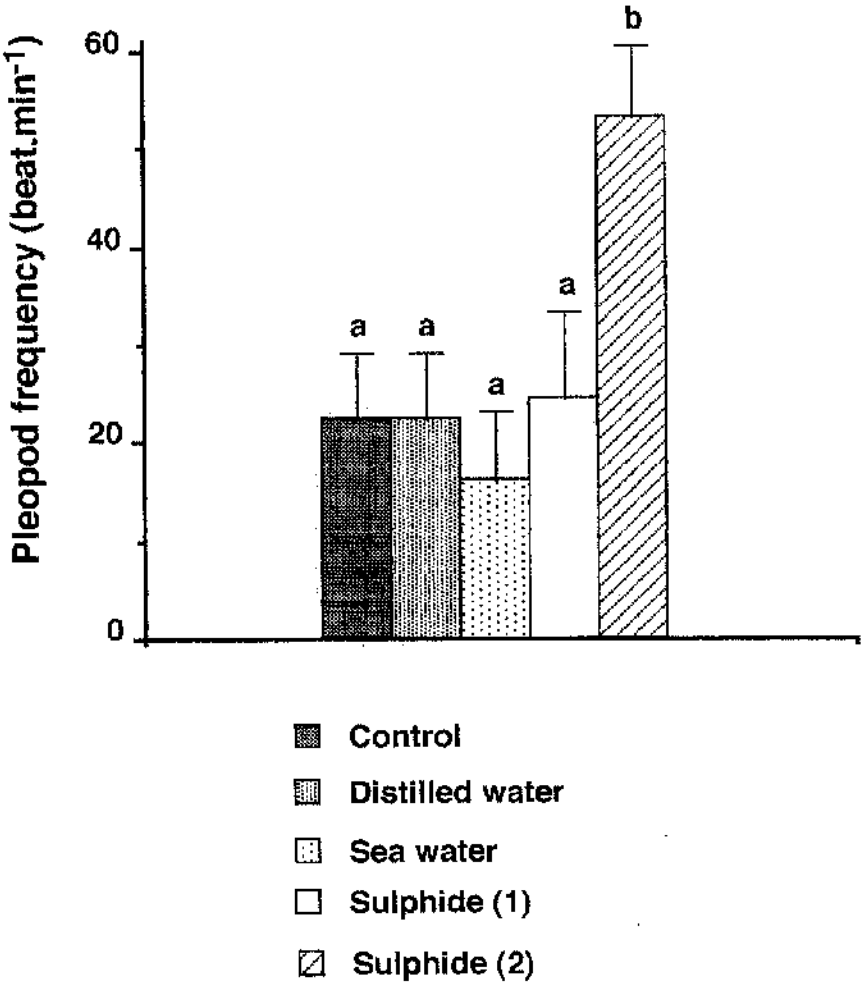

Figure 2.10 The effect of sulphide on the pleopod beat frequency (beats. min^{-1}) of *Calocaris macandreae* maintained in a glass burrow in the laboratory. Sulphide 1 = 0.36 ± 0.32 mM, Sulphide 2 = 6.25 ± 4.82 mM. Values are means ± SE, n = 7 - 8. ANOVA + Tukey $p < 0.05$, a/b = significant difference.

2.4. Discussion

2.4.1. Sulphide measurement

The colorimetric, chromatographic, potentiometric and iodometric methods used throughout this study to determine sulphide were found to give comparable and equally reproducible results. The colorimetric method offers practicality and simplicity which allows the determination of relatively large numbers of samples in the laboratory. In addition, it is particularly suitable for fieldwork since the samples can be easily fixed on site by the addition of a known sample volume to a pre-prepared tube containing alkaline zinc acetate prior to subsequent analysis in the laboratory.

In contrast, the chromatographic technique was found to be a fairly time-consuming procedure. Samples must be derivatized for 30 minutes in the dark at room temperature and each takes 35 minutes to analyse using an HPLC system. Nevertheless, this technique has a number of distinct advantages the most important of which is that it is possible to measure simultaneously a range of reduced thiols including sulphide, sulphite, thiosulphate, glutathione and cysteine in both water and tissue samples. After the initial derivatization process the samples can be stored for over a year without deterioration. This method is also the most sensitive technique (high nanomolar concentrations can be detected).

The electrode technique was found to be too cumbersome for field use due to the need for the accurate additional measurements such as pH, salinity and temperature. In the laboratory, however, the method proved to be a valuable technique for routine determinations.

Iodometric titration is not very suitable for the analysis of large numbers of samples because of the length of time taken for each titration. It is also impractical for use in the field because of the requirement for considerable quantities of glassware and access to laboratory facilities. However, because of its inherent accuracy and precision this technique was used to standardize the

sulphide solutions used for calibration of the other three methods.

2.4.2. Sulphide exposure in the field

The results from the analyses of the concentrations of sulphide in the burrow water of *Callianassa subterranea* together with indirect evidence from the accumulation of thiosulphate in the haemolymph of trawled *Calocaris macandreae* indicate that mud-shrimps can be exposed to significant concentrations of sulphide in their burrows. Sulphide was not detected, however, in the burrows of *Upogebia deltaura* sampled from around the Isle of Cumbrae. This may be because obtaining water samples from deep within the Y shaped burrow is very difficult; sampling is hindered by the small openings to the burrows and the convoluted shafts which prevent the insertion of the catheter sampler deep into the burrow where sulphide may be present. *Upogebia* spp. also tend to irrigate their burrows frequently as they are primarily suspension feeders (Nickell & Atkinson, 1995). This is in contrast to species of mud-shrimps, such as *C. subterranea* and *Jaxea nocturna*, that are primarily deposit feeders. The ingress of oxygenated sea water drawn into a burrow by feeding currents would clearly prevent sulphide from accumulating in the burrow.

There are very few recorded measurements of the concentrations of sulphide from the burrows of other crustaceans and marine invertebrates. Sulphide has been recorded, however, in the burrows of callianassids in Bermudan sediments (Waslenchuk *et al.*, 1983) and in the burrow water of the echiuran worm, *Urechis caupo*, and of the polychaete worm, *Arenicola marina* (Table 2.3). These authors noted that the concentrations recorded were very variable and that in a large number of burrows sulphide could not be detected (Arp *et al.*, 1992; Völkel *et al.*, 1995). This was also found to be the case in the present study.

Table 2.3 Comparison of the sulphide concentrations in the burrows of four marine invertebrates and in the interstitial water of the sediment. Values are means ± SD and the range is also given. The values of n are given in brackets.

Location	Species	Burrow sulphide	Pore water sulphide	Source
Loch Sween (Scotland)	*Callianassa subterranea*	37 μM ± 44, 0 - 206 μM (37)	34.1 ± 185.5 0- 1.6 mM (86)	This study
Coot Pond (Bermuda)	*Neotrypaea* (as *Callianassa*) spp.	8.4 μM ± 8.9, 2 - 26 μM (6)	0.1 - 1 mM	Waslenchuk *et al.*, 1983
St Pol de Léon (France)	*Arenicola marina*	0 - 32 μM (94)	0 - 252 μM	Völkel *et al.*, 1995
Elkhorn Slough, California (USA)	*Urechis caupo*	41 ± 7 μM 10 - 66 μM (10)	2.6 ± 0.3mM	Arp *et al.*, 1992

In all the species investigated to date, however, despite the presence of high micromolar sulphide concentrations in the surrounding interstitial water, the concentrations of sulphide were found to be considerably lower in the burrow water. This suggests that sulphide may diffuse into the burrow from the surrounding interstitial water despite the steep gradients which may exist between anoxic and aerobic conditions at the burrow wall. Some of the sulphide penetrating the burrow will, however, be oxidized even at the low oxygen tensions in the burrow water (see section 1.5). Additionally, during burrowing activity these animals may also be exposed to the higher sulphide concentrations that are present in the interstitial water, even though the duration of this exposure is likely to be relatively brief.

In this study the sulphide concentrations in the interstitial water at Loch Sween were found to be very variable (0 - 1.6 mM). The mean concentrations

of sulphide in the burrow water and in the sediment interstitial water were, however, very similar. Considerable difficulty was encountered, however, in accurately determining the concentration of sulphide in the interstitial water in Loch Sween. Attempts to use the suction interstitial water sampler failed (see section 2.2.3) as the sampler became quickly blocked by the very fine sediment particles present (median size = 4 μm, Anderson, 1989). The centrifugation technique as described above (2.2.3.) was therefore adopted to separate the interstitial water from the sediment (Vetter *et al.*, 1989). Under laboratory conditions the nitrogen filled glove bag was found to contain virtually no oxygen. In the field some oxygen may have been entered the glove bag during handling, manipulation, and centrifugation of the core. The determination of interstitial water sulphide in this study may therefore be an underestimate of the true values.

Exposure to significant amounts of sulphide may be a common problem facing animals that inhabit marine mud sediments. Significant concentrations of sulphide may also occur in the water column under certain conditions, such as during eutrophication events and during the stratification of the water column (Kitching *et al.*, 1976; Aure & Stigebrand, 1989; Mann & Lazier, 1991). Such effects typically occur in sea lochs and fjords (Kitching *et al.*, 1976; Rosenburg, 1980) where water movement is severely restricted. Hypoxia, anoxia and sulphidic conditions may occur in the deeper water beneath the thermocline. The mud-shrimp *Calocaris macandreae* is a common inhabitant of Loch Hyne, Eire and can be found in the vicinity of the thermocline (Kitching *et al.*, 1976). It would be interesting to establish how these animals cope with the possible exposure to sulphide and to hypoxia as the thermocline develops during the summer months

In the present study low micromolar concentrations of thiosulphate and sulphite were found in the haemolymph of every freshly-caught mud-shrimp examined and provides further evidence that they are exposed to sulphide in their

natural environment. Similar low micromolar concentrations of thiosulphate have been recorded in the coelomic fluid of freshly caught *Arenicola marina* from the intertidal mud flats at St. Pol de Léon, France (Völkel *et al.*, 1995). Thiosulphate concentrations in the coelomic fluid collected during July were significantly higher than those collected in October, suggesting a seasonal trend in sulphide exposure. The sulphide concentrations in the coelomic fluid of *A. marina* increased significantly during tidal emersion. but this was not reflected by an increase in thiosulphate concentration. Similarly, thiosulphate has been recorded in the haemolymph of the hydrothermal vent crab *Bythograea thermydron* (Gorodezky & Childress, 1994). Furthermore, the thiosulphate concentrations in these animals at the same site measured over a period of 6 months were similar suggesting that there may be rather little variation in the concentrations of sulphide to which they are exposed. There were, however, considerable inter-site differences in the concentrations of thiosulphate in the haemolymph. No indication was given, however, of the presence of sulphide in the haemolymph of freshly-captured crabs. In the present study the concentrations of thiosulphate in the haemolymph of *Calocaris macandreae* taken from the Clyde were significantly higher in summer than in winter. This suggests that these animals may be exposed to higher concentrations of sulphide throughout the summer.

The concentration of sulphide in any exposure must also be considered in terms of pH as this significantly alters sulphide toxicity. At physiological and environmental pH values, (pH approximately 7 - 8) H_2S and HS^- are the predominant species of sulphide (see section 2.1.1.). Since H_2S is uncharged and freely diffuses across membranes it is the most toxic form of sulphide (Bagarinao, 1992). Therefore, the lower pHs found in the burrows of mud-shrimps constructed in the laboratory compared with those of the overlying sea water are significant since more of the toxic H_2S will be present. The decrease in pH presumably results partly from the animal's respiratory activities which

may cause the burrow environment to become hypercapnic and hypoxic (Astall, 1993). The blood of marine animals typically has a lower pH than sea water (pH = 7.8 - 7.9 in decapods). The pH values of intra-cellular fluids are also approximately 0.5 pH units lower than that of the blood (Vetter *et al.*, 1991). The buffering capacity of blood and cells may compensate for this decrease in the pH of the burrow water. The fraction of total sulphide present as the toxic H_2S species, however, will be considerably higher at these lower pHs and hence more toxic conditions will prevail.

The Eh values of the sediments at the Caol Scotnish site inhabited by *Callianassa subterranea* and *Jaxea nocturna* were found to be negative at depths in excess of 5 cm throughout the year. Eh was found to decrease rapidly in the shallow surface layers (0 - 20 cm). This trend is typical of many marine sediments (Meadows & Campbell, 1988). The boundary between oxidized and reduced conditions, where oxygen is depleted and sulphide is produced by sulphate reducing bacteria, occurs at Eh values of less than +200 mV. At Eh values of less than +100 mV molecular oxygen is absent although an oxygen buffer system exists which results in positive Eh values despite the absence of oxygen (Pearson & Stanley, 1979; Meadows & Campbell, 1988; Nickell, 1992). The Eh values at the site in the Firth of Clyde, inhabited by *Calocaris macandreae*, were found to be negative during the summer and positive during the winter. The negative Eh values of reduced anoxic sediments recorded in this study therefore provide indirect evidence for the presence of sulphide in Loch Sween and in the Clyde. These data also indicate, however, that there is considerable temporal variability in sediment sulphide concentrations.

The concentrations of sulphide in the water of *Callianassa subterranea* burrows were greater in winter than in summer. In addition, Eh values were significantly more negative during winter. This contrasts with the data of Thiermann *et al.*, (1996) who showed that the highest concentrations of sulphide in intertidal mud flats occurred during the summer. The concentrations

of thiosulphate, used as an indicator of sulphide exposure (Gorodezky & Childress, 1994) in the tissues of *Calocaris macandreae*, were higher in summer than in winter suggesting that these animals may be exposed to higher concentrations of sulphide at this time. The contrast between the two sets of data from the present study may be attributable to inter-site variability (see Thiermann *et al.*, 1996). Large quantities of leaf material and detached macroalgae may accumulate in Loch Sween during the autumn and winter (Atkinson, 1987) and the degradation of this material may be responsible for the higher sulphide concentrations recorded during the winter months. In contrast, at the sites around the Isle of Cumbrae where *C. macandreae* were obtained, the spring-early summer plankton bloom may account for the main organic input to the sea bed which could result in elevated sulphide concentrations during the summer.

2.4.3. The effect of sulphide on the behaviour and distribution of marine invertebrates.

Another aspect which has received relatively little attention, despite its clear importance to invertebrates inhabiting marine sediments, is the effect of sulphide on behaviour. Whilst the physiological mechanisms of sulphide 'oxidation' have been established in a variety of marine invertebrates and are now believed to be relatively common, very few investigations into the behavioural responses of marine invertebrates to sulphide have been reported. The intertidal lug worm *Arenicola marina* has, however, been shown to reduce the ventilation of its burrow during exposure to sulphide (Wohlgemuth, 1995). Miron & Kristensen (1993a) investigating the behavioural responses of three nereid polychaetes to the injection of sulphide into their burrows reported that *Neanthes* (as *Nereis*) *virens* reacted strongly to sulphide by increasing the duration of periods of irrigation. The behaviour of *Nereis succinea* was similar to *N. virens* although a less pronounced reaction was seen. In contrast *Hediste* (as *Nereis*) *diversicolor* was largely unaffected by sulphide except at high sulphide concentrations. In this

study, the burrow irrigation behaviour of the mud-shrimp *Calocaris macandreae* was investigated during exposure to sulphide. The intensity and duration of burrow irrigation (pleopod beats) were not affected by sulphide until concentrations reached millimolar concentrations. General activity within the burrow and irrigation behaviour may be infrequent even under hypoxic conditions (Astall, 1993; Astall *et al.*, 1997, Forster & Graf, 1992; 1995). These results, as has been reported in the polychaete worms *H. diversicolor* and *A. marina* (Miron & Kristensen, 1993a; Wohlgemuth, 1995) suggests that the mud-shrimp *C. macandreae* tolerates exposure to sulphide. At low millimolar concentrations of sulphide, however, the mud-shrimp was considerably more active. This increase in irrigation activity may reduce the concentration of sulphide in the burrow by drawing in water from above the sediment. Whether the mud-shrimps are exposed to these higher concentrations of sulphide in their natural environment, however, seems unlikely (see section 2.4.1).

It has also recently been established that the lug worm *Arenicola marina* can detect sulphide in its environment (Toulmond *et al.*, 1995). Clearly the ability to detect and avoid sulphide would be advantageous. This has also been established in the burrow-dwelling amphipod *Corophium volutator* which actively avoids sulphide (Meadows *et al.*, 1981). This may be a common feature of mobile marine invertebrates which are not tolerant to sulphide allowing them to avoid exposure to potentially toxic concentrations of sulphide.

There is clearly considerable variation in the concentrations of sulphide in marine sediments. How does sulphide affect the distribution of marine invertebrates? It has recently been established that the spatial distribution of three species of nereid polychaetes was strongly correlated to presence of sulphide in interstitial water (Vismann, 1990; Miron & Kristensen, 1993b). *Nereis succinea* was found in high sulphidic conditions whereas the distribution of *Neanthes* (as *Nereis*) *virens* was restricted to lower sulphidic areas. *Hediste* (as *Nereis*) *diversicolor*, however, showed a broader distribution to variations in

interstitial water sulphide. Tiermann *et al.* (1996) studying a range of abiotic factors, including sulphide, at different sites in the Wadden Sea, established that the faunal composition was dominated by annelids and showed little variation despite large differences in the concentrations of sulphide. Although this aspect has not been specifically addressed in this study there may be a strong correlation between the distribution of mud-shrimps and the concentration of sulphide in their environment. This study has clearly established, however, that mud-shrimps may be routinely exposed to low micromolar concentrations of sulphide, although there appears to be considerable spatial and temporal variability. This agrees with the data for a variety of other marine invertebrates. The presence of sulphide in the marine environment is often overlooked and yet it must be of considerable ecophysiological significance to marine fauna that inhabit sedimentary biotopes.

2.4.4. Summary of Chapter 2

The main points of Chapter 2 in relation to mud-shrimps are summarized below:

a. A comparison of four techniques for the measurement of sulphide was conducted to determine their accuracy, precision and compatibility. The advantages and disadvantages of each method are discussed.

b. Reduced and sulphidic sediments may be inhabited by mud-shrimps and they may be exposed to low micromolar concentrations of sulphide in their burrows throughout the year. The concentration of sulphide may also change seasonally.

d. Thiosulphate, an 'oxidation' product of sulphide is commonly found in the haemolymph of freshly captured mud-shrimps and indicates exposure to ambient sulphide.

e. Micromolar concentrations of sulphide were found not to affect the irrigation behaviour of the mud-shrimp *Calocaris macandreae* suggesting tolerance of sulphide exposure.

3. Sulphide tolerance, 'oxidation' and metabolism in mud-shrimps.

3.1. Introduction

3.1.1. Sulphide tolerance

Animals inhabiting sulphide rich environments tend to have an increased tolerance of sulphide in comparison with species that are less likely to be exposed to it, and to have a range of adaptive strategies to counter its potentially toxic effects (Bagarinao, 1992). Despite the fairly extensive literature on sulphide tolerance in a range of marine and freshwater invertebrates, very few studies have been conducted on the sulphide metabolism of Crustacea (Vetter *et al.*, 1987; Vismann, 1991b; Astall, 1993; Hagerman & Vismann, 1993; Gorodezky & Childress, 1994). In comparison with other benthic invertebrates, it is generally believed that crustaceans have a low tolerance of sulphide and that they may use behavioural adaptations to overcome its toxic effects (Theede *et al.*, 1969; Vargo & Sastry, 1978; Meadows *et al.*, 1981; Vismann, 1991a).

Vetter *et al.* (1987), however, investigated the sulphide tolerance and metabolism of a range of decapod Crustacea native to habitats associated with varying degrees of sulphide. Perhaps not unexpectedly they discovered that the hydrothermal vent crab *Bythograea thermydron* was significantly more tolerant of sulphide poisoning than three non-vent crabs, *Cancer antennarius*, *Pachygrapsus crassipes* and *Portunis xantusii*. *B. thermydron* is directly exposed to very high levels of hydrogen sulphide emitted from the vents (Tunnicliffe, 1991) and lacks chemolithoautotrophic endosymbiotic bacteria that have been observed in a number of animals (Hand & Somero, 1983; Powell & Somero, 1986b; Powell & Arp, 1989; Somero *et al.*, 1989; Childress *et al.*, 1991; Tunnicliffe, 1991). In addition, the heart rate of *B. thermydron* was remained constant during exposure to ambient concentrations of 1400 μM, whilst the three non-vent crabs were severely affected when sulphide levels

reached 300 μM. The non-vent crabs did, however, show some tolerance of sulphide and this was found to be correlated in an incremental manner with their likely environmental exposure to sulphide (see below). A small number of other crustaceans such as the Baltic isopod *Saduria entomon* have also been found to tolerate sulphide (Vismann, 1991b).

Mud-shrimps have been shown to be exposed to sulphide in their natural environment (see Chapter 2). The aims of the studies reported in this chapter were to examine the tolerance of the different species to sulphide exposure and to investigate the mechanisms by which this is achieved.

3.1.2. Sulphide 'oxidation' and metabolism

Vetter *et al.* (1987) established that the hydrothermal vent crab's tolerance of sulphide could be explained by their discovery of an active sulphide 'oxidation' mechanism. Sulphide was not excluded from the animal and it was suggested that the hepatopancreas was the site of enzymatic sulphide 'oxidation' offering protection from its toxic effects. Thiosulphate was the major end-product that accumulated in the haemolymph to concentrations > 1 mM. Interestingly, the three non-vent crabs exhibited similar adaptations although they were significantly more sensitive to sulphide poisoning. *Pachygrapsus crassipes*, which burrows in sulphide rich sediments in salt marshes, was less affected by sulphide than *Cancer antennarius*, which lives on sand and rocks and *Portunus xantusii*, which is free swimming, was the most susceptible to sulphide. Vetter *et al.* (1987) suggested, therefore, that this mechanism of sulphide 'oxidation' also occurs in sulphide-tolerant crustaceans.

Vismann (1991b), using similar techniques to Vetter *et al.* (1987), established that the isopod *Saduria entomon* was also unable to prevent sulphide from entering its body and also detoxified sulphide in an oxygen-dependant mechanism in the hepatopancreas and muscle tissues (Hagerman & Vismann, 1993). The end-products were found to be thiosulphate and sulphite. Vismann

(1991b) also demonstrated an oxygen independent binding of sulphide by iron in the hepatopancreas, accounting for 40% of the sulphide removed. The crustacean hepatopancreas accumulates iron, and that of *Saduria entomon* turns black with sulphide exposure (Gibson & Barker, 1979; Vismann, 1991b).

Uncharacterized sulphide oxidases have been suggested as the biochemical system responsible for sulphide 'oxidation' in a range of marine invertebrates and fish (Powell & Somero, 1985; Vetter *et al.*, 1987; Bagarinao & Vetter, 1989; Powell & Arp, 1989; Vismann, 1990). The possibility of a sulphide 'oxidation' mechanism in the thalassinidean mud-shrimp *Calocaris macandreae* was investigated during the present study.

3.1.3. Symbiotic chemoautotrophic sulphur bacteria

Many marine invertebrates that inhabit sulphidic environments have been found to harbour symbiotic sulphide-oxidizing bacteria (see Chapter 1). These bacteria have a dual function; they supply the host with chemosynthetically fixed carbon (Felbeck, 1985) and, in addition, 'detoxify' sulphide preventing the inhibition of the host's aerobic metabolism (Arp *et al.*, 1995). Whilst this form of association has been found to be relatively common among many animals from sulphidic environments it has not been observed in crustaceans. Since there have been relatively few studies of the sulphide metabolism of Crustacea, the occurrence of this symbiotic association with chemoautotrophic sulphur bacteria in some species may have gone undetected. The possible presence of symbiotic sulphur bacteria in the mud-shrimps, *Callianassa subterranea* and *Calocaris macandreae* was therefore investigated.

3.1.4. Sulphur amino acid metabolism

Sulphur amino acid metabolism is an essential aspect of protein construction. Only two amino acids contain sulphur, methionine and cysteine. These amino acids are required by eukaryotic organisms but only cysteine can be synthesized; methione has to be obtained from their food (Baker, 1987). Eukaryotic sulphur amino acid metabolism, however, may differ significantly between protozoans and metazoans (Thong & Coombs, 1985a; 1985b). In virtually all eukaryotic organisms, cysteine is manufactured from methionine-based precursors during the trans-sulphuration pathways. The direct incorporation of hydrogen sulphide into these metabolic pathways has until recently not been observed in eukaryotes. It has, however, now been described in eukaryotic parasitic protozoans, in some gastro-intestinal nematode worms and in mammalian cells during the little-studied reversible reaction between serine and hydrogen sulphide producing cysteine (Walker & Barrett, 1992; Thong & Coombs, 1985b). This reaction is catalysed enzymatically by L-serine sulphydrase (EC 4.2.1.22). In addition, homocysteine another important sulphur-containing compound in the trans-sulphuration pathways of parasitic protozoans, can also undergo enzymatic desulphuration with the production of hydrogen sulphide, ammonia and a ketobutyrate. This resaction is catalyzed by the enzyme homocysteine desulphurase (EC 4.4.1.1.2) which has been found to have very high activitities in some parasitic protozoans (Thong & Coombs, 1985a).

The possibility exists, therefore, that the incorporation of sulphur into the amino acid cysteine, and subsequently into the sulphur amino acid trans sulphuration pathways, could act as a way of removing sulphide that has entered the body of an aquatic invertebrate. During this study the mud-shrimp *Calocaris macandreae* was investigated for the presence of the enzymes L-serine sulphydrase and homocysteine desulphurase in its tissues.

3.2. Materials and methods

3.2.1. Animal collection and maintenance

Calocaris macandreae Bell were collected from depths of approximately 80 m in the vicinity of the Isle of Cumbrae, Firth of Clyde, Scotland (55° 46.7' N, 004° 58.8' W) using an Agassiz trawl. *Callianassa subterranea* (Montagu) and *Jaxea nocturna* Nardo were collected from depths of 10 - 30 m in Loch Sween, west coast of Scotland (56° 02.6' N, 005° 35.5' W) using an anchor dredge and by using a mechanical diver-operated suction sampler (Alvey 'king size' bait pump), using a technique similar to that described in Manning (1975). *Upogebia deltaura* Leach were collected from depths of approximately 20 m in White Bay, Isle of Cumbrae (55° 47.8' N 004° 54.4' W) by anchor dredging. Immediately after capture, the animals were returned to the University of Glasgow where they were maintained under a 12:12 h light : dark regime in a recirculating sea water aquarium (salinity = 35 ± 1 ‰; temperature 10 ± 1 °C). Preliminary observations had indicated that these mud-shrimps frequently engage in agonistic behaviour which may occasionally result in the death of one of the animals. To prevent this, the mud-shrimps were kept individually in small perforated tubes in larger aquarium tanks through which sea water was continuously circulated. The mud-shrimps were used within one month of capture and during this time they were not fed.

3.2.2. Sulphide tolerance of mud-shrimps

Specimens of *Calocaris macandreae, Callianassa subterranea* and *Jaxea nocturna* were exposed to a range of sulphide concentrations under both normoxic and hypoxic conditions to assess their tolerance to sulphide. The animals (fresh weight range = 1.0 - 2.5 g; n = 7 - 30) were placed in individual perforated tubes (to prevent aggression between individuals) in a glass aquarium (volume = 6 l) in a temperature-controlled room (10 ± 1 °C). Sea water having the required sulphide concentration (0.6 - 1 mM) was produced using two

peristaltic pumps (Watson Marlow 101U and 501U) to pump a concentrated sulphide stock solution and sea water (salinity = 35 ± 1 ‰) into a small, continuously-stirred mixing chamber (Figure 3.1). A flow-through technique (flow rate approximately 160 ml.min^{-1}) was used to establish an equilibrium in the concentration of sulphide during each exposure. The sulphide-laden sea water then passed via a UV sterilizer (to suppress bacterial growth) into the exposure tank which was continuously stirred (Underwater stirrer, Rank Brothers). The tank was covered with a piece of polystyrene to reduce sulphide volatilization to the atmosphere. The sulphide-laden sea water, however, was allowed to overflow out of the tank into a sump before being drained away.

The Po_2 of the water was normally reduced to below normoxic levels due to the chemical oxidation of sulphide. Since the reduction in Po_2 was generally < 10 %, no attempt was made to increase the Po_2. Exposure to sulphide under hypoxic conditions was carried out by bubbling a gas mixture (supplied by a precision gas mixing system) having the required Po_2 through the water in the tank. The pH of the water was controlled by prior adjustment of the stock solution and by the addition of CO_2 to the gas mixture, creating a naturally buffered bicarbonate system, to ensure that any variability in pH was reduced (8.19 ± 0.08).

The Po_2 of the water was monitored continuously using an oxygen electrode and meter (Strathkelvin 1302 oxygen electrode & oxygen meter model 781) connected to a pen recorder. The anode of the oxygen electrode was gold plated prior to use to protect it from sulphide poisoning (Revsbeck & Ward, 1983; O'Brien & Vetter, 1990) (see section 3.2.5.). The oxygen electrode was calibrated prior to use against aerated sea water at the experimental temperature of 10 ± 1 °C and against a solution having a Po_2 of zero Torr (sodium sulphite in 0.01 M sodium tetraborate).

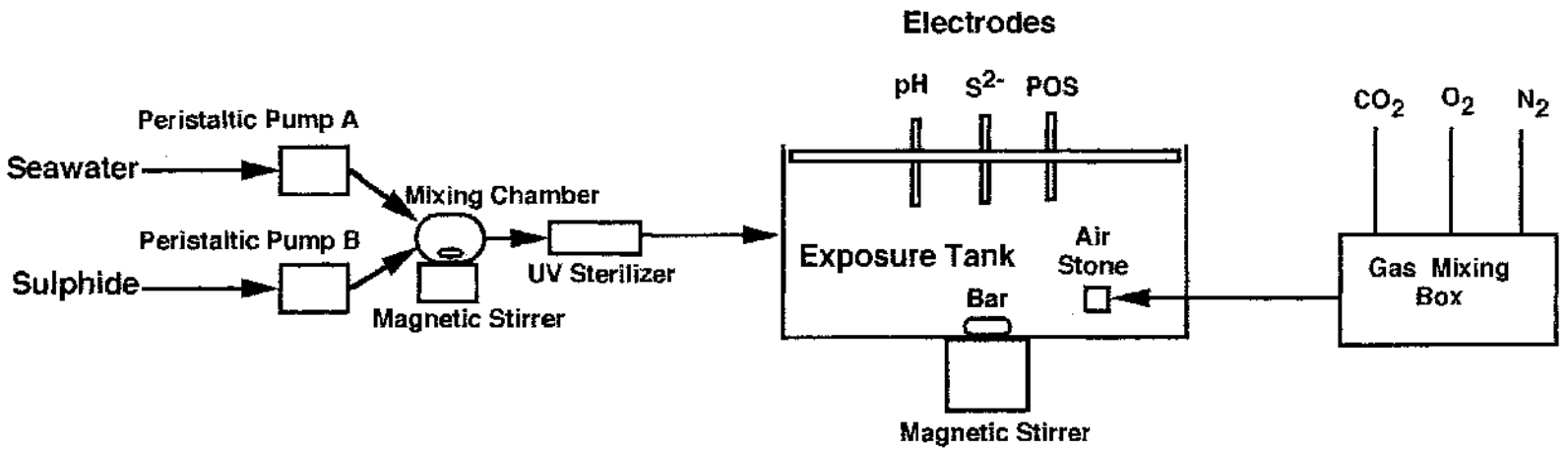

Figure 3.1 Diagram of the flow through system used to expose mud-shrimps to specific sulphide concentrations, oxygen tensions and pHs. These parameters were monitored using sulphide (S^{2-}), oxygen (POS) and pH electrodes.

Sulphide concentrations were continuously measured with a sulphide electrode (Russell silver/sulphide ion selective electrode ISE94-4169/11 combined with a Radiometer calomel reference electrode, Copenhagen) (see section 2.2.1c). The sulphide concentration was also confirmed at hourly intervals using the spectrophotometric method of Gilboa-Garber (1971). Full details of this technique are given in Chapter 2. The pH of the water was also monitored hourly (Russell pH electrode, Auctermuchty, Fife & Jenway 3020 pH meter). The survival of the three species of mud-shrimps was determined during exposure to a range of sulphide concentrations. Mortality was assessed by observing individual animals at regular intervals (1 hour intervals during the initial phase of the experiment but at 4 - 10 hour intervals thereafter). Animals were classified as dead if the pleopods no longer showed any signs of activity and the animals failed to respond to gentle tactile stimulation. This was confirmed by placing these animals in fresh sea water to observe whether they recovered.

3.2.3. Sulphide 'oxidation' and metabolism in mud-shrimps

Specimens of *Calocaris macandreae* were exposed to 87 ± 14 μM sulphide over 24 hours, under near normoxic conditions (Po_2 > 140 Torr) to examine the metabolic effects of sulphide exposure. The exposure apparatus is described above (see section 3.2.1.). The pH of the exposure medium, however, was reduced to 8.00 ± 0.05 to increase the relative proportion of the toxic H_2S present. At this pH the relative amount of toxic H_2S represents 8 % of the total sulphide present (Goldhaber & Kaplan, 1975). The concentrations of accumulated thiol metabolites in the haemolymph of *Calocaris macandreae* (n = 9 - 11) were determined using the technique described in Chapter 2, (see section 2.2.1b.). Blood samples were taken after 1, 6, 12, 18 and 24 hours and then analysed by high performance liquid chromatography (HPLC) following derivatization with monobromobimane (see section 2.1.1b.).

The concentrations of reduced thiols in hepatopancreas and abdominal muscle tissue were also determined at the same time intervals as the blood samples. Approximately 10 - 20 mg of hepatopancreas and abdominal muscle (taken from the first abdominal segment) were immediately dissected from an animal after removal from the sea water containing sulphide. The tissue samples were then placed in pre-weighed 1.5 ml Eppendorf tubes containing 10 μl of 46mM monobromobimane, 100μl of 160 mM HEPES / EDTA 16 mM pH 8 and 50μl of acetonitrile. These were re-weighed to establish the accurate weight of the tissue and then the tissue was homogenized in the tube for 2 minutes using a plastic homogenizer. The sample was then placed in the dark at room temperature for 30 minutes to allow derivatization of the thiols to occur before 100 μl of 65 mM methansulphonic acid was added. The samples were centrifuged for 10 minutes at 11500 g and 200 μl of the subsequent supernatant were pipetted into another 1.5 ml tube. The samples were then stored at -70 °C in darkness before analysis by HPLC. Care was taken throughout the assays to reduce the exposure of the tissue to light which is known to degrade the fluorescing reagent monobromobimane.

A preliminary experiment was also carried out to assess the reproducibility and accuracy of the HPLC thiol assay. Five replicate samples of hepatopancreas and abdominal muscle tissue were taken from the same animals which had previously been exposed to a sulphide concentration of 150 ± 11.5 μM sulphide for 24 hours. The samples were then assayed simultaneously using the procedure described above and the results compared.

3.2.4. Symbiotic chemoautotrophic sulphur bacteria and mud-shrimps

Hepatopancreas and gill filaments from freshly caught *Calocaris macandreae* and *Callianassa subterranea* were fixed for electron microscopy to establish if these species harbour symbiotic bacteria involved in sulphide 'oxidation'. This involved immediately placing the dissected tissues from

freshly-caught animals (< 30 min) in 1 % glutaraldehyde, 2 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 with 2 % sucrose and 1.5 % sodium chloride. On return to the laboratory the fixed tissues were rinsed in 0.1 M phosphate buffer containing 4 % sucrose, then post-fixed in 1 % osmium tetroxide in phosphate buffer for 1 h. Specimens were washed in several changes of distilled water and block stained with 0.5 % aqueous uranyl acetate for 1 h. Following dehydration through an ethanol series, the tissues were embedded in Spurr resin (Spurr, 1969), using propylene oxide as a transitional solvent. Thin sections of suitable areas were cut and mounted on uncoated 300 mesh copper/palladium grids and stained with uranyl acetate (methanolic) and lead citrate. Thin sections were examined with a Zeiss 902 transmission electron microscope operating at 80 kV.

3.2.5. Sulphur amino acid metabolism in mud-shrimps

Experiments were carried out to try to establish whether any activity of the enzymes L-serine sulphydrase and homocysteine desulphurase could be detected in the hepatopancreas of *Calocaris macandreae*. The activity of both enzymes can be assessed spectrophotometrically by the formation of sulphide which then reacts with lead acetate to form a brown colloidal suspension. In the case of L-serine sulphydrase, a reversible reaction is catalysed by this enzyme whereby cysteine is desulphurated to form serine and hydrogen sulphide. The desulphuration of homocysteine to form hydrogen sulphide, ammonia and alpha ketobutyrate catalysed by homocysteine desulphurase, can be followed in the same manner as described above.

A specimen of *Calocaris macandreae* was dissected and samples of hepatopancreas tissue resuspended in 0.1 M Tris pH 8.5 in combination with proteinase inhibitors at final concentrations of 1 μM E 64 trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane, 4 μM pepstatin A, 1 μM phenylmethylsulphonylfluoride and 2 μM 1,10 phenanthroline in a final volume

of 0.5 ml. The solution was maintained on ice and homogenized in a 1.5 ml Eppendorf tube with a plastic homogenizer for 5 minutes. Following centrifugation at 11500 g for 5 minutes, the resulting supernatant was used as a potential source of the enzymes. Homocysteine desulphurase and L-serine sulphydrase were assayed spectrophotometrically at 15 °C (Thong & Coombs, 1985a; 1985b). The reaction mixture for the L-serine sulphydrase assay consisted of 500 μl of 0.33 mM lead acetate, 300 μl of 4 mM L- cysteine, 650 μl of 0.1 M Tris adjusted to pH 8.5 and 50 μl of homogenate to give a final assay volume of 1.5 ml. The reaction mixture for homocysteine desulphurase assay was identical except that it contained D, L-homocysteine at a final concentration of 3.3 mM instead of L-cysteine. The reactions were monitored spectrophotometrically for 5 minutes at 15 °C by determining the production of lead sulphide at a wavelength of 360 nm. Control assays were also run with both enzymes to confirm the viability of the technique. In addition, to control for any free sulphide that may have been present in the hepatopancreas sample the assay was also run in the absence of the two substrates (L- cysteine and D, L-homocysteine).

3.2.6. Potentiometric oxygen sensors and sulphide interference

Polarographic oxygen sensors may be poisoned by sulphide (Hale, 1983). Since the Po_2 needed to be recorded in the experiments carried out during this study the affect of sulphide on potentiometric oxygen sensors was investigated. The platinum cathodes of Clarke type oxygen electrodes have been found to react with sulphide (Hale, 1983). The cathode of these electrodes can be protected from sulphide, however, by electroplating gold onto it (Revsbeck & Ward, 1983; O'Brien & Vetter, 1990). During the present study, this was achieved by first etching the cathode for 3 minutes in a solution of saturated KCN in a fume cupboard. This cleans the surface to enable efficient electroplating to occur. The cathode was then washed in 0.1 N HCl. Gold was electroplated onto the

cathode from a solution of 5 % $KAu(CN)_2$ in 0.2 M ammonium citrate adjusted to pH 6.3. A simple electrolysis system was created by applying 1.5 V d.c., negative at the cathode, with a 680 Ω resistor in series, and a graphite rod in the $KAu(CN)_2$. This prevented the anode from being exposed to the plating solution. The electrodes were then placed in the solution for 5 minutes.

O'Brien & Vetter (1990) state that electrodes modified in this way were largely insensitive to sulphide concentrations < 1 mM and had similar oxygen sensitivities to those of untreated platinum cathode electrodes. Throughout this study, the sulphide concentrations used in any experiment were generally in the low micromolar range. To confirm insensitivity to sulphide under these experimental conditions the gold cathode electrodes were always recalibrated, as described below, before and directly after use to establish whether any deterioration in the electrode's performance had occurred that may have been induced by sulphide exposure.

A series of empirical sulphide exposure experiments were also carried out to investigate the effect of millimolar sulphide concentrations on the calibration parameters of the electrodes. Gold-plated cathode and standard oxygen electrodes were initially calibrated (see section 3.2.2) and placed in 200 ml beaker of distilled water maintained at 10 °C (Grant temperature controlled water bath combined with a Techne tempette TE-8D cooler, refrigerated bath RB-5) for a control period of 24 hours and then recalibrated to confirm the electrode's performance (< 1% drift acceptable). Both electrodes were then placed in a range of sulphide solutions (nominally 1 mM, 5 mM and 50 mM, see section 2.2.1a.) also maintained at 10 °C. The electrodes were then removed at 24 hour intervals, thoroughly rinsed with distilled water, and placed in a solution of oxygen-saturated distilled water to establish if any deterioration in the electrode's performance had occurred.

In addition, an experiment was conducted to assess the extent to which sulphide and thiol compounds were able to diffuse across the membrane into the

electrolyte. A standard, untreated oxygen electrode and an electrode in which the cathode had been gold plated were connected to two oxygen meters (Strathkelvin Instruments) and placed in a sulphide solution (final concentration = 543 ± 113 μM). Prior to this the electrodes were calibrated against air-saturated sea water and in a stream of nitrogen in a small sealed container. The electrodes were left in the sulphide solution at 10 °C (as described above) for a total of 96 hours after which the electrodes were dis-assembled and samples (50 μl) of electrolyte were taken and the concentrations of reduced thiols in the electrolyte determined by HPLC.

3.3. Results

3.3.1. Sulphide tolerance of mud-shrimps

All three species of mud-shrimp were tolerant of sulphide. The survival curves for animals exposed to high concentrations of sulphide were approximately sigmoidal (Figure 3.2 & 3.3). The LT_{50} values for *Calocaris macandreae* and *Callianassa subterranea* exposed to 1.0 ± 0.1 mM sulphide under near normoxic conditions (Po_2 > 140 Torr) were similar; 29 h and 24 h respectively. *Jaxea nocturna*, however, showed an even greater tolerance of sulphide (LT_{50} = 62 h) even when exposed to sulphide (0.62 ± 0.14 mM) under hypoxic conditions (Po_2 38.5 ± 14.4 Torr). Control animals suffered little mortality (< 10 % over 7 days) throughout the experiments.

Due to the difficulty in collecting *C. subterranea* and *J. nocturna*, insufficient mud-shrimps were available to allow the determination of their tolerance of anoxia. However, the LT_{50} for *C. macandreae* under anoxic conditions was 30 h (Figure 3.2). The survival curve of *C. macandreae* maintained under anoxia was very similar to that obtained when animals were exposed to 1 mM sulphide and normoxia.

It is interesting to note that the pleopods, mouthparts and branchial chambers of mud-shrimps exposed to high concentrations of sulphide became blackened with what is believed to be metallic sulphides.

3.3.2. Sulphide 'oxidation' and metabolism in mud-shrimps

Exposure to sulphide (87 ± 14 μM) under normoxic conditions for 24 h resulted in a significant (ANOVA + Tukey, Man-Whitney & Mood tests, $p < 0.05$) accumulation of thiols in the haemolymph and tissues of *Calocaris macandreae* (Figure 3.4, 3.5, & 3.6). Although low concentrations of sulphide were found in the haemolymph and hepatopancreas these were well below the exposure concentration. The concentrations of sulphide in the hepatopancreas did increase significantly (Mood, $p < 0.05$) after 12 hours but did not increase

significantly (Mann-Whitney, $p > 0.05$) in the abdominal muscle tissue (Figure 3.6).

Most of the sulphide entering the haemolymph and tissues was oxidized to thiosulphate (ANOVA + Tukey, $P < 0.05$). The concentrations of thiosulphate in the haemolymph and the tissues increased rapidly during the first few hours of exposure to sulphide. In both the hepatopancreas and the haemolymph, the concentration of thiosulphate reached an equilibrium after 1 - 6 h. The concentration of thiosulphate in the abdominal muscle reached its highest value after 6 h exposure but then decreased significantly (Mood, $p < 0.05$). Sulphite occurred as a secondary 'oxidation' product in the haemolymph but did not accumulate significantly in the hepatopancreas and muscle tissue during the experiment (ANOVA, $p > 0.05$; Mann-Whitney, $p > 0.05$). The concentrations of cysteine and glutathione in the haemolymph, hepatopancreas and abdominal muscle of animals exposed to sulphide for 24 h, were found not to differ significantly (*t* - test, Mann-Whitney, Mood, $p > 0.05$) from their respective controls (Figure 3.7 & 3.8). Sulphite and thiosulphate were detected in the water at the end of the 24 h exposure period due to the chemical oxidation of sulphide but only at very low concentrations (< 10 μM). Concentrations of cysteine and glutathione in the exposure water were below the limit of detection (< 1 μM).

The preliminary experiment carried out to assess the reproducibility and accuracy of the HPLC thiol assay for muscle and hepatopancreas tissue found the repeatability of the assay to be good. When exposed to 150 ± 11.5 μM sulphide under normoxic conditions for 24 h the concentrations of sulphite, thiosulphate and sulphide in the abdominal muscle tissue from a single specimen of *Calocaris macandreae* (6 replicates) were found to be 0.116 ± 0.019 μmol. g^{-1}, 2.119 ± 0.370 μmol.g^{-1} and 0.178 ± 0.059 μmol.g^{-1}, respectively. The concentrations of sulphite, thiosulphate and sulphide from three replicate samples of the hepatopancreas of a single specimen were 0.111 ± 0.112 μmol.g^{-1}, 0.889

$\pm$ 0.148 μmol.g^{-1} and 0.450 $\pm$ 0.242 μmol.g^{-1}, respectively.

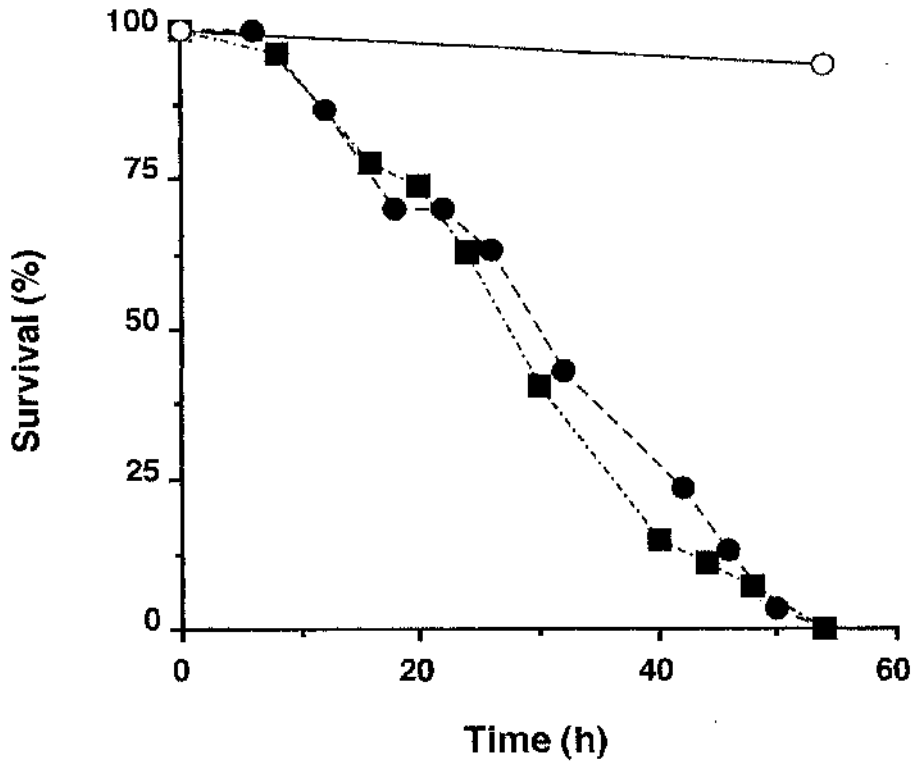

Figure 3.2 Cumulative survival (%) over time of *Calocaris macandreae* (n = 27 - 30) during exposure to normoxia (open circles), anoxia (closed circles) and 1.0 ± 0.1 mM sulphide under near normoxic (Po_2 > 140 Torr) conditions (closed squares).

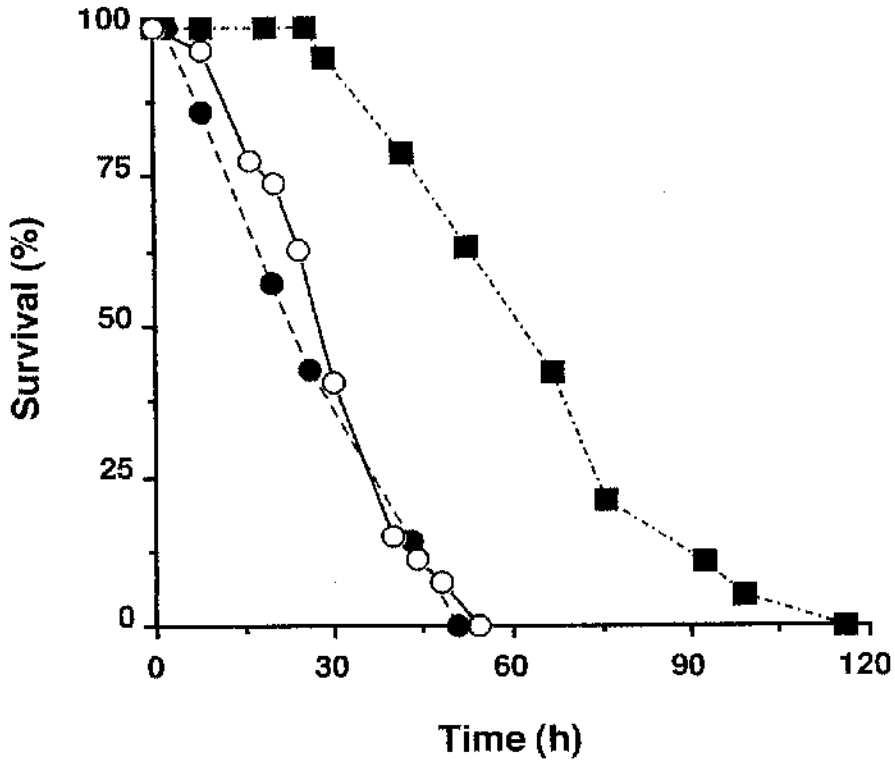

Figure 3.3 A comparison of the cumulative survival of *Calocaris macandreae* (open circles, n = 29) and *Callianassa subterranea* (closed circles, n = 7) exposed to 1.0 ± 0.1 mM sulphide under near normoxic conditions (Po_2 > 140 Torr) and *Jaxea nocturna* (closed squares, n = 19) exposed to 0.62 ± 0.14 mM sulphide under hypoxic conditions (Po_2 = 38.5 ± 14.4 Torr).

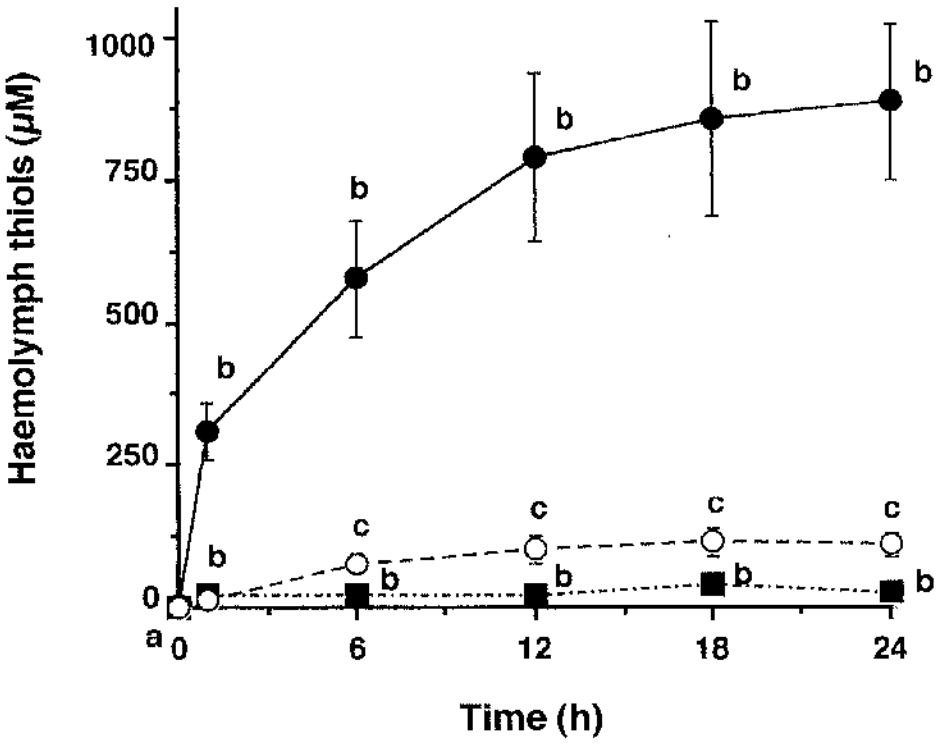

Figure 3.4 The accumulation of the thiols, thiosulphate (closed circles), sulphite (open circles) and sulphide (closed squares) in the haemolymph of *Calocaris macandreae* following 24 h exposure to 87 ± 14 μM sulphide under near normoxic conditions (Po_2 > 140 Torr). Values are means ± SE (n = 8 - 11). a/b/c = significant difference (ANOVA + Tukey, Mood or Mann-Whitney, $p < 0.05$).

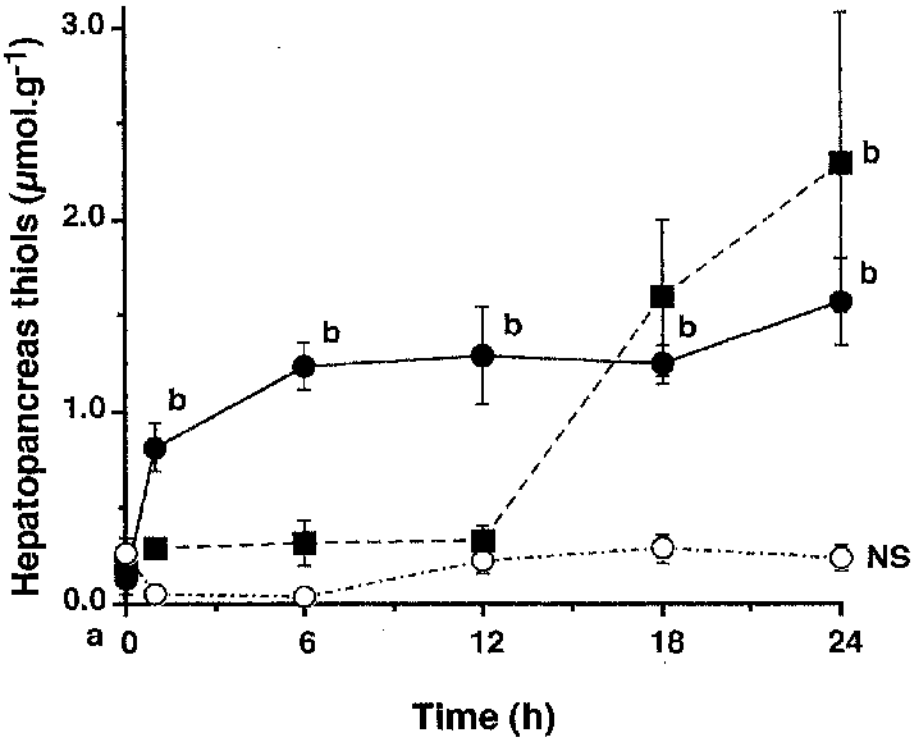

Figure 3.5 The accumulation of the thiols, thiosulphate (closed circles), sulphite (open circles) and sulphide (closed squares) in the hepatopancreas of *Calocaris macandreae* following 24 h exposure to 87 ± 14 μM sulphide under near normoxic conditions (Po_2 > 140 Torr). Values are means ± SE (n = 8 - 11). a/b = significant difference (ANOVA + TUKEY, Mood or Mann-Whitney, $p < 0.05$).

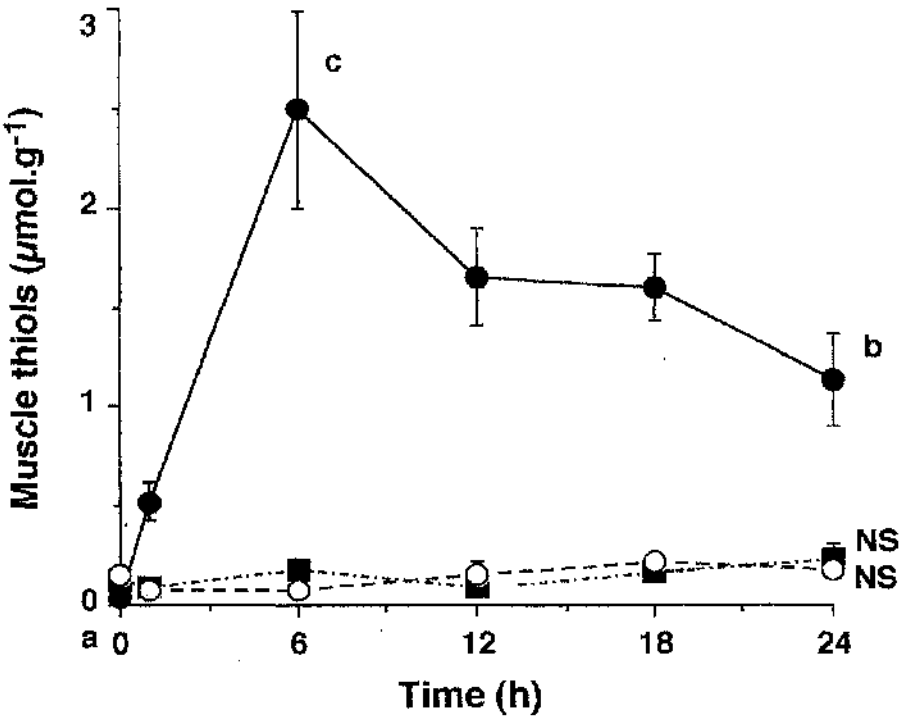

Figure 3.6 The accumulation of the thiols, thiosulphate (closed circles), sulphite (open circles) and sulphide (closed squares) in the abdominal muscle of *Calocaris macandreae* following 24 h exposure to 87 ± 14 μM sulphide under near normoxic conditions (Po_2 > 140 Torr). Values are means ± SE (n = 8 - 11). a/b = significant difference (ANOVA + TUKEY, Mood or Mann-Whitney, $p < 0.05$).

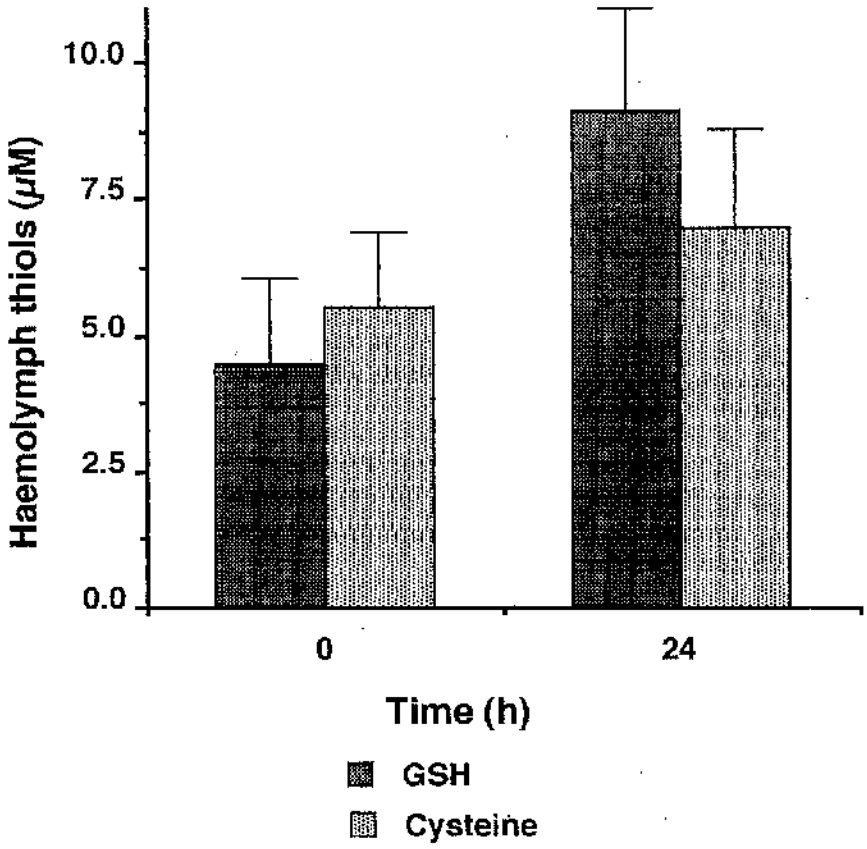

Figure 3.7 The concentrations of the organic thiols cysteine and glutathione (GSH) in the haemolymph of *Calocaris macandreae* following 24 h exposure to 87 ± 14 μM sulphide under near normoxic conditions (Po_2 > 140 Torr). Values are means ± SE (n = 8 - 11). There were no significant differences between the initial and final concentrations (*t* - test, Mood or Mann-Whitney, p > 0.05).

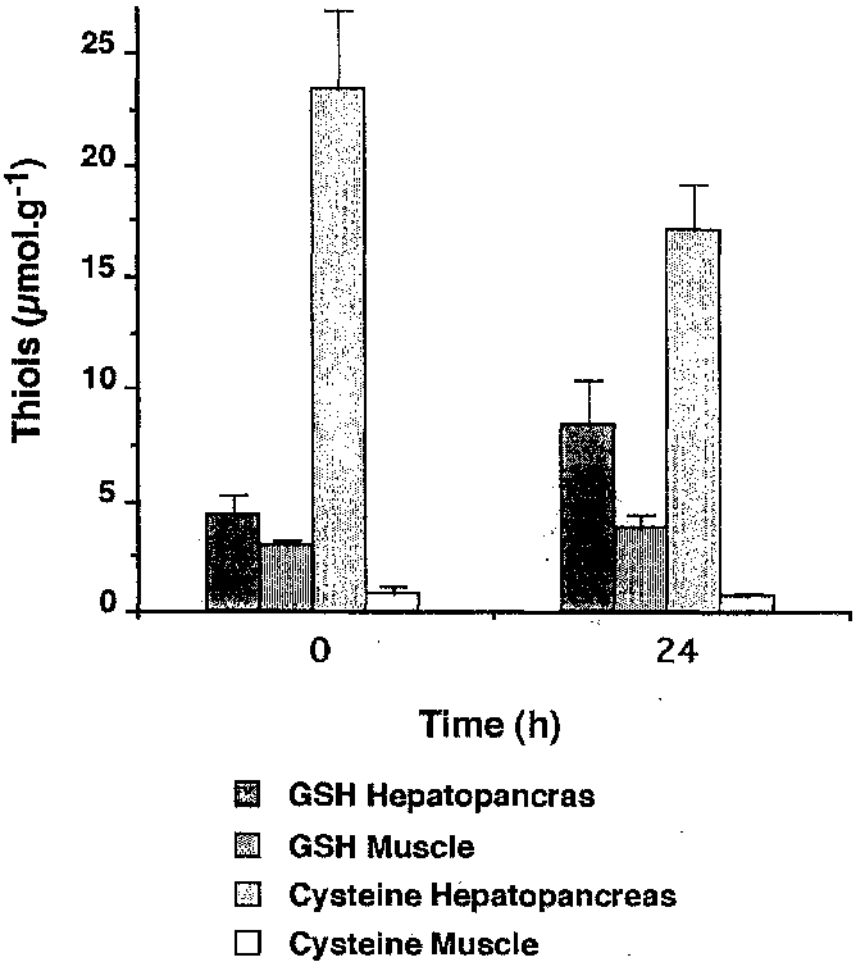

Figure 3.8 The concentrations of the organic thiols cysteine and glutathione (GSH) in the hepatopancreas and abdominal muscle of *Calocaris macandreae* following 24 h exposure to 87 ± 14 μM sulphide under near normoxic conditions (Po_2 > 140 Torr). Data point values are means ± SE (n = 8 - 11). There were no significant differences between the start and final concentrations (*t* - test, Mood or Mann-Whitney, p > 0.05).

3.3.3. Symbiotic chemoautotrophic sulphur bacteria and mud-shrimps

Careful examination by transmission electron microscopy did not reveal any evidence of bacterial symbionts in either the hepatopancreas or gill tissue from freshly-captured *Callianassa subterranea* (n = 2 for each tissue) and *Calocaris macandreae* (n = 2 for each tissue).

3.3.4. Sulphur amino acid metabolism in mud-shrimps

The enzymes L-serine sulphydrase and homocysteine desulphurase were not detected in the hepatopancreas (n = 1) of *Calocaris macandreae*. Control cuvettes were also run to establish the presence of any sulphide in the hepatopancreas, none was detected. To ensure this was a genuine negative result the enzymes were also added to the assay mixture to confirm its viability.

3.3.5. Potentiometric oxygen sensors and sulphide interference

Comparisons of the performance of the two types of electrode showed that both the untreated and gold plated electrodes were unaffected by sulphide concentrations up to at least 6.0 ± 0.99 mM since, after prolonged exposure to sulphide (144 h), they could still be recalibrated to within 1 % of their original values. At higher sulphide concentrations (37.7 ± 2.7 mM) the standard POS could not be recalibrated after 24 hours, suggesting sulphide poisoning. The gold-plated electrode, however, could be calibrated within 1% of its original calibration after 72 hours after which it was also poisoned by the sulphide. In addition, in the anoxic sulphide solutions both electrodes consistently recorded a Po_2 of zero Torr in each of the three sulphide solutions.

When exposed to concentrations of 543 ± 113 μM sulphide for 96 hours only very low micromolar concentrations of sulphite, thiosulphate and sulphide could be detected in the electrolyte of the untreated and gold plated electrodes (Fig. 3.9). Since the standard electrolyte does not contain any of these

compounds this may indicate that sulphide slowly diffuses across the membrane into the electrolyte and is oxidized to thiosulphate and sulphite.

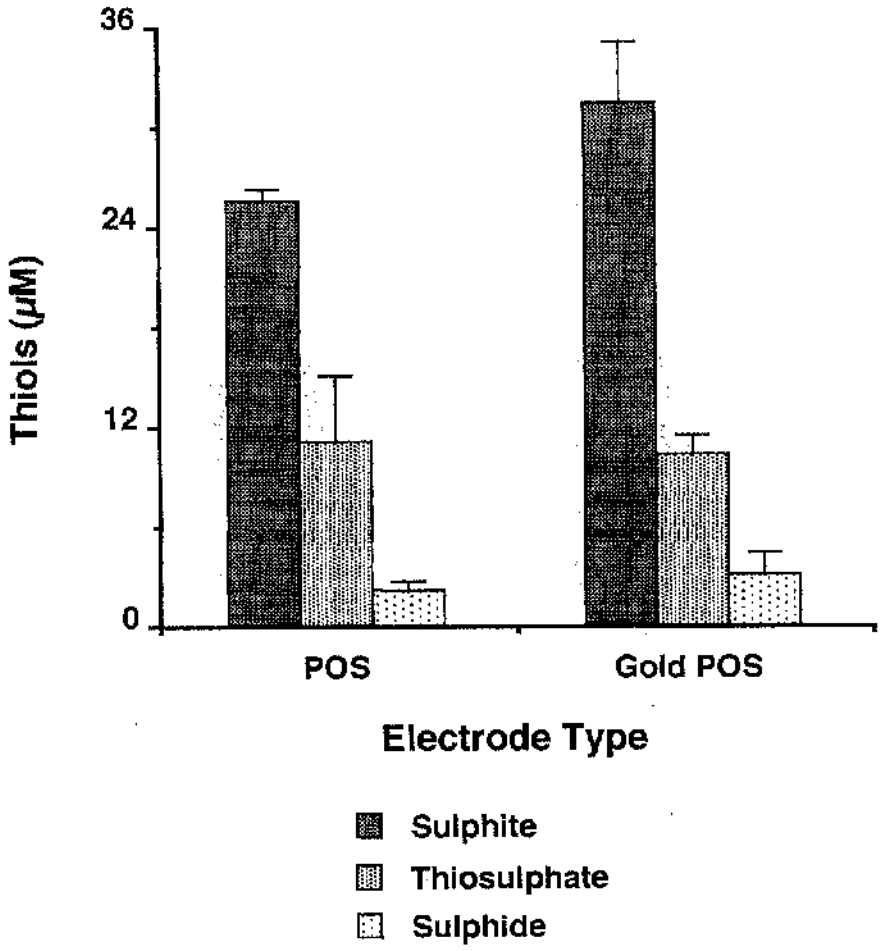

Figure 3.9 The accumulation of sulphite, thiosulphate and sulphide in the electrolyte of an untreated Strathkelvin oxygen electrode and one in which the cathode had been gold plated following exposure to a solution of 543 ± 113 μM sulphide for 96 hours (n = 3). Cysteine and glutathione were not detected in the electrolyte. The standard electrolyte was found not to contain any of the above reduced sulphur compounds. Values are means ± SD.

3.4. Discussion

3.4.1. Sulphide tolerance in mud-shrimps

The considerable ability of the mud-shrimps studied here to survive sulphide exposure may be related to their burrowing lifestyle which frequently exposes them to significant concentrations of sulphide (see section 2.3.2.). Although there are very few comparative data on sulphide tolerance in crustaceans, the available evidence indicates that most species have a poor tolerance of sulphide. For example, the larval stages of the white shrimp *Metapenaeus monoceros* and the blue crab *Portunus trituberculatus* have been shown to be very intolerant of low micromolar concentrations of sulphide (Kang *et al.*, 1993; Kang & Matsuda 1994). The survival rate of adult blue crabs *P. trituberculatus* was also found to decrease significantly when exposed to similar conditions (Kang *et al.*, 1995). Similarly, Hagerman & Vismann (1995) reported that the shrimp *Crangon crangon* suffered high mortalities when exposed to sulphide concentrations as low as 5 μM. In addition, when exposed to sulphide concentrations of 1 mM, the swimming crab *Liocarcinus depurator* suffered 50 % mortality within 11 hours (Astall, 1993). These species are benthic but are not burrow dwellers although they may periodically bury themselves in the surface sediment. Their non-burrowing lifestyle may explain their low tolerance of sulphide.

Much greater tolerances of sulphide exposure have been demonstrated, however, in the hydrothermal vent crab *Bythograea thermydron* (Vetter *et al.*, 1987) and in the Baltic isopod *Saduria entomon* (Hagerman & Vismann, 1993) which may bury in sediment. There is also evidence, based on choice chamber experiments, that species such as the burrow-dwelling amphipod, *Corophium volutator,* may actively avoid high concentrations of sulphide by behavioural means (Meadows *et al.*, 1981). Similarly, burrow irrigation behaviour may reduce the concentration of sulphide in the burrow water (see section 2.4.3.) Thus, suspension feeding mud-shrimps such as *Upogebia* spp. might be

expected to experience lower sulphide concentrations in their burrows than species such as *Calocaris macandreae* and *Callianassa subterranea* that are primarily deposit feeders. The similar trends in mortality of *C. macandreae* between those animals exposed to anoxia and those exposed to sulphide suggest the total reliance on anaerobic metabolism whilst exposed to millimolar concentrations of sulphide despite the presence of oxygen at near normoxic concentrations.

3.4.2. Sulphide 'oxidation' and metabolism in mud-shrimps

The presence of low concentrations of sulphide in the tissues of *Calocaris macandreae* exposed to sulphide in the laboratory indicates that they were unable to prevent the diffusion of sulphide into the body. This is in agreement with data from studies on some other crustacean species (Vetter *et al.*, 1987; Vismann, 1991b; Hagerman & Vismann, 1993). *C. macandreae* was able, however, to maintain the concentrations of sulphide within the body tissues much lower than those in the exposure medium. This was achieved in part by the 'oxidation' of sulphide to form thiosulphate which may be considered as a possible 'detoxification' mechanism. Internal pH may also be significant because during hypoxia a reduction in pH (acidosis) of body fluids and tissues may occur. This results in an increase in the internal concentration of undissociated hydrogen sulphide (Pörtner, 1987). In the event of an equilibrium between the internal and external hydrogen sulphide concentrations the total sulphide concentration will be lower within the animal (Groenendaal, 1981; Völkel & Grieshaber, 1992; Grieshaber *et al.*, 1996). The sulphide concentration in the haemolymph may also be reduced by an auxiliary 'oxidation' mechanism such as the immobilization of sulphide by proteins in the haemolymph acting as a sulphide buffer. This mechanism has been described in other marine invertebrates (Arp *et al.*, 1987; Doeller *et al.*, 1988; Powell & Arp, 1989; Vismann, 1990; Childress *et al.*, 1991; Oeschger & Vetter, 1992; Grieshaber *et al.*, 1996) and is

investigated and discussed in Chapter 6.

Thiosulphate accumulated as the major 'oxidation' product in *Calocaris macandreae* with sulphite accumulating as a significant secondary metabolite and possible intermediate product (O'Brien & Vetter, 1990). The production of thiosulphate is energetically more efficient than sulphate synthesis since 1 mole of sulphide is removed for every 1.5 moles of oxygen whereas the 'oxidation' of one mole of sulphide to form sulphite and sulphate requires three and four moles of oxygen, respectively.

It has been suggested that, in crustaceans, the hepatopancreas is the primary site of sulphide 'oxidation' (Vetter *et al.*, 1987). In the present study, both the muscle and hepatopancreas tissues accumulated similar concentrations of thiosulphate when the mud-shrimps were exposed to sulphide. It is unlikely, therefore, that in mud-shrimps sulphide 'oxidation' is confined to the hepatopancreas. It would appear that a possible alternative mechanism for the removal of sulphide via the direct incorporation of sulphide into sulphur amino acid metabolism does not exist in *Calocaris macandreae* since the enzymes L-serine sulphydrase and homocysteine could not be detected in the hepatopancreas.

How millimolar concentrations of thiosulphate are removed by the animal remains to be addressed. Thiosulphate has a poor ability to pass through biological membranes and has consequently been used a blood marker (Holmes & Donaldson, 1969). In the lug worm, *Arenicola marina,* however, the body wall has been found to be permeable to thiosulphate (Hauschild & Grieshaber, 1995). It has been suggested, therefore, that the metabolites of sulphide 'oxidation' such as thiosulphate which may accumulate to millimolar concentrations in the tissues, may diffuse out of the animal into the burrow water where thiosulphate has been shown to be present only at low micromolar concentrations (Völkel *et al.*, 1995). It is possible that the cuticle of crustaceans is permeable to both sulphide and to 'oxidation' products such as thiosulphate. It

has yet to be established, however, whether thiosulphate can be lost from the body by passive diffusion or whether an active transport mechanism exists.

Vetter *et al.* (1987), however, found no evidence that thiosulphate was rapidly cleared from the blood of the hydrothermal vent crab *Bythograea thermydron* and also found that the gills were not freely permeable to thiosulphate. They proposed that thiosulphate may either be excreted after being converted to sulphate or by the direct disposal of thiosulphate via the antennule gland (Vetter *et al.*, 1987; Astall, 1993).

It is has been established that, for some invertebrate species, chemoautotrophic bacteria have an important role in the 'oxidation' of sulphide entering the body (Somero *et al.*, 1989; Tunnicliffe, 1991; Vismann, 1991a; Southwood, 1994) and also in the provision of chemoautotrophically fixed CO_2. In addition, recent studies on some marine nematodes have shown that sulphide-oxidizing bacteria on the cuticle may also restrict the amount of sulphide that diffuses into the body (Ott *et al.*, 1991; Polz *et al.*, 1992). The electron microscope studies on *Calocaris macandreae* and *Callianassa subterranea*, however, failed to establish the presence of such bacteria associated with either the gill or hepatopancreas tissues. This suggests that the only 'oxidation' mechanism that exists in *C. macandreae*, and possibly in other mud-shrimps, is mediated solely by the animal's tissues.

Electron microscopy studies of other upogebiid and callianassid mud-shrimps, however, have found the mid-gut to be extensively coated with filamentous bacteria which were absent in the hind-gut (Harris *et al.*, 1991, Pinn, 1995). These bacteria appear to be very different from those ingested during feeding and it would be interesting to determine whether they may possibly be involved in sulphide 'oxidation'. It has been established that sulphur-oxidizing bacteria are present in the gut of the sediment-dwelling echinoderm, *Echinocardium cordatum* (Temara *et al.*, 1993). These symbiotic filamentous bacteria, morphologically similar to *Thiothrix*, a sulphide-oxidizing

species, thrive in the intestinal caecum of this deposit feeding animal and have been shown to oxidize sulphide and thiosulphate.

Sulphide 'oxidation' by mitochondria linked to the electron transport chain has been demonstrated in the gill and foot tissue of the bivalve *Solemya reidi* (Powell & Somero, 1986a; O'Brien & Vetter, 1990), in the liver of the Californian killifish, *Fundulus parvipinnis* (Bagarinao & Vetter, 1990), in the body wall of the priapulid, *Halicryptus spinulosus* (Oeschger & Vetter, 1992) and in the polychaete, *Heteromastus filiformis* (Oeschger & Vismann, 1994). Sulphide 'oxidation' and the concurrent production of ATP has been found to be stimulated at low micromolar sulphide concentrations in these species but at higher sulphide concentrations (in excess of 50 μM) complete inhibition of the respiratory chain occurred. It is believed, therefore, that sulphide 'oxidation' is linked with the respiratory chain by cytochrome c oxidase, which could be the sulphide oxidase, transferring electrons to oxygen (Powell & Somero, 1986a).

Recent studies of the intertidal worm *Arenicola marina* have indicated, however, that mitochondrial sulphide 'oxidation' is not inhibited by sulphide (Völkel & Grieshaber, 1994, 1995) and, recently, the possible existence of an alternative terminal oxidase appears to have been demonstrated (Völkel & Grieshaber, 1996). The biochemical mechanism and characterization of this proposed alternative cytochrome-*c* oxidase remains to be fully established. It seems highly unlikely that *A. marina* should be the only sulphide tolerant marine invertebrate to contain mitochondria with this alternative enzyme but further studies are required to establish its presence in other species.

The concurrent synthesis of ATP during mitochondrial sulphide 'oxidation' has been established in a small number if marine invertebrates (Oeschger & Vetter, 1992; Oeschger & Vismann, 1994; Völkel & Grieshaber, 1994, 1995; Völkel *et al.*, 1995; Völkel & Grieshaber, 1996). This energy resource may be very significant in providing the animal with a supplementary supply of ATP whilst under sulphide stress.

A small number of conceptual models have been designed to integrate the different features of sulphide 'detoxification'. They seem to indicate that the diffusion rate of sulphide into the cell and diffusion barriers may be important in preventing sulphide from reaching inhibitory concentrations at the site of cytochrome c oxidase on the inner membrane of mitochondria (Bagarinao & Vetter, 1993, Arp *et al.*, 1995). For this to be possible a balance between the rate at which sulphide diffuses towards the enzyme, dependent on pH and external sulphide concentration, and the rate at which sulphide is detoxified by external agents to the enzyme (such as the oxidising capacity of the respiratory pigment) must occur.

It would appear, therefore, that mud-shrimps are adapted physiologically to tolerate exposure to sulphide in their natural habitat and that the mitochondria may be responsible for the 'oxidation' of sulphide to thiol metabolites during 'detoxification'. These animals may therefore be added to those marine invertebrates so far reported to 'detoxify' sulphide. It seems likely that the underlying sulphide 'oxidation' mechanism is mitochondrial and that the ATP produced at this time may be used as a supplementary energy source (Oeschger & Vetter, 1992; Oeschger & Vismann, 1994; Völkel & Grieshaber, 1994, 1995; Völkel *et al.*, 1995; Völkel & Grieshaber, 1996).

3.4.3. Potentiometric oxygen sensors and sulphide interference

The experimental evidence from this study indicates that gold plated and standard potentiometric oxygen sensors are largely insensitive to micromolar and very low millimolar concentrations of sulphide and is in agreement with O'Brien & Vetter, (1990). The electrodes could be recalibrated after sulphide exposure for a considerable number of hours without any apparent loss of performance (6.0 ± 0.99 mM for 144 h). In contrast, at higher millimolar sulphide concentrations (37.7 ± 2.7 mM) both types of electrode suffered considerable reductions in their performance. The gold plated electrode, however, appeared to

maintain its calibration for at least 72 hours whereas the standard electrode was apparently more susceptible to sulphide in that it could not be recalibrated after 24 hours exposure to sulphide.

Low concentrations of thiols were also detected in the electrolyte even after prolonged exposure to sulphide (543 ± 113 μM sulphide for 96 hours). The membrane, which seals the cathode and anode within the electrode body, is therefore apparently largely impermeable to sulphide and prevents sulphide diffusion into electrode electrolyte. Sulphide poisoning may not therefore be a problem until mM concentrations of sulphide are reached.

3.4.4. Summary of Chapter 3

The findings of Chapter 3 in relation to mud-shrimps are summarized below:

a. Mud-shrimps appear to be physiologically adapted to tolerate elevated levels of sulphide that they may encounter in their natural habitat.

b. Sulphide diffusing into the body tissues is 'oxidized' primarily to thiosulphate which may act as a possible 'detoxification' mechanism.

c. Sulphite accumulates as a secondary and possible intermediate sulphide 'oxidation' metabolite

d. Symbiotic sulphide oxidizing bacteria were not detected in the gills or hepatopancreas in mud-shrimps, although their possible presence in the gut remains to be investigated.

e. Sulphur amino acid metabolism seems not be directly implicated in sulphide 'oxidation'.

f. Cathode gold plated oxygen electrodes are insensitive to micromolar concentrations of sulphide.

4. Aerobic and anaerobic metabolism of mud-shrimps during exposure to sulphide.

4.1. Introduction

4.1.1. Anaerobic metabolism in marine invertebrates

Many marine invertebrates which inhabit environments that can become hypoxic or anoxic may rely on anaerobic metabolism to survive these conditions. Anaerobic mechanisms during exposure to environmental anoxia have been described in a variety of marine invertebrates (Zebe, 1982; de Zwaan & Putzer, 1985; Hochachka, 1986; Gäde & Grieshaber 1986, Bryant, 1991; de Zwaan, 1991; Grieshaber *et al.*, 1992, 1994). The biochemical adaptations seen in these animals which enable them to survive the effects of hypoxia or anoxia depend primarily on the duration of the period of exposure and the energy requirements of the animal.

As soon as severely hypoxic or anoxic conditions prevail, adenosine triphosphate (ATP) may be provided by the exploitation of phosphagens, such as creatine phosphate and phospho-L-arginine (Grieshaber *et al.*, 1994). In addition, the synthesis of ATP also occurs in the cytosol during anaerobic glycolysis. To maintain the glycolytic flux and cytosolytic redox balance, NADH, which is produced during the reaction of glyceraldehyde 3 phosphate to form 3 phosphoglycerate, must be reoxidized to NAD^+. This involves the reduction of pyruvate to form D, L-lactate or opines by the pyruvate oxydoreductases (Grieshaber & Gäde, 1986) and results in the accumulation of metabolic end-products such as L-lactate and opines which are functionally analogous (Fields, 1983). The rate of ATP production in these reactions can be very high because the glycolytic flux may increase, although biochemical efficiency (ATP yield per mol glucose or glycogen) is low since they involve the incomplete oxidation of glycogen (Shick, 1991; Grieshaber *et al.*, 1992, 1994, 1996). Anaerobic glycolysis results in the production of only 3 moles of ATP

per mole of glycogen, whereas during aerobic metabolism the yield of ATP is considerably higher at 38 moles of ATP per mole of glycogen (Eckert *et al.*, 1988). Anaerobic glycolysis, however, may be more important during exercise or functional anaerobiosis, requiring a higher rate of ATP production, than during environmental anaerobiosis which, in contrast, involves low energy expenditure. Anaerobic glycolysis may therefore have been selected for periods of high power output during the course of evolution. For example, the escape responses of many marine invertebrates to avoid predation, such as the tail-flipping escape response of many decapod crustaceans and the strong muscular contractions of the polychaete worm *Arenicola marina* during burrowing, and the fast white muscle of fish, which require a high rate of ATP production (Grieshaber *et al.*, 1992, 1994).

Over long periods of anoxia or severe hypoxia, however, ATP can be produced more efficiently by anaerobically-functioning mitochondria than during anaerobic glycolysis in the cytosol (Grieshaber *et al.*, 1994). The glucose-succinate pathway ferments glycogen and produces succinate as a transient end-product (which cannot be excreted) in the mitochondria. Succinate is further metabolised to propionate and acetate which may then be excreted (Grieshaber *et al.*, 1994). These end-products have been found in a range of marine and limnic invertebrates, particularly in molluscs and annelids. The succinate-propionate pathway is energetically more efficient than anaerobic glycolysis because approximately 5 moles of ATP are produced in comparison to the 3 moles of ATP produced during anaerobic glycolysis. Less substrate is, therefore, used for a given amount of energy production. Depletion of glycogen, during long periods of ambient oxygen reduction, may be even further reduced by the attenuation of the metabolic rates, to approximately 5 - 20% of normoxic values.

4.1.2. Anaerobic metabolism in Crustacea

The Crustacea tend to have low tolerances of anoxia (Zebe, 1982, 1991). This is strongly correlated with their exposure to reductions in oxygen concentrations in their environment which for many decapod crustaceans is unlikely. In general they are also highly mobile, which allows them considerable scope to avoid anoxic or hypoxic conditions. Almost without exception, therefore, the crustaceans rely entirely on anaerobic glycolysis (see section 4.1.1) if they encounter short periods of anoxia and hypoxia (Zebe, 1982; de Zwaan & Putzer, 1985, Gäde *et al.*, 1986). The production of only glycolytic end-products (D, L-lactate and D, L alanine) during crustacean anaerobic metabolism rather than any other metabolic end-products contrasts strongly with other groups of marine invertebrates which tend to use a greater variety of anaerobic pathways (Zebe, 1982; Fields, 1983; Grieshaber *et al.*, 1992, 1994). As has been described above, this may in part be an evolutionary reflection of the possibly more important role of anaerobic glycolysis in crustaceans. The supply of oxygen or the aerobic capacity of muscle tissues during functional anaerobiosis are limited for short periods during which escape responses which involve intense muscular activities occur (see above). The increased demand for ATP can be quickly met by anaerobic glycolysis with the potential of a 100 fold increase in flux rates (de Zwaan, 1977).

A small number of crustaceans however, may be able to survive anaerobically for longer periods than most species by producing succinate in addition to the anaerobic end-products D, L-lactate and Alanine (de Zwaan & Skjoldal 1979; Zebe, 1982; Carlson & Gäde, 1986; Hagerman & Szaniawska, 1990). These include the isopods *Natatolana borealis, Saduria entomon* and the thalassinideans *Upogebia pugettensis* and *Neotrypaea* (as *Callianassa) californiensis.* In the two thalassinidean mud-shrimps, however, both alanine and succinate were found to be minor end-products and were produced at only very low concentrations during exposure to anoxia over 24 hours (Zebe, 1982).

Phosphagen transphosphorylation and anaerobic glycolysis leading to the formation of ATP and L-lactate in these two invertebrates is therefore the major mode of anaerobic metabolism.

4.1.3. Sulphide-induced anaerobic metabolism in marine invertebrates

The presence of sulphide in the water column or in interstitial water is strongly correlated with anoxic or hypoxic conditions (Theede *et al.*, 1969; Fenchel & Riedl, 1970). Sulphide is highly toxic by mainly inhibiting cytochrome c oxidase (Nicholls, 1975; National Research Council, 1979) and may therefore prevent aerobic respiration in many marine invertebrates (Bagarinao, 1992). Sulphide-tolerant animals, however, may be able to sustain their aerobic metabolic pathways, defending the cytochrome c oxidase in the electron transport chain within their mitochondria, through a range of mechanisms (Vetter *et al.*, 1991; Vismann, 1991a; Bagarinao, 1992). In addition, these mechanisms may also be involved in the synthesis of ATP which could act as a supplementary energy source during sulphide stress (Powell & Somero, 1986a; Völkel & Grieshaber, 1996).

The use of anaerobic metabolism may allow an animal to survive exposure to sulphide (Vetter *et al.*, 1991). Sulphide-induced anaerobiosis in the presence of oxygen has been reported in a number of marine invertebrates and is believed to be relatively common (Anderson *et al.*,1990; Oeschger & Vetter, 1992; Völkel & Grieshaber, 1992, 1994; Hagerman & Vismann, 1993, 1995; Grieshaber *et al.*, 1996). The maintenance of aerobic metabolism during exposure to sulphide before resorting to anaerobic metabolic pathways has, however, been shown to be very variable and dependent on oxygen provision as well as ambient sulphide concentrations (Hagerman & Vismann, 1993, 1995; Völkel *et al.*, 1994).

4.1.4. Sulphide-induced anaerobic metabolism in mud-shrimps

Mud-shrimps show a number of adaptations to declining oxygen tensions which include behavioural responses such as irrigating the burrow to draw in oxygenated sea water (Anderson 1989; Astall, 1993; Astall *et al.*, 1997). In addition, in an attempt to ensure oxygen delivery is not compromised, physiological adaptations have been shown, such as increasing ventilation, maintaining a constant heart rate, reducing metabolism to reduce energy expenditure and the possession of a high affinity respiratory pigment (Anderson, 1989; Astall, 1993; Nickell, 1992, Anderson *et al.*, 1994). The mud-shrimp *Calocaris macandreae* is also able to maintain its oxygen consumption constant over a wide range of oxygen partial pressures down to a critical Po_2 (Pc) of approximately 20 Torr (Anderson *et al.*, 1991). Similar Pc values have been recorded in several other species of mud-shrimp (Atkinson & Taylor, 1988; Paterson & Thorne, 1995; Astall *et al.*, 1997). *C. macandreae* has been shown to maintain aerobic metabolism during moderate hypoxia above the P_c ($Po_2 > 20$ Torr) and survive periods of severe hypoxia (below the Pc) and anoxia for many hours (Anderson *et al.*, 1994). Similarly, other species of mud-shrimp appear to have an even greater tolerance of anoxia than many other species of decapod crustaceans. For example, *Neotrypaea* (as *Callianassa) californiensis* and *Lepidophthalmus louisianensis* (as *Callianassa jamaicense*), can survive anoxia for 3 - 4 days (Thompson & Pritchard, 1969; Felder, 1979).

In addition to low partial pressures of ambient O_2 mud-shrimps may also be exposed to sulphide in their burrows and in the surrounding reduced sediments. They tolerate sulphide oxidizing it to thiosulphate and sulphite (see section 3.3.2 and 3.4.2). Since they rarely, if ever, leave their burrows (Nickell & Atkinson, 1995) it would be interesting to establish their oxygen consumption requirements when exposed to sulphide. Of additional importance is under what sulphidic conditions do the animals resort to anaerobic metabolism? The oxygen consumption and onset of anaerobiosis at various sulphide concentrations and

different ambient oxygen partial pressures were therefore investigated in the mud-shrimp *Calocaris macandreae*.

4.2. Materials and methods

4.2.1. Collection and maintenance of animals.

Calocaris macandreae were collected by Agassiz trawling in the Clyde and returned to the University of Glasgow where they were maintained under a 12:12 h light : dark regime in a recirculating natural sea water aquarium (salinity = 35 ± 1 ‰; temperature 10 ± 1 °C). Further details are given in section 3.2.1. The animals were not fed and allowed at least 48 h to acclimate to these conditions before any physiological measurements were made.

4.2.2. Whole animal oxygen consumption during sulphide exposure

The rates of oxygen consumption of *Calocaris macandreae* under varying oxygen partial pressures (Po_2) and sulphide concentrations were determined by flow-through respirometry (Figure 4.1). Eight mud-shrimps of similar weight (fresh weight range = 1.5 - 2.0 g) were placed in individual 40 ml clear Perspex tubes through which natural sea water (salinity = 35 ± 1 ‰, pH 7.99 ± 0.08, temperature 10 ± 1° C) flowed at a constant rate of 75 $ml.h^{-1}$. An additional two tubes without animals acted as controls to allow the rates of microbial oxygen consumption and chemical oxidation of sulphide to be calculated. In addition, to reduce the effects of bacterial respiration during the experiment, the sea water was sterilized using ultra-violet light (Tropical Marine). Exposure to different sulphide concentrations (0 - 300 μM) and normoxic and hypoxic, 47 ± 7 Torr conditions was controlled as described in 3.2.2. The animals were left undisturbed overnight to acclimatize and to allow the experimental conditions to reach equilibrium.

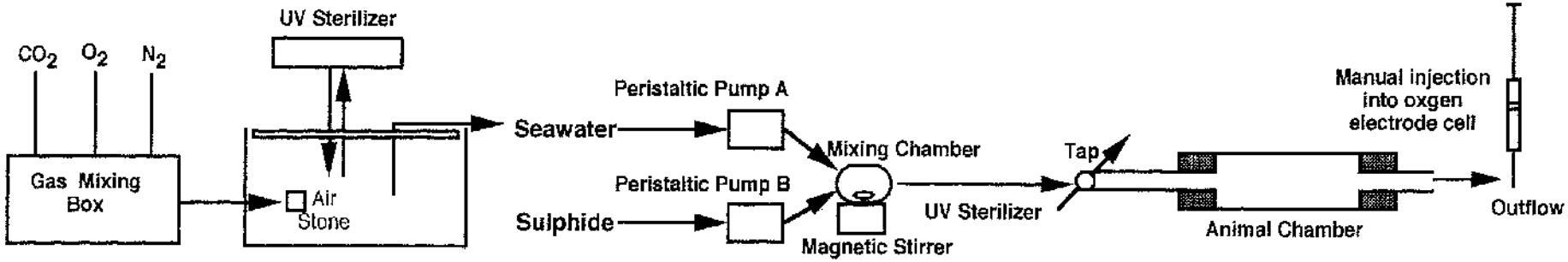

Figure 4.1 Diagram of the flow-through respirometer used to determine the oxygen consumption of *Calocaris macandreae* when exposed to sulphide under normoxic and hypoxic conditions.

The exact flow rate and the Po_2 of the sea water entering and emerging from all ten tubes was then determined. Samples were taken from each tube at 70 minute intervals to ensure > 90 % exchange of water within the tubes (Steffensen, 1989). Water samples (0.75 ml) were withdrawn from the outflow of each tube using a 1 ml syringe and immediately injected into an oxygen cell (EH100, Strathkelvin Instruments, Glasgow) thermostatted at 10 ± 1 °C, containing a sulphide-insensitive oxygen electrode (1302, Strathkelvin Instruments, Glasgow) connected to an oxygen meter (Model 781, Strathkelvin Instruments, Glasgow) and pen recorder. The anode of the electrode was gold plated prior to use to protect it from sulphide poisoning (see section 3.2.2). The concentration of sulphide in the water from the control tubes was determined as described below. Prior to each experiment the respirometers and associated tubing were sterilized with a solution of sodium hypochlorite to reduce bacterial respiration and then thoroughly rinsed with distilled water.

Weight specific rates of oxygen consumption ($\dot{M}o_2$) were calculated after subtraction of the rates of microbial respiration recorded in the control tubes. The mass of the mud-shrimps used was intentionally limited to a narrow range (1.5 - 2.0 g) to reduce size-related effects which affect oxygen consumption estimates (Bridges & Brand, 1980). It was noted that a reduction of the Po_2 in the control tube (by approximately 6%, depending on the concentration of sulphide used) occurred and was attributable to both microbial respiration and the chemical oxidation of sulphide.

The mean oxygen consumption rate of each individual animal (μmol O_2. $g^{-1}.h^{-1}$) was then calculated, from at least five Po_2 measurements taken during the course of the exposure. The oxygen partial pressures were initially converted into concentrations of oxygen (in μmol $O_2.ml^{-1}$) at a salinity of 35 ‰ and a temperature of 10 °C (Bayne *et al.*, 1985). Mo_2 was then determined using the equation described below.

$$\dot{M}o_2 = ([O_2]_{control\ tube} - [O_2]_{animal\ tube}) \times flow\ rate / mass$$

where $\dot{M}o_2$ = oxygen consumption (μmol $O_2.g^{-1}.h^{-1}$), $[O_2]_{control\ tube}$ and $[O_2]_{animal\ tube}$ = concentration of oxygen (μmol.ml^{-1} , temperature = 10 °C and salinity = 35 ‰) , mass = mass of animal (g) and flow rate = flow rate ($ml.h^{-1}$). Values for the mean and standard error from the total number of animals used during the exposure (normally 8) were then calculated.

4.2.3. Anaerobic metabolism under sulphidic conditions

In a series of experiments, specimens of *Calocaris macandreae* (usually 10) were exposed to different sulphide concentrations (0 - 200 μM) under normoxic and hypoxic conditions (20 ± 1 Torr) for 24 h. Each experimental exposure (see section 3.2.2.) was conducted at 10 ± 1°C, pH = 8.0 and salinity = 35 ± 1 ‰. After each exposure the concentrations of L-lactate in the haemolymph and abdominal muscle tissue of each mud-shrimp were determined and subsequently used to assess the extent to which anaerobic metabolism was used by the animal during the exposure.

4.2.3a. Preparation of haemolymph and tissue samples using perchloric acid extraction

Haemolymph and abdominal muscle samples were obtained for L-lactate determination by the techniques described below. Approximately 50 μl of haemolymph was removed from the animal by inserting a narrow gauge needle (25 G) into the pericardium of the mud-shrimp and withdrawing the haemolymph using a 1 ml syringe. The haemolymph sample was then placed into a 1.5 ml Eppendorf tube and maintained on ice. The whole animal was then immediately immersed in liquid nitrogen for 1 minute and subsequently stored at -20 °C before the tissue preparation was carried out.

For analysis, 50 μl of the haemolymph sample was then pipetted into

another 1.5 ml Eppendorf tube containing 50 μl of chilled 0.6 M perchloric acid. After mixing thoroughly, the samples were centrifuged for 20 minutes at 11000g (Heraeus Sepatech Biofuge A or MSE Microcentaur) and the resulting supernatant removed. This was then neutralized by the drop-wise addition of 2 M potassium bicarbonate. The pH of the solution was monitored during this process using narrow range pH paper (6 - 8). After cooling on ice for 10 minutes, the precipitated potassium perchlorate was removed from the solution by a further 10 minutes of centrifugation. The supernatant was then separated from the pellet and frozen at - 20 °C until required (normally the next day).

Frozen tissue samples were prepared for L-lactate determination by perchloric acid extraction in a similar manner. The abdomen of the frozen animal was initially dissected from the cephlothorax and the pleopods and telson removed. The muscle tissue was then removed and placed in a mortar filled with approximately 20 ml of liquid nitrogen and ground into a fine powder using a pestle. Once the liquid nitrogen had evaporated away, approximately 50 mg of the powder was transferred into a pre-weighed 1.5 ml Eppendorf tube. The exact mass of tissue was then estimated. 500 μl of chilled 0.3 M perchloric acid were then added to the Eppendorf tube. After mixing (Miximatic, Jencons [Scientific] Limited), the sample tubes were centrifuged for 10 minutes at 11000g. The resulting initial supernatant was then separated and stored on ice. A further 500 μl of 0.3 M perchloric acid were then added to the pellet and the pellet resuspended and centrifuged for another 20 minutes. The supernatant from this second extraction was then added to the supernatant taken from the initial extraction. The total volume of supernatant from both extractions was then neutralized with potassium bicarbonate, as described above, and centrifuged for a further 10 minutes. The final supernatant was removed and stored at - 20 °C until required (normally the next day).

4.2.3b. Method for the analysis of L-lactate

The concentrations L-lactate in the haemolymph and muscle samples were estimated using the method of Gutmann and Wahlefeld (1974) as modified by Engel & Jones (1978). The principle of the assay is based on the NAD^+ dependent oxidation of lactate to form pyruvate catalysed by lactic dehydrogenase (EC 1.1.1.27) according to the reaction:

$$\text{L-lactate} + NAD^+ \Leftrightarrow \text{Pyruvate} + NADH$$

The subsequent formation of NADH can be measured spectrophotometrically at an absorbance wavelength of 340 nm.

The following reagents were mixed in a 1.5 μl Eppendorf tube: 50 μl of 40 mM NAD^+, 50 μl of sample or standard, 5 μl of L-lactate dehydrogenase, and 1000 μl of glycine-hydrazine buffer. The buffer was prepared by adding 3.75 g of glycine, 0.5 g of EDTA and 2 ml of hydrazine-hydrate to 98 ml of distilled water. The EDTA was included in the buffer to remove any free copper ions which have been shown to interfere with the end point of the reaction (Engel & Jones, 1978). The pH of buffer solution was adjusted to 9.0 using 1 M NaOH. The reagent mixture was incubated for 2 hours in a water bath at 37 °C. The absorbance of the reaction mixture at 340 nm was then determined (Philips PU 8700 or Shimazdu UV - 1201).

Individual calibrations were prepared for each batch of samples by the serial dilution of a standard 2 mM Lactic acid solution. The subsequent calibration curve was found to be linear over the absorbance range of at least 0 - 0.7. Any samples that were found to be outside this range were diluted with distilled water and re-assayed. Blanks were also prepared by substituting 50 μl of distilled water for the sample or standard. Throughout the whole extraction and assay procedure disposable plastic gloves were worn to prevent any lactate contamination during handling (from skin). The total concentration of L-lactate

in the original haemolymph and abdominal muscle tissue samples was then calculated from the calibration curve.

4.2.4. Accumulation of thiols during exposure to sulphide during both aerobic and anaerobic metabolism.

The concentrations of thiols in the haemolymph, hepatopancreas and abdominal muscle of specimens of *Calocaris macandreae* were also determined using the procedures detailed in section 3.2.2 following experimental exposure to different sulphidic concentrations under normoxia and hypoxic conditions (as described above).

4.3. Results

4.3.1. Whole animal oxygen consumption

The animals generally remained inactive in the respirometers, although they were occasionally observed to beat their pleopods and to move in the chambers. The rates of oxygen consumption determined were therefore assumed to represent the quiescent rates of metabolism. The effect of sulphide on the rate of oxygen consumption of *Calocaris macandreae* is shown in Figure 4.2. The animals continued to respire aerobically, as indicated by the continued uptake of oxygen and the absence of lactate accumulation (see below) despite the presence of sulphide in micromolar concentrations. Although there was an apparent increase in the mean $\dot{M}o_2$ during exposure to low (<100 μM) concentrations of sulphide under both normoxic and hypoxic conditions (Po_2 = 47 ± 7 Torr), this increase was not significant (ANOVA, $p > 0.05$). Exposure to sulphide concentrations greater than 100 μM under normoxia resulted in a pronounced decrease in $\dot{M}o_2$. This decrease did not become significant (ANOVA + Tukey, $p < 0.05$), however, until the mud-shrimps were exposed to sulphide concentrations greater than 180 μM. A similar reduction in $\dot{M}o_2$ occurred when the mud-shrimps were exposed to sulphide under hypoxic conditions although this reduction occurred at significantly lower sulphide concentrations (ANOVA + Tukey, $p < 0.05$). In addition, even at concentrations between 200 - 300 μM, Mo_2 did not approach zero indicating that some animals continued to consume oxygen .

4.3.2. Anaerobic metabolism under sulphidic conditions

Exposure of *Calocaris macandreae* to increasing sulphide concentrations for 24 h under near normoxic conditions, resulted in a gradual but significant accumulation of L-lactate in the abdominal muscle (Figure 4.3). When exposed to the same range of sulphide concentrations under hypoxic conditions (Po_2 = 20.1 ± 1.3 Torr), however, there was a pronounced and significant (ANOVA +

Tukey, $p < 0.05$) increase in the L-lactate content in the muscle when sulphide concentrations exceeded 25 μM (Figure 4.3). A further increase in L-lactate muscle content at higher sulphide concentrations (25 - 160 μM) was also seen, although this was not significant (ANOVA + Tukey, $p > 0.05$).

Similarly, the concentration of L-lactate in the haemolymph of mud-shrimps exposed to sulphide under near normoxic conditions increased significantly (ANOVA + Tukey, $p < 0.05$) only at sulphide concentrations greater than 150 μM (Fig 4.4). Under hypoxic conditions, however, L-lactate accumulated significantly (ANOVA + Tukey, $p < 0.05$) at much lower sulphide concentrations (35 μM). During exposure to anoxic conditions L-lactate was seen to accumalte in the haemolymph (21.0 ± 2.9 μM) and abdominal muscle (32.8 ± μmol.g^{-1}) at similar rates to the higher concentration sulphide exposures.

4.3.3. Accumulation of thiols during exposure to sulphide under normoxic and hypoxic conditions

The concentrations of thiols that accumulated in the haemolymph of *Calocaris macandreae* during exposure to sulphide under normoxic and hypoxic conditions differed (Figure 4.5 & 4.6). There was a large significant (ANOVA + Tukey, $p < 0.05$) increase in the concentration of thiosulphate in the haemolymph at sulphide concentrations up to 35 μM under hypoxic conditions but, at higher sulphide concentrations, the concentration of thiosulphate was significantly (ANOVA + Tukey, $p < 0.05$) lower (Figure 4.5). Under normoxic conditions there was also a significant increase (ANOVA + Tukey, $p < 0.05$) in the concentration of thiosulphate in the haemolymph but this did not decrease significantly (ANOVA + Tukey, $p > 0.05$) at higher sulphide concentrations (Figure 4.6). Under both sets of conditions, however, low but significant (ANOVA + Tukey, $p < 0.05$) concentrations of both sulphide and sulphite were recorded in the haemolymph.

Similar trends in the accumulation of thiols during sulphide exposure under

normoxia and hypoxia were also observed in the hepatopancreas of *Calocaris macandreae*. When exposed to hypoxia, thiosulphate accumulated significantly (ANOVA + Tukey, $p < 0.05$) in the hepatopancreas at an exposure concentration of 7 μM sulphide (Figure 4.7). Under normoxic conditions, however, the increase in thiosulphate occurred only at higher sulphide concentrations. This trend did not become significant (ANOVA + Tukey, $p < 0.05$) until an exposure sulphide concentration of 63 μM was reached (Figure 4.8). Sulphite did not accumulate significantly (ANOVA, $p > 0.55$) in the hepatopancreas under hypoxic conditions but did increase significantly (ANOVA + Tukey, $p < 0.05$) under normoxia at sulphide exposure concentrations in excess of 150 μM sulphide. Sulphide was seen to increase significantly (ANOVA + Tukey, $p < 0.05$) to low micromolar concentrations under both normoxic and hypoxic conditions.

The concentrations of sulphite and sulphide in the abdominal muscle tissue of *Calocaris macandreae* were not found to increase significantly (ANOVA + Tukey, $p > 0.115$) when exposed to sulphide under both normoxic and hypoxic conditions (Figure 4.9 & Figure 4.10). In contrast, the concentration of thiosulphate was found to increase significantly (ANOVA + Tukey & *t* - tests, $p < 0.05$) during exposure to sulphide. A significant (ANOVA + Tukey, $p < 0.05$) decrease in the concentration of thiosulphate in the muscle tissue was found to be coincident with the decrease of thiosulphate in the haemolymph (see above) at sulphide concentrations in excess of 35 μM under hypoxic conditions. This trend, however, was not found to be significant (pooled *t* - test, $p > 0.12$) during exposure to sulphide under normoxic conditions.

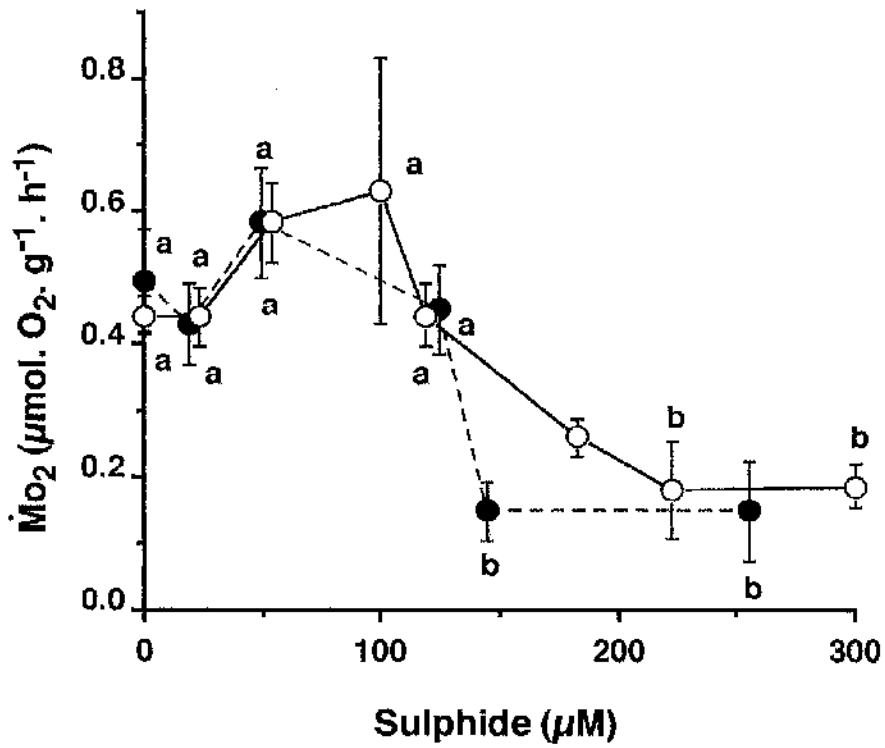

Figure 4.2 The effect of sulphide on the rate of oxygen consumption ($\dot{M}o_2$) of *Calocaris macandreae* under near normoxic (Po_2 > 140 Torr) (open circles) and under moderate hypoxic conditions (Po_2 = 46.9 ± 7.2 Torr) (closed circles). Values are means ± SE (n = 6 - 16). a/b = significant difference (ANOVA + Tukey, $p < 0.05$)

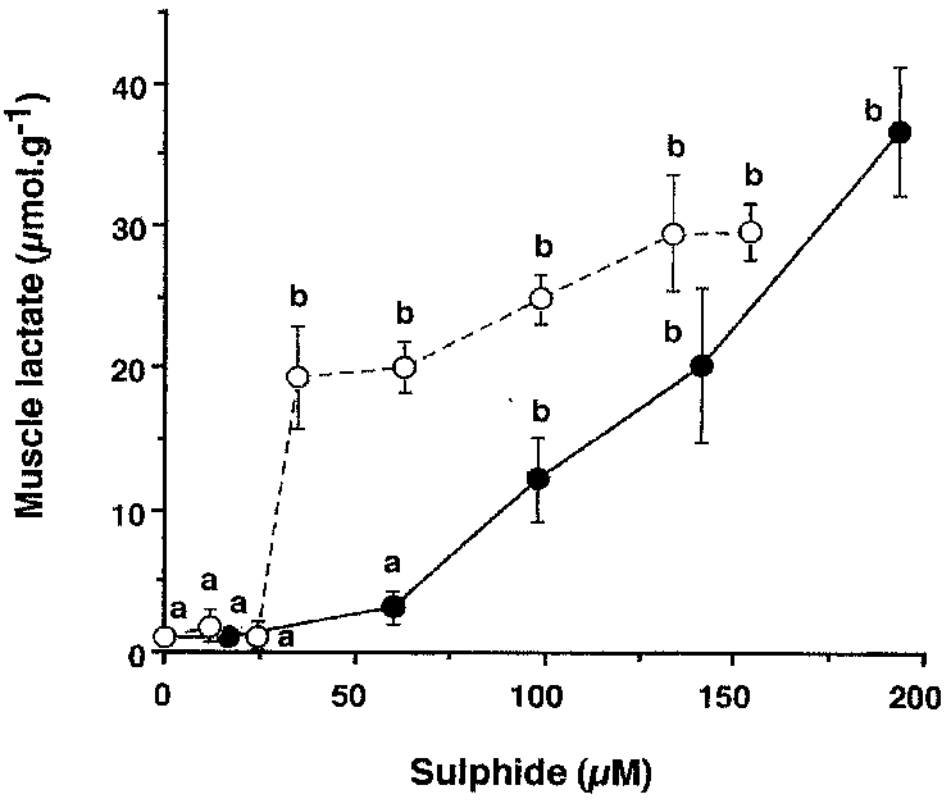

Figure 4.3 The concentration of L-lactate in the abdominal muscle of *Calocaris macandreae* following 24 hour exposure to differing sulphide concentrations under near normoxia (Po_2 > 140 Torr) (closed circles) and severe hypoxia, (Po_2 = 20 ± 1 Torr) (open circles). Values are means ± SE (n = 6- 10). a/b = significant difference (ANOVA + Tukey, $p < 0.05$)

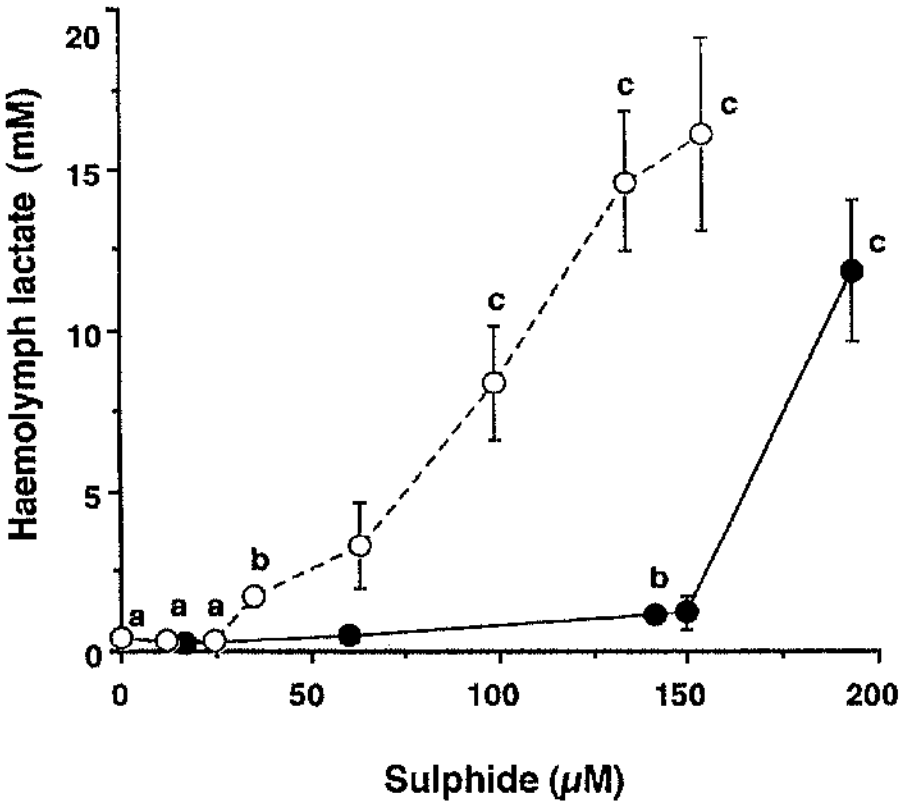

Figure 4.4 The concentration of L-lactate in the haemolymph of *Calocaris macandreae* following 24 hour exposure to differing sulphide concentrations under near normoxia (Po_2 > 140 Torr) (closed circles) and severe hypoxia, (Po_2 = 20 ± 1 Torr) (open circles). Values are means ± SE (n = 6 - 10). a/b/c = significant difference (ANOVA + Tukey or Mood test , $p < 0.05$)

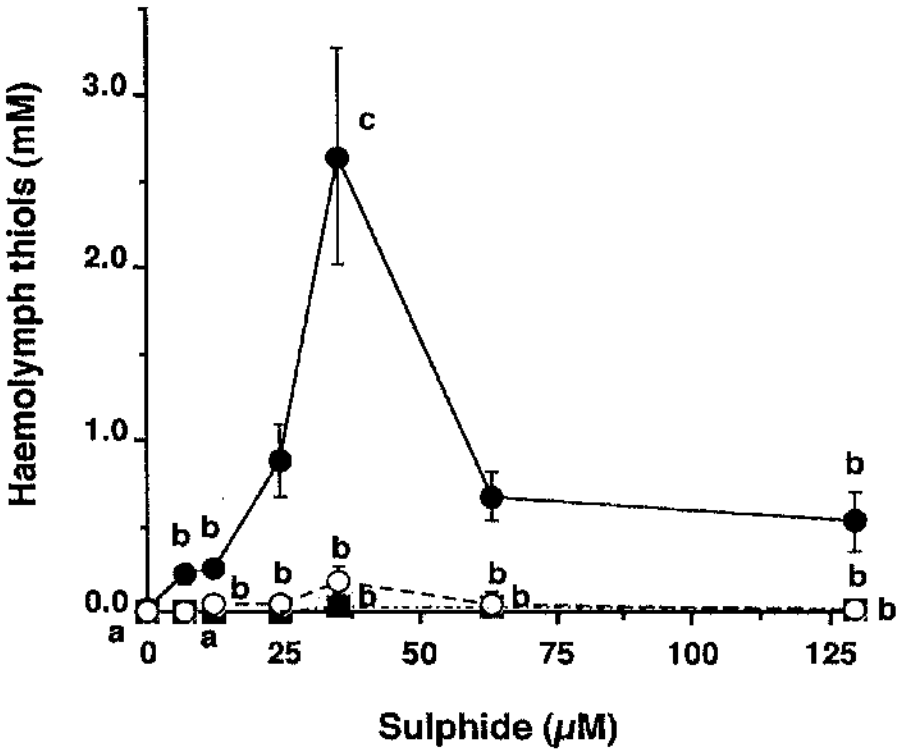

Figure 4.5 The concentrations of thiosulphate (closed circles), sulphite (open circles) and sulphide (closed squares) in the haemolymph of *Calocaris macandreae* following 24 h exposure to differing sulphide concentrations under severe hypoxia (Po_2 = 20 ± 1 Torr). Values are means ± SE (n = 6 - 10). a/b/c = significant difference (ANOVA + Tukey, $p < 0.05$).

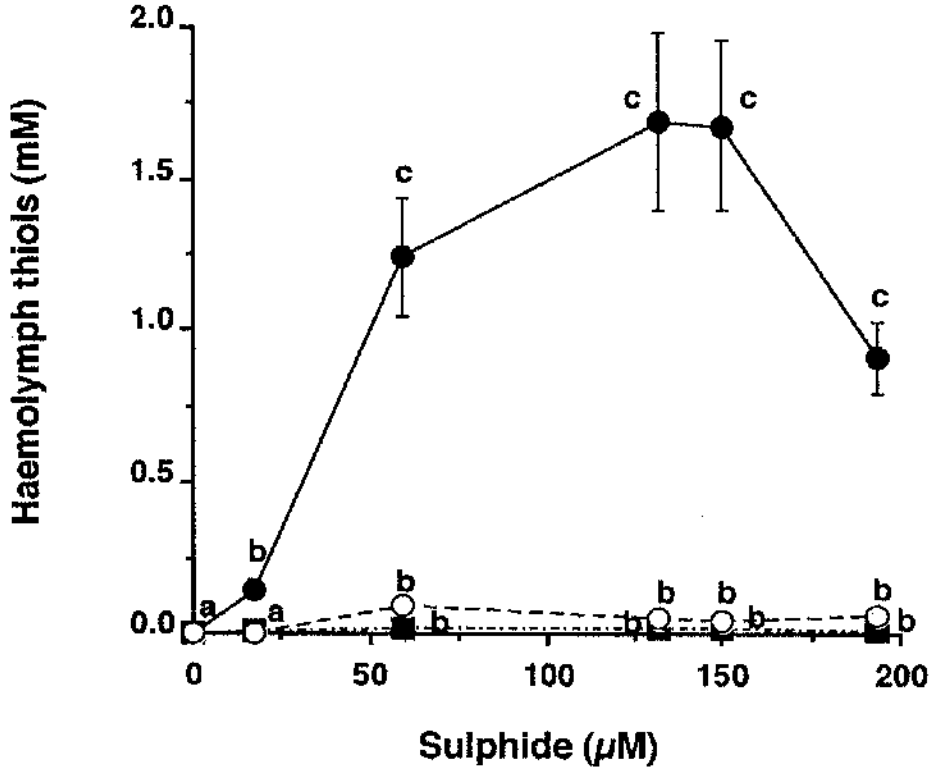

Figure 4.6 The concentrations of thiosulphate (closed circles), sulphite (open circles) and sulphide (closed squares) in the haemolymph of *Calocaris macandreae* following 24 h exposure to differing sulphide concentrations under normoxia (Po_2 > 140 Torr). Values are means ± SE (n = 6 - 10). a/b/c = significant difference (ANOVA + Tukey, $p < 0.05$)

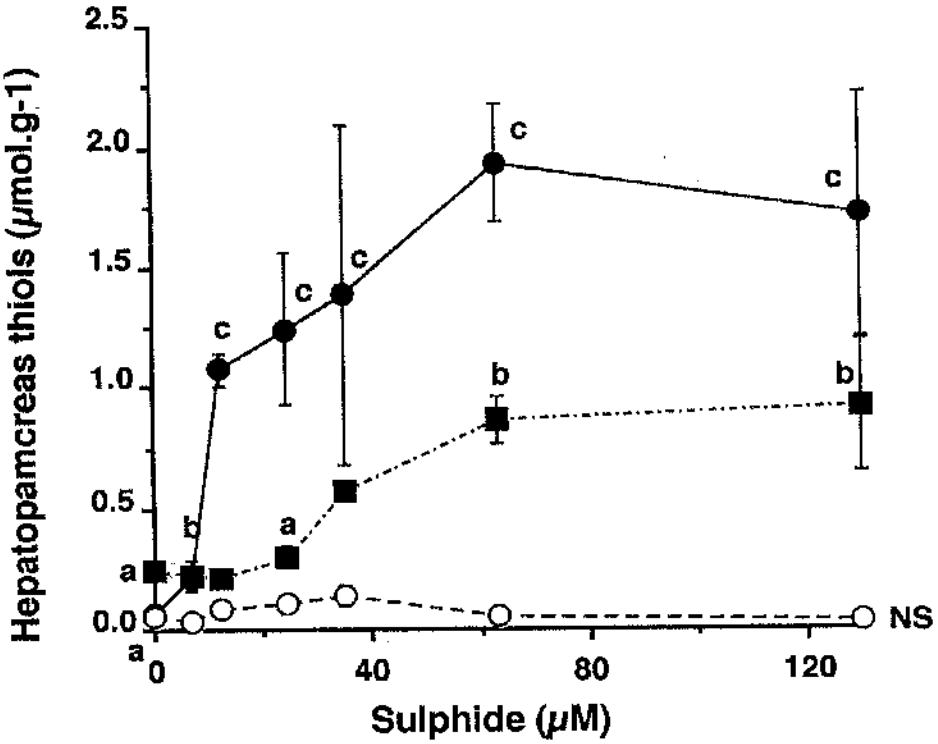

Figure 4.7 The concentrations of thiosulphate (closed circles), sulphite (open circles) and sulphide (closed squares) in the hepatopancreas of *Calocaris macandreae* following 24 h exposure to differing sulphide concentrations under severe hypoxia (Po_2 = 20 ± 1 Torr). Values are means ± SE (n = 6 - 10). a/b/c = significant difference (ANOVA + Tukey, *t*, Mann-Whitney or Mood Tests, NS = not significant, $p < 0.05$).

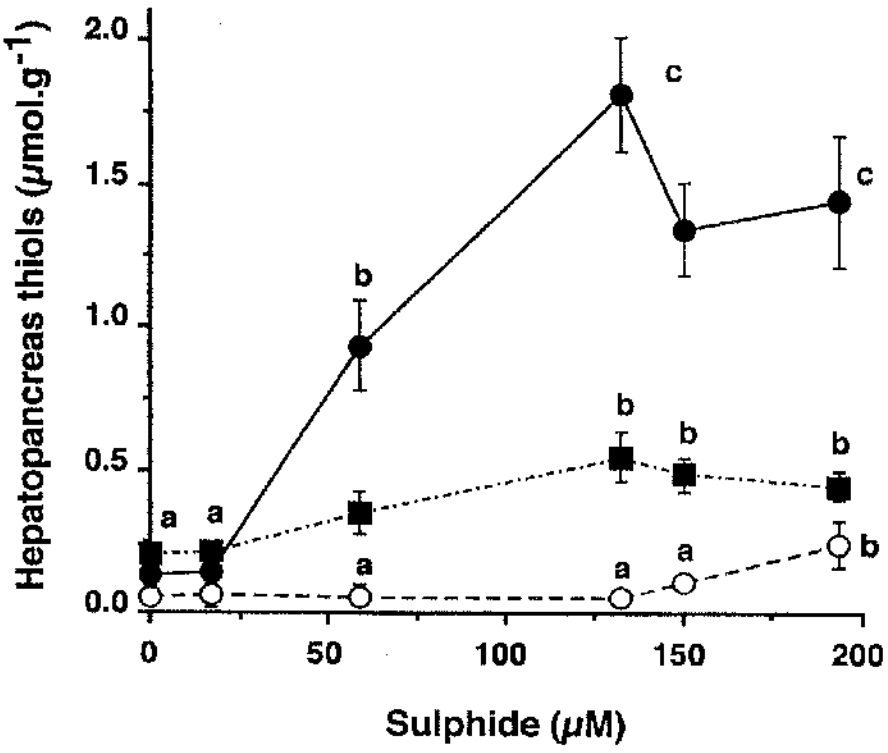

Figure 4.8 The concentrations of thiosulphate (closed circles), sulphite (open circles) and sulphide (closed squares) in the hepatopancreas of *Calocaris macandreae* following 24 h exposure to differing sulphide concentrations under normoxia (Po_2 > 140 Torr). Values are means ± SE (n = 6 - 10). a/b/c = significant difference (ANOVA + Tukey, $p < 0.05$).

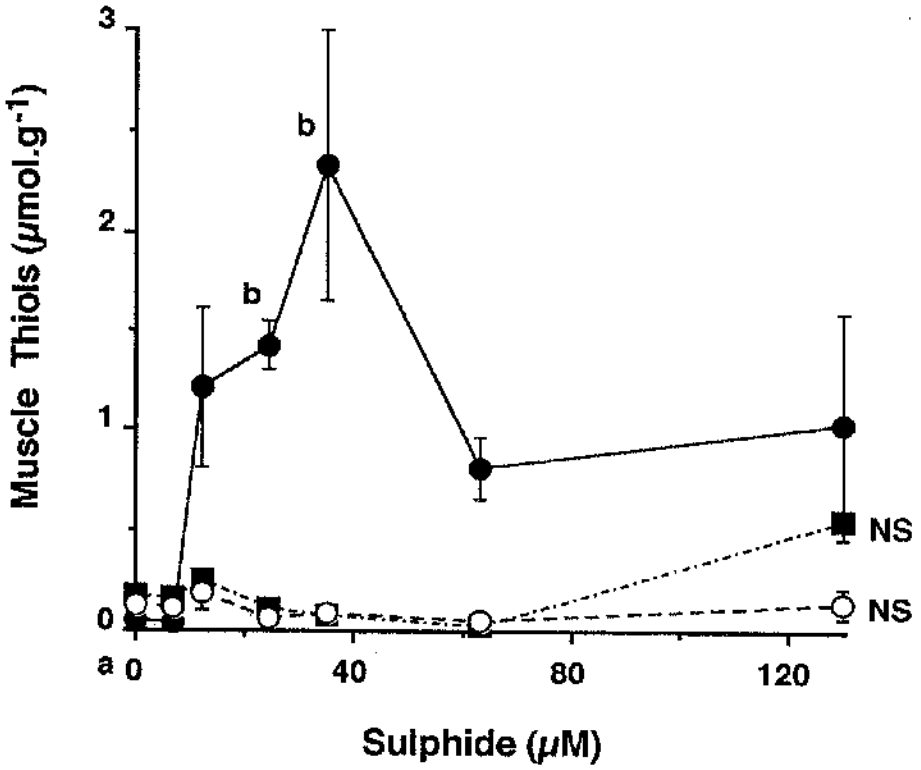

Figure 4.9 The concentrations of thiosulphate (closed circles), sulphite (open circles) and sulphide (closed squares) in abdominal muscle tissue of *Calocaris macandreae* following 24 h exposure to differing sulphide concentrations under severe hypoxia (Po_2 = 20 ± 1 Torr). Values are means ± SE (n = 6 - 10). a/b = significant difference, NS = not significant, ANOVA + Tukey, $p < 0.05$.

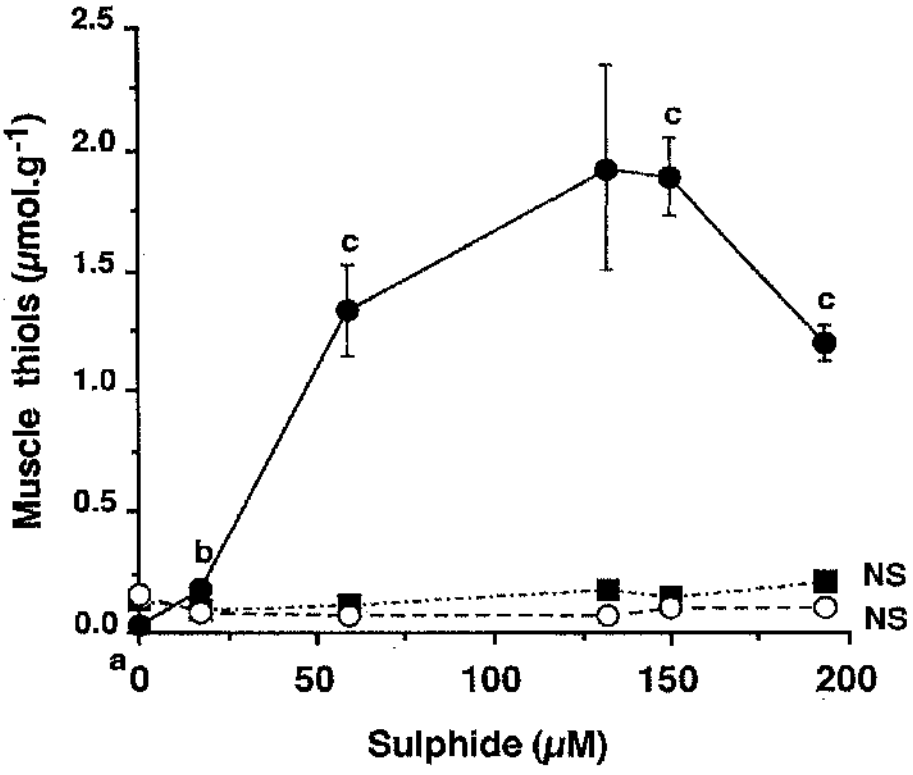

Figure 4.10 The concentrations of thiosulphate (closed circles), sulphite (open circles) and sulphide (closed squares) in abdominal muscle tissue of *Calocaris macandreae* following 24 h exposure to differing sulphide concentrations under normoxia (Po_2 > 140 Torr). Values are means ± SE (n = 6 - 10). a/b = significant difference (ANOVA + Tukey or *t* - tests, NS = not significant, $p < 0.05$).

4.4. Discussion

4.4.1. Whole animal oxygen consumption

The effect of sulphide on whole animal oxygen consumption has been investigated in relatively few marine invertebrates (Childress & Mickel, 1982; Chen *et al* ., 1987; Anderson *et al* ., 1987, 1990; Eaton & Arp, 1993). Animals such as the bivalves *Solemya reidi* and *S. velum* that inhabit reduced sediments, and which contain endosymbiotic sulphide-oxidizing bacteria, appear capable of maintaining their rates of oxygen consumption over a range of sulphide concentrations, before resorting to anaerobic metabolism at higher sulphide concentrations (Chen *et al* , 1987; Anderson *et al* , 1987, 1990). This has also been shown to occur in the deep sea hydrothermal vent clam, *Calyptogena pacifica* (Childress & Mickel, 1982). Similarly, the intertidal echiuran worm, *Urechis caupo* and the polychaete worm *Arenicola marina* have also been shown to maintain their oxygen consumption when exposed to low micromolar concentrations of sulphide, before resorting to anaerobic metabolism at higher sulphide concentrations (Eaton & Arp, 1993; Grieshaber *et al.*, 1996). The rate of oxygen consumption of coelomic fluid from *U. caupo*, however, increased significantly when exposed to low micromolar concentrations of sulphide. This trend, however, was not established during the determination of the whole animal oxygen consumption of *U. caupo.*

There are very few published reports on the direct effects of sulphide on oxygen consumption in Crustacea. Vetter *et al.* (1987) observed that both the heart and scaphognathite rates of the hydrothermal vent crab *Bythograea thermydron* were maintained during exposure to high sulphide concentrations (> 700 μM). This has been interpreted as indicating continued oxygen consumption under these conditions (Eaton & Arp, 1993). In another study, Gorodezky & Childress (1994), showed that the oxygen consumption of *B. thermydron* increased both during and after exposure to sulphide but the reasons for this remain unclear.

Kochevar & Childress (pers. comm. in Eaton & Arp, 1993), suggest that the callianassid mud-shrimp *Neotrypaea* (as *Callianassa) californiensis* could not regulate its oxygen consumption during sulphide exposure. In the present study, however, the calocaridid mud-shrimp *Calocaris macandreae* was apparently able to maintain its oxygen consumption when exposed to sulphide concentrations in excess of 150 μM under normoxic conditions and in excess of 100 μM sulphide under hypoxia. This suggests that the sulphide 'detoxification' mechanism is oxygen-dependent. Further evidence to support this hypothesis is discussed below and in Chapter 7.

The increase in oxygen consumption at low sulphide concentrations, although not significant, may indicate a trend that is masked by variation in the responses of individual animals, since additional oxygen will be required to oxidize sulphide to thiosulphate. This trend has apparently been observed in other experimental studies of benthic marine invertebrates (Eaton & Arp, 1993, Grieshaber *et al.*, 1996). It has not however, been clearly demonstrated (significantly). This may be due to the high degree of variation which is typically observed in this type of whole animal experiment. Despite the statistical non-significant inference seen in these data, sulphide entering the tissues is apparently oxidized to thiosulphate. Therefore additional oxygen may be required if aerobic respiration is maintained.

The oxygen consumption of the mud-shrimp *Calocaris macandreae* was seen to increase during exposure to sulphide concentrations of less than 100 μM sulphide. This may be interpreted as the oxygen required to produce the 'oxidation' metabolites thiosulphate and sulphite, in addition to maintaining normal aerobic metabolism. At higher sulphide concentrations, however, oxygen consumption declined and may reflect oxygen provision within the tissues become limiting as the amount of oxygen required to oxidize sulphide to thiosulphate and sulphite increases. When the 'oxidation' mechanism becomes saturated in this manner as the oxygen supply becomes limited and intracellular

sulphide concentrations become toxic the mud-shrimps resort to anaerobiosis (which may operate concurrently with aerobic metabolic pathways, see Chapter 5).

The 'oxidation' mechanism may therefore allow aerobic metabolism to be maintained despite the presence of sulphide, although the mud-shrimp's sensitivity to sulphide is apparently greater at lower oxygen concentrations. Sulphide therefore appears to have a concentration-dependent capacity to either stimulate or inhibit aerobic metabolism in mud-shrimps which is also related to the oxygen availability. Aerobic metabolism appears to increase during exposure to sulphide, when oxygen is available, and can be maintained even under severe hypoxic and sulphidic conditions.

An increase in oxygen consumption following exposure to sulphide has also been observed in the priapulid worm *Halicryptus spinulosis* (Oeschger *et al.*, 1992), the hydrothermal vent crab *Bythograea thermydron* (Gorodezky & Childress, 1994) and in the isopod *Saduria entomon* (pers. comm. Hagerman). This may in part be due to the re-oxidation of anaerobic metabolites such as L-lactate (Oeschger *et al.*, 1992) which can accumulate during exposure to sulphide through the initiation of anaerobic metabolic metabolism (see below).

The reduction in oxygen consumption during exposure to sulphide concentrations greater than 150 μM, under both oxygen saturation treatments, suggests the use of anaerobic metabolism under these conditions. The mean rates of oxygen consumption, however, did not approach zero as might be expected if all mud-shrimps were respiring anaerobically during exposure to sulphide. This may be explained by examining the data for individual mud-shrimps and not just the mean values. Many animals were clearly not consuming oxygen indicating that the 'oxidation' mechanism was saturated (reached its maximum rate) and anaerobic metabolism was initiated. A small but significant number of mud-shrimps, however, continued to consume measurable quantities of oxygen which indicates that there may be variation between individuals in

their ability to cope with exposure to such high sulphide concentrations.

Interestingly, if the concentrations of sulphide in the burrows of *Calocaris macandreae* are similar to or lower than those found in the burrows of *Callianassa subterranea,* (low micromolar, see section 2.3.2.) these oxygen consumption data indicate that aerobic metabolism can be maintained and regulated in the burrow environment. An ambient sulphide-oxygen environmental threshold, however, seems to exist which dictates the bias between aerobic and anaerobic metabolism. It may also be possible for aerobic and anaerobic metabolisms to function concurrently. This question is addressed in Chapter 5.

4.4.2. Sulphide-induced anaerobic metabolism

At some critical ambient sulphide concentration and oxygen tension when either the availability of oxygen to 'oxidize' sulphide is limited or the maximum rate of 'oxidation' mechanism is reached, a switch to anaerobic metabolism seems to occur. Anaerobic metabolism has been observed in a small number of marine invertebrates during sulphide-induced anaerobiosis (Anderson *et al.*, 1990; Oeschger & Vetter, 1992; Völkel & Grieshaber, 1992, 1994; Hagerman & Vismann, 1993, 1995; Grieshaber *et al.*, 1996). These include the priapulid worm *Halicryptus spinulosus* which has been found to accumulate the anaerobic metabolite succinate in the haemolymph and body wall tissue after 4 days exposure to 200 μM sulphide during normoxic incubation (Oeschger & Vetter, 1992). This suggests that the worms could not maintain their aerobic metabolism during long-term exposure to sulphide. The intertidal polychaete worm *Arenicola marina* has been observed to maintain aerobic metabolism during exposure at to least 100 μM sulphide before initiating anaerobic metabolic pathways that produce succinate as the major end-product (Völkel & Grieshaber, 1992, 1994). In the absence of sulphide the accumulation of succinate commenced at 10 kPa (equivalent to 75 Torr), although when exposed to 200

μM sulphide for 8 hours under normoxia, succinate was found to accumulate. A similar trend in the increase of accumulation of succinate has also been seen in the peanut worm *Sipunculus nudus*. When exposed to 200 μM sulphide under normoxia the animal resorted to anaerobic metabolism (Völkel & Grieshaber, 1992). Similarly, the brackish water isopod *Saduria entomon* resorted to anaerobic metabolism when exposed to a concentration of 150 μM sulphide under hypoxic conditions between 4 - 6.7 kPa (equivalent to 30 - 50.3 Torr) during a 48 hour exposure (Hagerman & Vismann, 1993). L-lactate and alanine both accumulated significantly under these conditions. At these hypoxic oxygen tensions in the absence of sulphide the concentrations of these anaerobic metabolites did not differ significantly from the normoxic controls indicating anaerobiosis induced by sulphide during these sulphidic conditions. Exposure to only low micromolar concentrations of sulphide in another crustacean *Crangon crangon*, however, induced total anaerobic metabolism (indicated by the accumulation of L-lactate). This occurred at an oxygen saturation which would permit aerobic metabolism in the absence of sulphide (Hagerman & Vismann, 1995).

Direct comparisons between these studies is complicated by the duration of exposure to the various conditions since the initiation of anaerobic metabolism is also time dependent. Despite this, a clear trend in the initiation of anaerobic metabolism seems to exist and occurs at a critical sulphide-oxygen threshold when conditions become too severe for the animal. Clearly, differences in sulphide tolerance will affect the position of the critical point. This difference can be illustrated by two of the species of marine invertebrate described above. The brown shrimp *C. crangon* does not tolerate sulphide and must rely on its limited anaerobic metabolism at far lower sulphide concentrations than the polychaete worm *Arenicola marina* which does tolerate sulphide and can maintain its aerobic metabolism.

A critical transition point between anaerobic and aerobic metabolism (indicated by an accumulation of lactate in the abdominal muscle tissue and in the haemolymph) in the mud-shrimp *Calocaris macandreae* occurs when ambient oxygen is severely limited. When exposed to sulphide concentrations of up to 25 μM under severe hypoxia (Po_2 = 20 ± 1 Torr) aerobic metabolism can, however, be maintained. Under normoxic conditions a similar critical transition between aerobic and anaerobic metabolism is also apparent. The change to anaerobic metabolism, however, occurred at greater sulphide concentrations than under hypoxia. Interestingly, the transition under normoxia occurred over a far less defined range of sulphide concentrations. As was suggested above during the discussion of the whole animal oxygen consumption data, some variability in the tolerance of individual animals to sulphide seems to exist. This may account for the less well-defined initiation of anaerobic metabolic pathways when ambient oxygen is not limiting.

The earlier onset of anaerobiosis under hypoxic and sulphidic conditions is also seen in the accumulation of thiols in mud-shrimps. Lower concentrations of thiosulphate were produced under hypoxic conditions than under normoxia (Figure 4.5 - 4.10). In addition, when exposed to hypoxia, a significant decrease in the concentration of thiosulphate in the haemolymph occurred at low micromolar ambient sulphide concentrations, whereas under normoxia, thiosulphate in the haemolymph did not decrease significantly even during the highest sulphide exposures. The concentration of thiosulphate was also seen to increase significantly in the hepatopancreas and abdominal muscle tissues of *Calocaris macandreae* under both normoxic and hypoxic conditions. Low but significant concentrations of sulphite also accumulated in the haemolymph of *C. macandreae* under both oxygen treatments but sulphite was not observed to accumulate significantly in abdominal muscle tissues. The concentration of intra-cellular sulphide in the abdominal muscle tissue of the mud-shrimp *C.*

macandreae did not differ from control values despite exposure to severe sulphidic and hypoxic conditions. This suggests that a considerable degree of protection is afforded to this body compartment by the 'oxidation' mechanism since thiosulphate was found to be the only metabolite present. Differences in the concentrations of thiols were observed in the hepatopancreas. Although sulphite accumulated significantly under normoxia, no change was seen under hypoxic conditions. Interestingly, sulphide began to accumulate at a lower ambient sulphide concentration when the mud-shrimps were exposed to hypoxia than during normoxic conditions. This also appears to indicate the existence of an oxygen-dependent 'detoxification' mechanism. The reduced availability of oxygen required for the 'oxidation' of sulphide in the mitochondria during low oxygen tensions may prevent effective sulphide 'oxidation' (Völkel & Grieshaber, 1996). As has been discussed here and in previous chapters, it is clear that a number of factors, acting in a complex manner at different biological hierarchies, influence the sulphide 'oxidation' capacity of mud-shrimps. These mechanisms and in particular the requirement of oxygen to produce thiosulphate from sulphide in addition to aerobic metabolic processes will be discussed further in chapter 7

4.4.3. Summary of Chapter 4

The main points of Chapter 4 in relation to sulphide metabolism in mud-shrimps are summarized below:-

a. A sulphide 'oxidation' mechanism apparently allows aerobic metabolism to be maintained to a certain extent despite the potential inhibitory effects of sulphide.

b. The 'oxidation' mechanism is apparently oxygen-dependent and appears to operate 'oxidizing sulphide' below a critical transition point.

c. At sulphide concentrations in excess of the critical transition point the 'oxidation' mechanism becomes saturated (reaches its maximum rate). Mud-shrimps therefore rely on anaerobic metabolism to survive these conditions.

5. A preliminary calo-respirometric study of the sulphide metabolism in mud-shrimps.

5.1. Introduction

5.1.1. Metabolic adaptations to hypoxic, anoxic and sulphidic conditions

Sulphide has been shown to induce anaerobiosis in a variety of marine invertebrates (Anderson, 1990; Oeschger & Vetter, 1992; Völkel & Grieshaber, 1992; 1994; Hagerman & Vismann, 1993, 1995; Grieshaber *et al.*, 1996). An ambient sulphide-oxygen threshold seems to be apparent which dictates the bias between aerobic and anaerobic metabolism in these animals (Grieshaber *et al.*, 1996). There also appears to be considerable inter-specific differences in the ability of many marine invertebrates to maintain aerobic metabolic pathways during exposure to sulphide before resorting to anaerobic metabolism (see Chapter 4). During exposure to sulphide it may be possible for both aerobic and anaerobic metabolisms to run concurrently and also for specific tissues to function anaerobically while others remain aerobic.

For many years calorimetric techniques (the estimation of heat dissipation) have been widely used to determine the metabolic rates of many animals. This technique, however, has been restricted to large animals because of the precision required (Eckert *et al.*, 1988). More recently, the availability of micro-calo-respirometric techniques has enabled the metabolism of small invertebrates, giving out very little heat and having low oxygen consumption rates, to be investigated. Direct calorimetry (the rate of heat dissipation) allows the continuous determination of total metabolism, while simultaneous measurements of oxygen uptake (indirect calorimetry) monitor the changing rates of oxygen consumption (Gnaiger 1983b). When the rate of heat dissipation and oxygen consumption are compared, in equivalent units of energy ($mJ.h^{-1}.g^{-1}$), the relative proportions of aerobic and anaerobic metabolism can be calculated during

different experimental conditions (Pamatmat, 1983a; 1983b). The simultaneous determination of heat dissipation (direct) and oxygen consumption (indirect) therefore provides a value insight into aerobic and anaerobic metabolic rates (Gnaiger, 1983b, Gnaiger *et al.*, 1989). Using this technique, a small number of marine invertebrates have been shown to rely on both aerobic and anaerobic metabolism simultaneously. These include the bivalve mollusc *Mytilus edulis* (Pamatmat, 1983b; Shick *et al.*, 1986) and the sea anemone *Actina equina* (Shick, 1981) during exposure to air. In addition, a small number of marine invertebrates have also been described as having the ability to depress their metabolism to assist in their survival during a reduction of ambient oxygen tension. These include the sipunculid worm *Sipunculus nudus*, the priapulid *Halicryptus spinulosus* (Oeschger, 1992) the polychaetes *Marenzelleria viridis* and *Hediste diversicolor* (Fritzsche & von Oertzen, 1995) and the bivalve molluscs *Mytilus edulis*, *Modiolus demissus*, *Polymesdoa caroliniana* (Pamatmat, 1979, 1980), *Arctica islandica* and *Astarte borealis* (Oeschger, 1990). Although the mechanisms which underlie these metabolic adaptations are poorly understood, the ability of a variety of intertidal and benthic marine invertebrates to rely partially on anaerobic metabolism during periods of hypoxia, and to depress their metabolism during hypoxic and anoxic conditions, has been widely interpreted as ecophysiological adaptations to survive adverse environmental conditions (Oeschger, 1990, 1992; Guppy *et al.*, 1994). Only one study to date has investigated the relationship between aerobic metabolism and anaerobic metabolism during exposure to sulphide using calo-respirometric techniques (Schneider, 1996). Schneider has recently reported that, in contrast to the species described above, the metabolic rate of both the larvae and adults of the brackish water polychaete *Marenzelleria viridis* increased during exposure to sulphidic and hypoxic conditions.

Calo-respirometric analytical equipment, however, is rarely available for physiological work. During the course of this project an opportunity arose to

conduct a small calo-respirometric study at the Institut für Zoophysiologie in the Heinrich Heine Universität of Düsseldorf. Some preliminary calo-respirometric investigations into sulphide metabolism of mud-shrimps under normoxic, hypoxia and sulphidic conditions were therefore conducted and are presented here. The metabolic rates in the mud-shrimp *Calocaris macandreae* were measured respirometrically as oxygen consumption and calorimetrically as heat dissipation during exposure to different ambient oxygen concentrations and sulphidic conditions. A comparison of these aspects of metabolism was conducted to establish the effect of hypoxia and sulphide on the metabolic requirements of mud-shrimps and to determine the balance between aerobic and anaerobic mechanisms.

5.2. Materials and methods

5.2.1. Collection, transportation and maintenance of mud-shrimps

Approximately 30 mud-shrimps (*Calocaris macandreae*) were collected by Agassiz trawling in the Clyde Sea (see section 3.2.1) approximately one week before being transported by air to the Institut für Zoophysiologie, Heinrich Heine Universität in Düsseldorf. Prior to departure, the mud-shrimps were placed individually into small mesh containers (to prevent fighting) in a sea water filled cool box. On arrival in Düsseldorf, the animals were placed in a re-circulating sea water aquarium (salinity 35 ± 1‰; temperature 15 ± 1 °C) and allowed 48 hours to recover from any stress incurred during transit.

5.2.2. Direct and indirect calo-respirometry (the instrument)

Metabolic activity was determined simultaneously by a micro-calorimeter (Thermal activity monitor, Thermetric) and a twin flow respirometer (Cyclobios) containing two Orbisphere potentiometric oxygen sensors (set up Figure 5.1). The difference in heat detected between the animal and reference chamber represents the heat dissipation of the animal (direct metabolic rate). Sea water was drawn into the calorimeter through the animal chamber and reference chamber by a multi-channel peristaltic pump (Ismatec IPC) at a flow rate of 38 $ml.h^{-1}$. The sea water was supplied from a 500 ml reservoir immersed in a 20 l water bath which was maintained at 10 ± 0.1 °C (RC6 Lauda Cooler connected to a copper cold-finger and external thermistor). The 500 ml flask was continually refilled by a peristaltic pump (Ismatec IPC) from an external 5 l sea water reservoir.

Oxygen consumption was estimated using the twin flow respirometer (Cyclobios) containing two sulphide-insensitive gold cathode potentiometric oxygen sensors (Orbisphere) each connected to an oxygen monitor (Cyclobios).

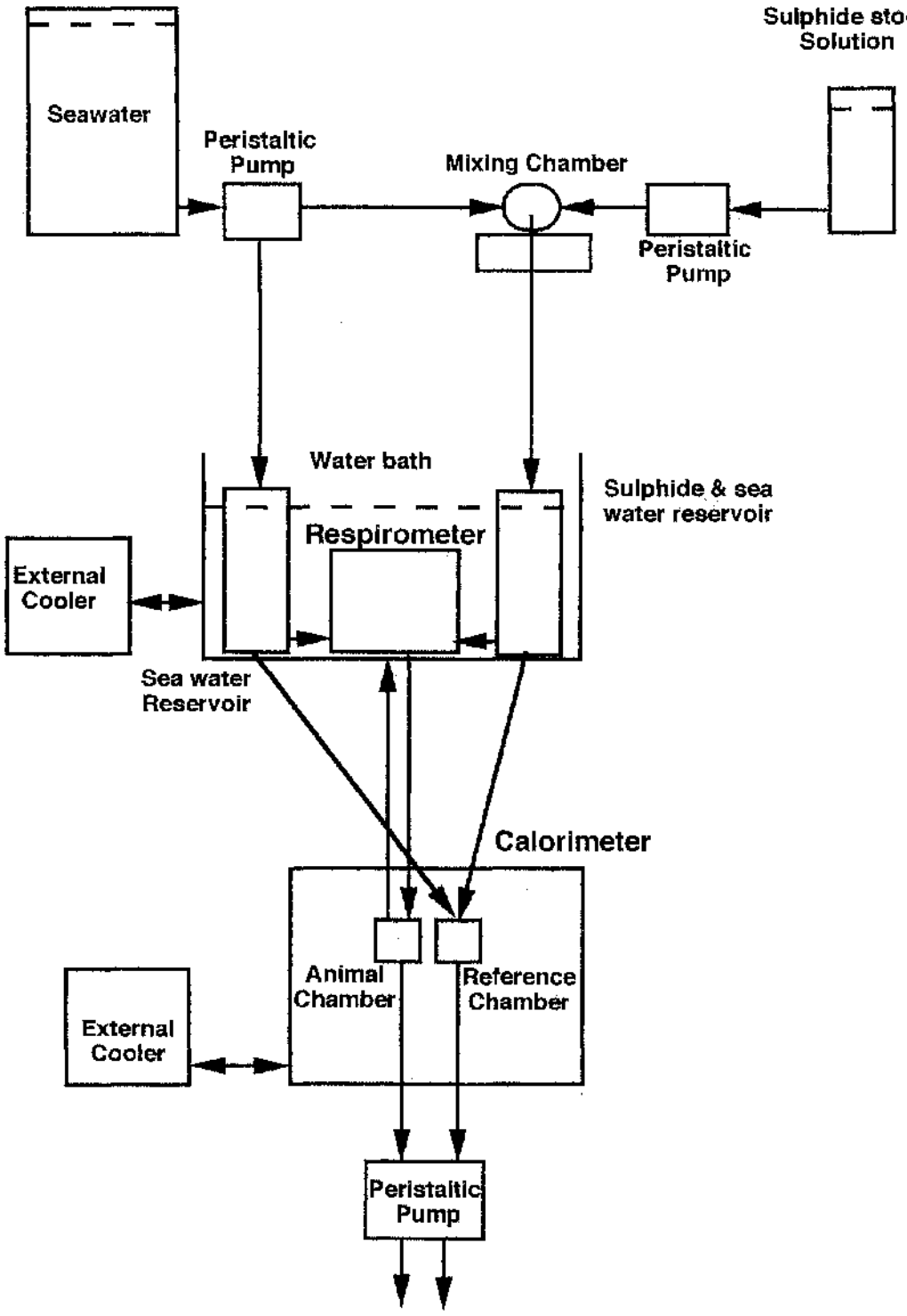

Figure 5.1 Diagram of the calorimetry and respirometry apparatus used to estimate simultaneously the metabolic heat dissipation ($_tQ = \mu W$) and oxygen consumption ($\dot{M}o_2 = \mu mol.O_2.g^{-1}.h^{-1}$) of the mud-shrimp *Calocaris macandreae* when exposed to normoxic, hypoxic, anoxic and sulphidic conditions.

This allowed the continuous measurement of oxygen tension in the water immediately before and directly after the animal chamber. The water in oxygen the electrode chambers within the respirometer was continuously mixed by two magnetic stirrers. The respirometer was immersed in the temperature controlled water bath, as described above, and connected to the 500 ml flask containing sea water. A valve, changing the flow route of the water from before the animal chamber to behind the animal chamber, was set to operate automatically every 6 hours, in order to eliminate any differences in the individual electrodes performance. The whole system was operated inside a small air-conditioned laboratory.

5.2.3. Preparation and Calibration

Prior to conducting the experiment the whole system was flushed through with ethanol for 6 hours to reduce bacterial respiration. The Wheaton tubing used throughout the system, to prevent diffusion, was also sterilized in a solution of sodium hypochlorite for 1 hour and then thoroughly rinsed in filtered Millipore water (Sartorius 0.45 μm). The calorimeter was dried with air passed through a column of silicate gel and cobalt chloride indicator and then calibrated internally over a range of 0 - 300 μW. The control rate of bacterial heat dissipation (blank) was also determined before and after the experiment. Calibration of the oxygen electrodes was carried out by passing water with a Po_2 of zero, supplied from the sea water reservoir which was bubbled with nitrogen, through the respirometer (Figure 5.1). The electrodes were then equilibrated in oxygen-saturated sea water supplied from a reservoir through which air was bubbled. Control runs were conducted to establish the residual bacterial rate of oxygen consumption in the absence of an animal immediately prior to the experiment and again on completion.

After the calibration and initial preparation of the calo-respirometer system, a single mud-shrimp (fresh weight approximately 2.0 g) was placed in a 20 ml

gold-plated stainless steel animal chamber containing sea water and connected to the calorimeter. The external surfaces of the animal and reference chambers were then thoroughly dried with a low pressure air line and slowly inserted into the calorimeter. This was done in 4 stages (approximately 15 minutes for each step). The operating temperature of the calorimeter was set at a temperature of 10 ± 0.01 °C and maintained by an external cooler (5510 Thermostat, Thermetric).

Artificial sea water was used throughout the experiments and prepared from Millipore water and Seamarine marine mix salts (Wiegandt GmbH). The salinity of the solution (salinity = 35 ‰) was carefully adjusted by adding very small amounts of the marine mix salts or Millipore water and confirmed using a refractometer (ATAGO S/MILL, Japan). The pH of the sea water was adjusted to 8.00 using 1 M HCl and pH meter. Prior to use the sea water was filtered through a 0.45 μm cellulose acetate filter (Sartorius). Finally, the pH and salinity of the sea water was confirmed and re-adjusted if necessary to pH 8.00 and 35 ‰.

5.2.4. Normoxic, hypoxic and sulphidic exposures

Two experiments were conducted on separate mud-shrimps. In the first, an animal (mass = 2.16 g) placed inside the chamber and exposed to normoxic conditions for 24 hours before the ambient oxygen tension was sequentially reduced to 20, 15, 10, 5 % saturation and, finally, to near anoxic conditions. The manipulation of ambient oxygen tension was achieved using a gas mixing pump (H. Wösthoff 2M301) supplied with nitrogen and oxygen from high pressure cylinders. The output from the pump was fed via an airline to an air-stone in the sea water reservoir. In the second experiment, a different animal (1.93 g) was initially exposed to normoxia for 24 hours and then to sulphidic conditions (71.9 ± 11.6 μM) under hypoxia (31.2 ± 6.4 Torr). The sulphide exposure was achieved by preparing 500 ml of a 5 mM sulphide solution (pH

adjusted to 8.00) using oxygen-free water (see section 2.2.1a.). 50 ml of the sulphide solution was then added to 450 ml of a sea water solution having a salinity of 39 ‰. The greater salinity of this solution was used to allow for the dilution effect during the addition of the sulphide solution. The sulphide-laden sea water was then used to fill a second 500 ml flask in the water bath (Figure 5.1). This solution was allowed to react in the flask and to equilibrate to 10 °C for an hour. A valve was then operated to allow the sulphide-laden sea water to supply the system instead of the normoxic sea water. In an attempt to maintain the concentration of sulphide in the sea water reservoir as constant as possible it was continuously refilled with the sulphide laden sea water. This was achieved using a magnetically-stirred mixing chamber supplied with sea water (39 ‰) and the sulphide stock (5 mM) solution by two peristaltic pumps (Ismatec IPC & Pharmacia LkB-Pump P1) at a combined flow rate of approximately 38 ml. h^{-1} and a dilution ratio of 10 sea water : 1 sulphide. The concentration of sulphide was determined every 2 hours, using the method described in section 2.2.1a. Samples were taken from the reservoir immediately before the system and at both the animal chamber and reference chamber outlets. The pH and salinity of the sea water was also determined at two-hourly intervals. Since it was not possible to measure the concentration of sulphide in the animal chamber during the experiment an indirect estimate was made. The concentrations of sulphide entering and leaving the system and when the animal was removed at the end of the experiment were determined. These values were used to determine a ratio which was subsequently used to obtain an estimate of the concentration of sulphide in the animal chamber throughout the experiment.

5.2.5. Calculation of metabolic rates

The output from the total heat dissipation rates ($_tQ = \mu W$) and oxygen saturations (%) before and after the animal chamber were recorded during the course of the experiment using two paper chart recorders (Kipp & Zonen) and also via an a/d converter to a PC (sampling rate = 4 min^{-1}). These data sets were subsequently analysed using a computer programme specifically developed for this purpose (DatGraf Analysis) and also manually from the chart paper. The heat dissipation ($_tQ = \mu W$) was adjusted to control for the mean bacterial blank heat dissipation rate, as described above. The mean heat signal during the normoxic, hypoxic and sulphidic exposures (6 - 24 h) was then integrated over time to give the direct specific rate of heat dissipation ($mJ.g^{-1}.h^{-1}$). An interval of at least 6 h was left between each treatment to allow the system to stabilize and the animal to acclimatize to the new exposure conditions. The mean oxygen consumption of the mud-shrimp ($\dot{M}O_2 = \mu mol.O_2.g^{-1}.h^{-1}$) during each experimental treatment was also calculated (see section 4.2.2.) from the mean of both oxygen electrodes after the subtraction of the relevant estimates of bacterial respiration under each experimental treatment. These data were then converted to an indirect estimate of $_tQ$ using a generalised oxycaloric equivalent of -450 $kJ.mol.O_2^{-1}$ (Gnaiger, 1983a; 1983b) and compared with the direct calorimetric heat dissipation determination. Some of the heat dissipation and oxygen consumption data, however, were quite variable (Figure 5.3 & 5.4). In these cases the data were analysed from stable hourly intervals (normally 5) when the animal was believed to be quiescent. The mean and standard deviation of these data were then calculated. Care was also taken to ensure the heat dissipation and oxygen consumption rates were taken from the same time points to ensure compatibility of the data.

5.3. Results

5.3.1. Direct and Indirect calorimetry

The rate of oxygen consumption of an animal may be converted to an equivalent heat production by using the standard oxycaloric ratio = -450 kJ.mol.O_2^{-1} (Pamatmat, 1983b). This ratio represents the amount of heat produced by the oxidation of mixed carbon substrates during wholly aerobic metabolism. The theoretical value of oxycaloric ratio may vary between -442 kJ.mol.O_2^{-1} ,when the substrate is purely fat, to -477 kJ.mol.O_2^{-1} when the substrate is protein. When oxygen and heat dissipation are determined simultaneously, an experimental estimate of the oxycaloric coefficient ($\Delta_t QO_2$) can be calculated. This is useful because the value should be within the theoretical range of -442 to -477 kJ.mol.O_2^{-1} when the animal is completely aerobic and therefore confirms the validity of the data. An experimental estimate of the oxycaloric ratio of the mud-shrimp *Calocaris macandreae* was therefore calculated from the rate of oxygen consumption ($\dot{M}O_2$) and heat dissipation ($_tQ$), of the animal under normoxic conditions (Gnaiger *et al.*, 1989).

$$\Delta_t QO_2\,(\text{kJ.mol.}O_2^{-1}) = {}_tQ\,(\text{kJ.g}^{-1}\text{.h}^{-1}) \,/\, \dot{M}O_2\,(\mu\text{mol.}O_2\text{.g}^{-1}\text{.h}^{-1})$$

The mean value of the experimentally determined oxycaloric ratio was -454.5 ± 11.3 kJ.mol.O_2^{-1} (n = 2). This value corresponds closely to the recognized generalized oxycaloric equivalent ($\Delta_t QHO_2$ = -450 kJ.mol.O_2^{-1}) for the respiratory oxidation of mixed carbon substrates (Gnaiger, 1983b). Despite the small degree of variation found, this is well within the accepted range (442 - 477 kJ.mol.O_2^{-1}). This ratio is routinely applied to calo-respirometric investigations of this nature because the proportions of different substrates oxidized are rarely known (Gnaiger, 1983a; 1983b; Shick, 1991). These may be experimentally determined, however, from the respiratory quotient (RQ) and nitrogen quotient (NQ) calculated from molar ratios of CO_2 and N excreted

compared with the oxygen consumed by the animal (Shick, 1991) No attempt was made to determine the values of these ratios for the mud-shrimp *Calocaris macandreae*. The generalized oxycaloric ratio was therefore used to convert oxygen consumption to equivalent heat production. This value could then be directly compared with the experimentally measured heat dissipation of the mud-shrimp.

The contribution of anaerobic metabolism to total metabolism can be seen as the difference between the total heat dissipation and oxygen consumption. If the energy dissipated during oxygen consumption is less than the experimentally determined heat dissipation rate, during periods of environmental or physiological stress, the difference must be due to anaerobic metabolism (Gnaiger, 1983b; Pamatmat, 1983b; Shick, 1991). Although only a few data points could be obtained during this study this was seen to be the case in the mud-shrimp *Calocaris macandreae* during exposure to hypoxia at oxygen saturations of below 20 % (Po_2 = approximately 31 Torr) (Figure 5.2). In addition, the reliance on anaerobic metabolism increased with decreasing ambient oxygen saturations. At an oxygen saturation of 15 % (Po_2 = approximately 23 Torr) anaerobic metabolism accounted for approximately one third of the mud-shrimp's total energy requirements. Furthermore, at an oxygen saturation of 5% (Po_2 = approximately 8 Torr) anaerobic metabolism increased, accounting for approximately three-quarters of the total energy expenditure.

A number of difficulties were encountered during the calo-respirometry involving sulphide. These problems are described below in relation to the results obtained and are discussed further in 5.4.1. Despite these problems it was, possible however, to assess the general trends in metabolism in the mud-shrimp *Calocaris macandreae* during exposure to sulphidic and hypoxic conditions. Three main factors complicate the energetic determination of aerobic and anaerobic metabolism, during these conditions, using this technique. Firstly, the maintenance of sulphide, oxygen and pH equilibria, at the very slow flow rates

required during calo-respirometry, presents many problems. This can be seen indirectly from the variable heat dissipation rate of the mud-shrimp (Figure 5.3f). A control estimate of oxygen consumption due to the chemical oxidation of sulphide is also required. The determination of this value was particularly problematic because of the difficulties in maintaining a sulphide equilibrium throughout the experiment. Finally, the reaction rate of sulphide oxidation was also found to alter within the calo-respirometric system. This can be seen under sulphidic control conditions as a variable heat dissipation rate (Figure 5.3h).

Due to the difficulties described above, the responses of the mud-shrimp to sulphidic and hypoxic conditions were rather variable and difficult to interpret (Figure 5.3 & Figure 5.4). Figure 5.3 describes the raw heat calorimetry data, as the rate of heat dissipation ($_tQ = \mu W$), of the mud-shrimp *Calocaris macandreae* during exposure to normoxia and also to sulphidic and hypoxic conditions. The calibration of the system occurred prior to determining the residual bacterial heat dissipation (a) which can be seen to be low. The calibration of the system was then confirmed between 0 - 100μW and is shown at (b). The mud-shrimp was then placed into the animal chamber and inserted into the calorimeter (c). The rate of heat dissipation of the mud-shrimp under normoxic conditions is shown at (d) and can be seen to be rather variable. During the sulphidic and hypoxic exposure even greater variability was seen in the heat dissipation rate (f) which may be due to the varying sulphidic conditions. The animal was then removed (g) and the heat dissipation rate under sulphidic and hypoxic conditions determined. The reaction rate of sulphide oxidation was also found to alter within the calo-respirometric system and can be seen by the variable heat dissipation rate (h). A control estimate of the bacterial residual heat dissipation rate after the experiment, in sea water, was then estimated and found to be small.

Despite the variation in heat dissipation and oxygen consumption during this experiment it was possible to establish some trends in the animal's

metabolism during exposure to hypoxic and sulphidic conditions ($[S^{2-}] = 71.9 \pm 11.6$ μM; $Po_2 = 31.2 \pm 6.4$ Torr & pH 7.86 ± 0.14). Oxygen consumption although variable, was strongly correlated with the heat dissipation rate (Figure 5.3f & 5.4b) and continued throughout the sulphide exposure at rates equivalent to normoxic conditions indicating the use of aerobic metabolism. The absolute value of oxygen consumption and heat dissipation, however, could not be integrated and compared energetically because of the difficulties described above in determining the rate of removal of oxygen from the system by the chemical oxidation of sulphide alone. Despite these difficulties a reduction in the rate of heat dissipation (17.5 % of normoxic values) of the mud-shrimp *C. macandreae* during exposure to both hypoxic and sulphidic conditions was seen (Figure 5.5). This trend and the partial reliance on anaerobiosis during hypoxic conditions is discussed below.

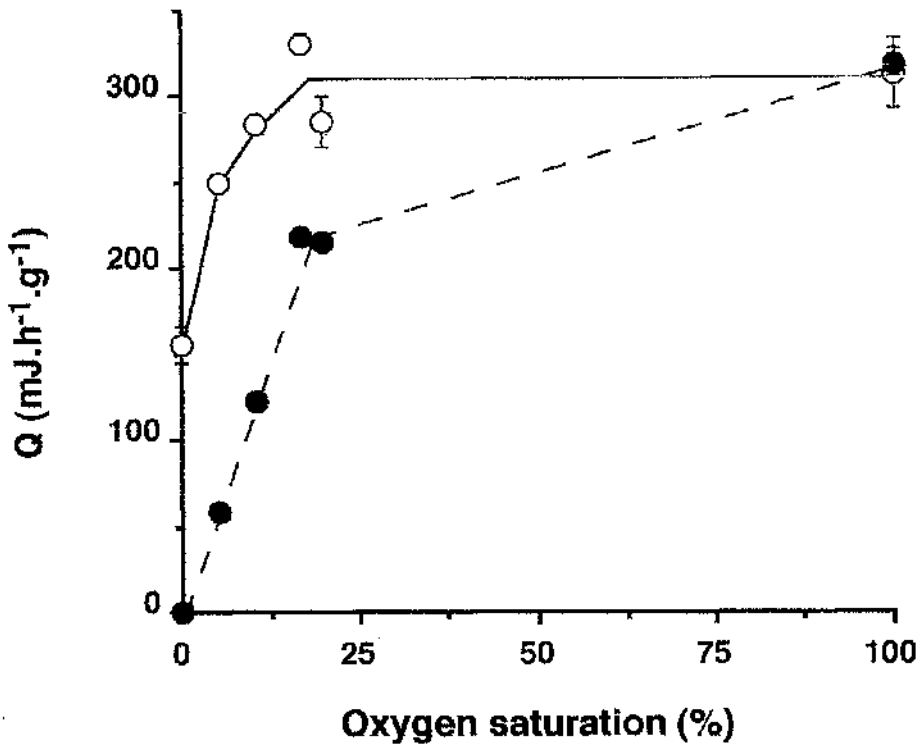

Figure 5.2 A comparison ($_tQ$ = mJ.g^{-1}.h^{-1}) between the total heat dissipation (open circles) and oxygen consumption (closed circles) of the mud-shrimp *Calocaris macandreae* under normoxic, hypoxic conditions and anoxic conditions assuming a generalized oxycaloric equivalent (Δ_kHo_2) of -450 kJ mol.O_2^{-1} (Gnaiger, 1983a; 1983b). (pH = 8.1 ± 0.1). Data points = means of pseudo-replicates ± SE, n = 1)

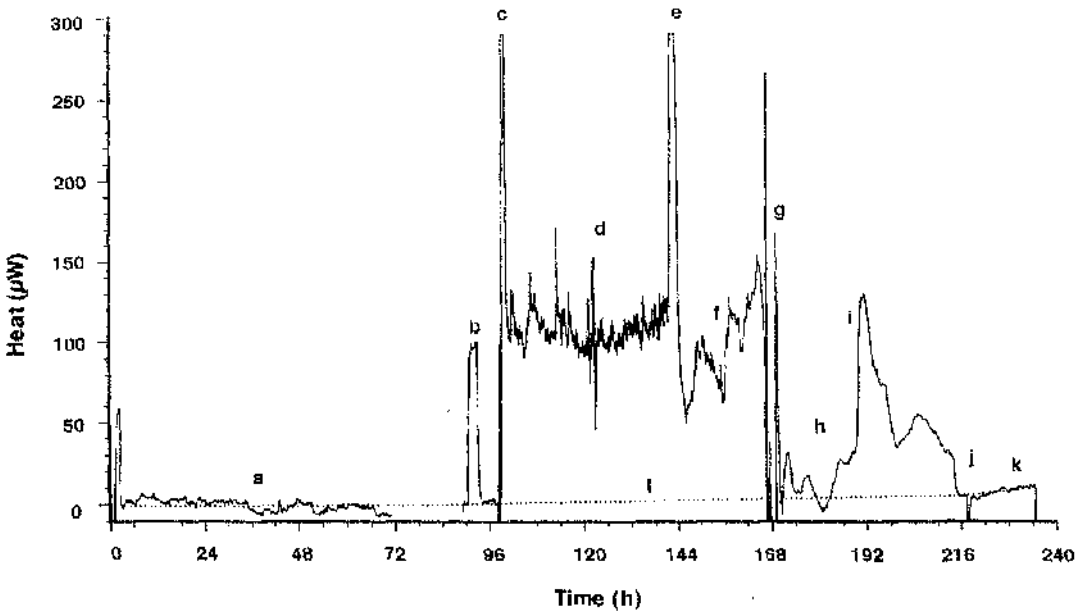

Figure 5.3 The rate of heat dissipation ($_tQ = \mu W$) of the mud-shrimp *Calocaris macandreae* during normoxia and under sulphidic/hypoxic conditions. **a)** blank heat dissipation prior to the experiment, **b)** internal calibration check, **c)** animal placed in chamber and inserted into the calorimeter, **d)** animal heat dissipation under normoxic conditions, **e)** start of the sulphide exposure, **f)** animal heat dissipation under sulphidic and hypoxic conditions, **g)** removal of animal, **h)** sulphide blank heat dissipation, **i)** sulphide reservoir refilled in error, **j)** sea water only, **k)** blank heat dissipation after experiment, **l)** interpolated blank heat dissipation.

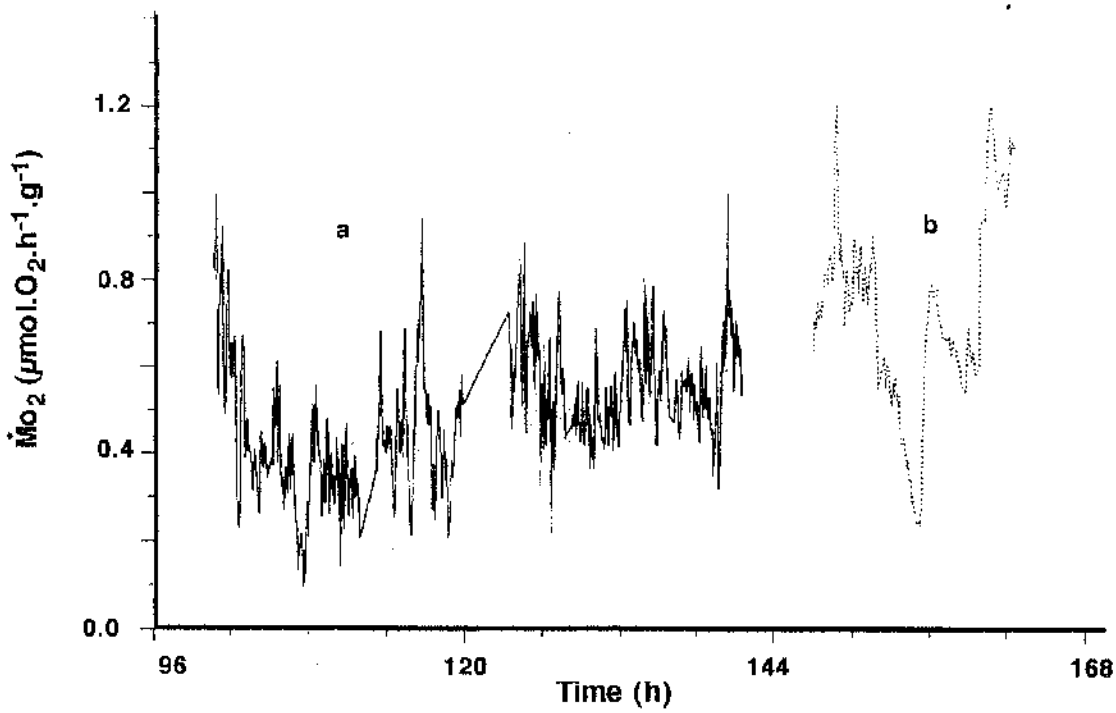

Figure 5.4 The rate of oxygen consumption ($\dot{M}o_2$ = μmol.O_2.g^{-1}.h^{-1}) of the mud-shrimp *Calocaris macandreae* under normoxia and during sulphidic/hypoxic conditions ($[S^{2-}]$ =71.9 ± 11.6 μM; Po_2 = 31.2 ± 6.4 Torr & pH 7.86 ± 0.14) . **a)** $\dot{M}o_2$ under normoxic conditions, **b)** $\dot{M}o_2$ under sulphidic and hypoxic conditions.

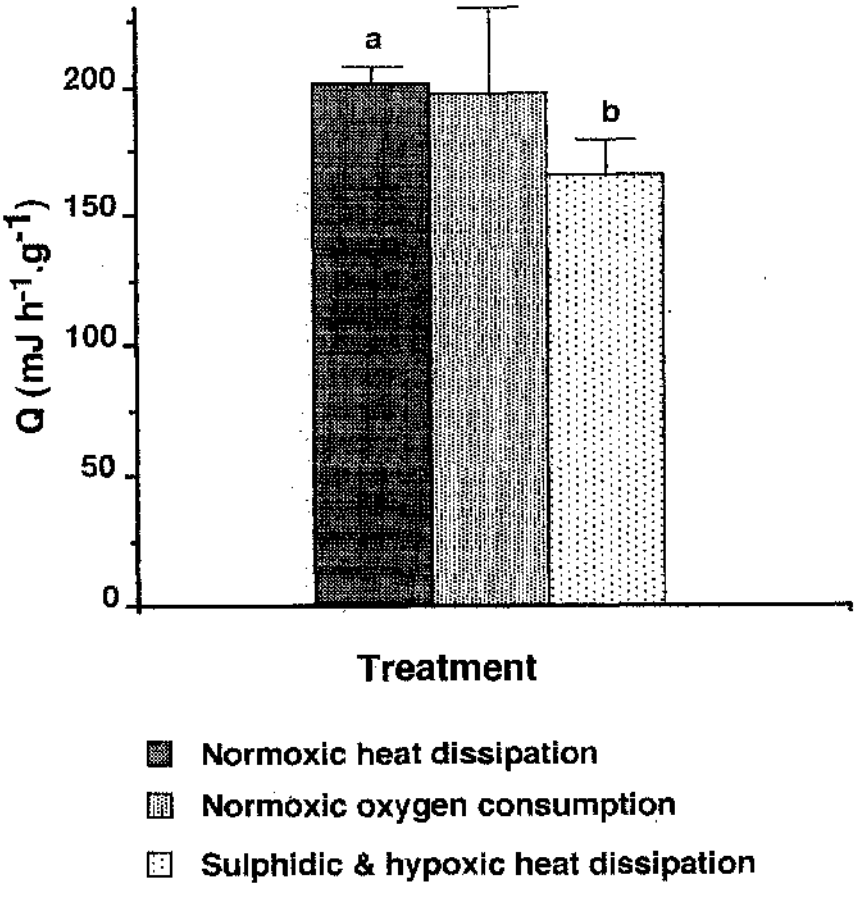

Figure 5.5 Total heat dissipation ($_tQ$ = mJ.g^{-1}.h^{-1}) of the mud shrimp *Calocaris macandreae* under normoxic (pH 7.9 = ± 0.04) and during sulphidic/hypoxic conditions ([S^{2-}] =71.9 ± 11.6 μM; Po_2 = 31.2 ± 6.4 Torr & pH = 7.86 ± 0.14) . An estimate of the mud-shrimp's oxygen consumption under normoxia assuming a generalized oxycaloric-caloric equivalent ($\Delta_k Ho_2$) of - 450 kJ.mol.O_2^{-1} (Gnaiger, 1983a; 1983b) is also included. Data points = means of pseudo-replicates ± SE, n = 1)

5.4. Discussion

5.4.1. Direct and indirect calorimetry

Calo-respirometry provides a valuable method for the investigation of the metabolic responses of animals during exposure to changing environmental conditions. Further work, however, needs to be conducted and the experimental protocol refined (see below) to confirm the metabolic trends of the mud-shrimp *Calocaris macandreae* during exposure to hypoxic, anoxic and sulphidic conditions seen from this calo-respirometric study. The sample sizes in these types of experiment are generally small (< 5), due mainly to the considerable technical problems that may arise and the length of time each exposure requires, Larger numbers of mud-shrimps, however, need to be investigated. The time to complete such an experimental series might take many months and is easily underestimated. Despite this, some very interesting aspects of the metabolism of the mud-shrimp *Calocaris macandreae* have been observed in this study. When the mud-shrimp *Calocaris macandreae* was exposed to decreasing oxygen tensions the animals became progressively more reliant on anaerobic metabolism. Anderson *et al.*, (1994) demonstrated an incremental increase in the concentration L-lactate in muscle tissue of *Calocaris macandreae* as oxygen tension decreased below 20 Torr indicating an increasing reliance on anaerobic metabolism under hypoxic conditions. Similarly, in this study, an incremental increase of the concentration of L-lactate was found when *C. macandreae* was exposed to increasing concentrations of sulphide and could also be interpreted as an increasing reliance on anaerobic metabolism (see section 4.3.2. & 4.4.2.). Similar results have also been reported in a small number of other marine invertebrates such as the sea anemone *Actina equina* and the bivalve mollusc *Mytilus edulis* (Shick *et al.*, 1983; Shick *et al.*, 1986).

Metabolic depression in response to reductions in ambient oxygen tensions has also been seen in a small number of marine invertebrates. This physiological adaptation may increase the ability of these animals to survive severe reductions

in oxygen tension in their environment. The sipunculid worm *Sipunculus nudus*, has been found to reduce its metabolism under anoxic conditions to 20 % of normoxic values (Hardewig *et al.*, 1991). In addition, the priapulid *Halicryptus spinulosus* has also been shown to exhibit metabolic depression during exposure to hypoxic and anoxic conditions to approximately 25 % and 2 %, respectively (Oeschger, 1992). The polychaetes *Marenzelleria viridis* and *Hediste diversicolor* may also suppress their metabolisms to around 20 % of normoxic values when exposed to anoxia (Fritzsche & von Oertzen, 1995). Bivalve molluscs appear to have an even greater capacity to reduce their metabolic energy requirements. Metabolic rate may be suppressed to less than 1 % of normoxic values in *Arctica islandica* and *Astarte borealis* under long periods of anoxia (Oeschger, 1990). *Mytilus edulis*, *Modiolus demissus* and *Polymesdoa caroliniana* have also been observed to reduce their metabolisms to rates as low as 5 % of normoxic values when exposed to anoxia (Pamatmat, 1979, 1980, 1983a; 1983b). Clearly reducing activity and remaining quiescent may significantly reduce the animal's energetic requirements. The mechanisms that allow metabolic depression below this level, however, remain largely unclear (Guppy *et al.*, 1994). Biological processes which require energy must, however, be decreased to facilitate this process. Carbohydrate energy production has been found to be actively depressed in a wide range of studies (Guppy *et al.*, 1994). The specific identity of energy-consuming processes which are reduced and the elucidation of how the cell homeostasis is maintained during metabolic depression however, require further investigation.

The reduction in metabolism of the mud-shrimp *Calocaris macandreae* during exposure to anoxic conditions appears to be similar to that of other marine invertebrates which depress their metabolism to survive extended periods of hypoxic and anoxic conditions. The mud-shrimp *Calocaris macandreae*, however, in contrast to the other marine invertebrates described above, has only a limited ability (a few hours or days) to survive periods of anoxia (Anderson *et*

al., 1994). This may be explained, in part, by the limited capacity for anaerobic metabolism in mud-shrimps. This involves the biochemically inefficient reduction of pyruvate to form L-lactate catalysed by L-lactate dehydrogenase which regenerates NAD^+ and allows the continued glycolytic production of ATP (see section 4.1.1.). The reduction in metabolism (by approximately 50 %) observed in this study is much smaller than has been reported in some molluscs and polychaetes which possess more efficient (see section 4.1.1.) anaerobic metabolic pathways (Grieshaber *et al.*, 1994). Despite the more limited anaerobic capacity and smaller depression in metabolism observed in the mud-shrimp *Calocaris macandreae* the ability to reduce metabolic activity must clearly be of importance during exposure to hypoxia and anoxia. Under the sulphidic and hypoxic conditions ($[S^{2-}]$ =71.9 ± 11.6 μM; Po_2 = 31.2 ± 6.4 Torr & pH = 7.86 ± 0.14) used in these experiments the mud-shrimp *C. macandreae* was apparently able to maintain its rate of oxygen consumption. The rate of Mo_2 were found to be comparable to values obtained for larger numbers of *Calocaris macandreae* when exposed to similar sulphide concentrations and oxygen partial pressures using multiple flow-through respirometry in Glasgow (see section 4.3.1). Only a small reduction in metabolism was observed during exposure to these conditions. This suggests that aerobic metabolism is maintained under these conditions although, due to the difficulties encountered in conducting the experiment and subsequently interpreting the data, it is not clear whether anaerobic metabolism was used by the mud-shrimp.

In contrast to this study, Schneider (1996) has recently shown that the metabolic rates of both the larvae and adults of the brackish water polychaete *Marenzelleria viridis* increased during exposure to sulphidic and hypoxic conditions. Schnieder suggests that the enhanced metabolic rates observed under these conditions may be due to sulphide 'oxidation' and may be coupled with ATP synthesis or possibly associated with bacterial symbionts (Schneider, 1996). It also appears that the metabolism of *Marenzelleria viridis* remained

aerobic during exposure to sulphidic conditions and hypoxic conditions (sulphide = 65 - 225 μM, Po2 = 8.4 - 14.3 kPa which is equivalent to 63 - 107 Torr). It seems likely that *Marenzelleria viridis* may reduce its metabolism at higher sulphide concentrations. This was found to be the case during exposure to anoxia (Fritzsche & von Oertzen, 1995; Schneider, 1996) and for the early larval stages (3 - 6 segments) of *Marenzelleria viridis* when exposed to sulphide (Schneider, 1996).

Further developmental work needs to be carried out in order to establish the oxidation rate of sulphide in the calo-respirometric system before any investigation into the animal's metabolism can be conducted and the trend seen in this study confirmed. A method needs to be established to maintain the sulphide concentration and oxygen tension under more stable conditions. This could be achieved by increasing the flow rate through the calo-respirometric system. A faster flow rate would allow less time for sulphide to oxidize and hence may allow the maintenance of a sulphide equilibrium to be established. This would undoubtedly assist in stabilizing the exposure conditions (see section 3.2.1). A maximum flow rate of 60 ml h^{-1} however, is recommended by Thermetric. This is because of the sensitivity of the oxygen electrodes to faster flow regimes and the time lag in detecting the dissipated heat. Once a satisfactory flow regime has been established a number of trials with sulphide flowing through one chamber and sea water through the other need to be conducted. This would establish the heat dissipation from the oxidative reaction of sulphide alone as it passes through the respirometer and calorimeter. It may be that the heat dissipation due to this reaction is small in relation to the signal produced by the animal. Once this has been determined under specific conditions, an alternative approach could be adopted by comparing the animal's heat dissipation when exposed to sulphidic conditions against a sea water reference. This may overcome the difficulties of maintaining constant sulphidic conditions in both the 'reference' and 'animal'

parts of the system which have different volumes, flow and mixing characteristics.

During the present study, differences were also observed in the total heat dissipation of the mud-shrimps. This may be explained, in part, by the differences in the mass of the animals and also because, once inside the calorimeter, the animal could not be observed. Consequently any activity by the mud-shrimps could not be easily determined. Although mud-shrimps fit inside the chamber they are unable to stand completely upright within it because of the chamber's narrow diameter. This is not ideal and may present the mud-shrimp with difficulties and possibly affect the animal's activity pattern. In its burrow environment it is unlikely that the animal's movement and posture would be restricted in such a manner. Ideally the animal needs to be observed in a sealed glass calorimetric chamber, equivalent to the gold-plated stainless steel chambers used during the experiment, to establish any inherent activity pattern.

Ultimately the approach of Schneider (1996) using H_2S, O_2 and N_2 gases via two gas mixing pumps to the reservoir solution to maintain the sulphide concentration and oxygen saturation constant may provide the best practical solution to maintaining an equilibrium for simultaneous determination of oxygen consumption and heat dissipation. Clearly considerable care would need to be taken when conducting such an experiment due to the toxicity of sulphide. Even using this technique, however, once the sulphide-laden sea water enters the calo-respirometric system the sulphide equilibrium is unlikely to be maintained because of the very slow flow rates. No estimate of the mean sulphide exposure in the animal chamber and its variability is given (Schneider, 1996). In addition, the potential variability of pH and the subsequent possible changes in sulphide toxicity (see sections 1.3 & 2.2.1) is apparently ignored by Schneider. Additionally, no indication of the heat dissipation from the oxidation of sulphide alone is presented. These difficulties must be addressed and resolved in any future experimental sulphidic calo-respirometric studies. Despite this, the use of

calo-respirometric techniques will undoubtedly provide a valuable insight into the effect of sulphide on the metabolic rates of marine invertebrates.

5.4.2. Summary of Chapter 5

The main points of Chapter 5 in relation to mud-shrimps are summarised below:

a) A preliminary attempt was carried out to determine the role of aerobic and anaerobic metabolism in the mud-shrimp *Calocaris macandreae* during exposure to sulphidic conditions.

b) The experiment, however, was only partially successful and revealed a number of problems which need to be overcome during any further studies of this nature.

c) Despite the difficulties encountered in this study it appears that mud-shrimps may show metabolic depression during exposure to hypoxic, anoxic and sulphidic conditions.

d) Partial anaerobiosis may occur in mud-shrimps during exposure to hypoxic and sulphidic conditions.

6. Specific effects of sulphide and thiosulphate on the oxygen affinity of the haemocyanin of decapod crustaceans.

6.1. Introduction

6.1.1. Haemocyanin oxygen transport properties

Respiratory gas transport involves the reversible binding of respiratory gases (O_2 and CO_2) to pigments such as haemoglobin, haemocyanin and hemerythrin. These respiratory pigments increase the oxygen carrying capacity of haemolymph and maintain a large diffusion gradient across the respiratory surfaces. Decapod crustacean haemocyanins are high molecular weight macromolecules which contain copper (0.17%), small amounts of carbohydrate (less than 4%), lipid and polypeptide sub-units (6 - 48) arranged in a complex hierarchy (Mangum, 1983b). Each sub-unit has a single active site which is composed of two copper atoms and has a molecular weight of 70 - 80 x 10^3 daltons (Mangum, 1983b). The degree of sub-unit polymerization affects the physical properties (particularly oxygen affinity) of the pigment (Mangum, 1983b). Considerable intra-specific variation in the number of polymerised sub-units also occurs, although typically these are found as 6 (hexamer) and 12 (dodecamer) sub-unit polymers in crustaceans (Mangum, 1983b). The haemocyanins of the thalassinideans, however, have been noted for their very high molecular weight polymers (Svedberg, 1933; Roxby, 1974; Miller, 1977; Mangum, 1983b) where as many as 24 sub-units may be combined (24 = eikositetramers).

For many years it has been known that pH significantly affects the oxygen affinity of haemocyanins (the Bohr shift). At higher physiological pHs the oxygen affinity of haemocyanin is increased to facilitate the transfer of oxygen from the environment to the respiratory pigment. At lower physiological pHs oxygen affinity is reduced to allow oxygen to be supplied to the tissues. More

recently, it has been discovered that the function of crustacean haemocyanins in terms of oxygen affinity may be 'fine tuned' by a range of modulators (temperature, divalent cations, L-lactate, urate, catecholamine and dopamine) which may act in response to changing environmental and physiological conditions (Mangum, 1983a; 1983b; Morris *et al.*, 1985; Bridges & Morris, 1986; Lallier & Truchot, 1989a; 1989b; Morris, 1991; Sanders & Morris, 1992).

6.1.2. The effect of sulphide on respiratory pigments

Marine invertebrates which encounter elevated concentrations of sulphide in their environment are likely to face the problem of sulphide diffusing through their respiratory surfaces, entering the haemolymph and possibly interacting with the oxygen-binding properties of the respiratory pigment. The effects of sulphide on haemocyanin have received very little attention and will be discussed later. The oxygen-binding properties of haemoglobin, however, have been extensively investigated and may show a number of specific chemical responses in the presence of sulphide (Powell & Arp, 1989; Hagerman & Vismann, 1993, Kraus *et al.*, 1996). When sulphide is added to the erythrocytic oxyhaemoglobin of many mammals and marine fish *in vitro* sulphide binds covalently to the pyrrole of the porphyrin ring causing the formation of sulphaemoglobins which are dysfunctional because their oxygen affinity is reduced several fold (Carrico, *et al.*, 1978 a & b; Chatfield *et al.*, 1987; Bagarinao, 1991). In a second reaction, sulphide may bind to the ferric haem iron of ferric haemoglobin such as in some cytoplasmic haemoglobins from symbiont-harbouring bivalves (Doeller *et al.*, 1988; Kraus & Wittenberg, 1990). Despite this, sulphide does not seem to impair the *in vivo* function of many haemoglobins (National Research Council, 1979; Bagarinao, 1991; 1992) such as the extra-cellular haemoglobin of the polychaete worms *Arenicola marina* (Patel & Spencer , 1963) and *Abarenicola affinis* (Wells & Pankhurst, 1980). In addition, the haemoglobin of these two

worms may also catalyse the oxidation of sulphide, although haematin, a ferrihaem hydroxide may be formed. Finally sulphide may react with haemoglobin at non-haem sites. In the haemoglobin of the vestimentiferan hydrothermal vent 'worm' *Riftia pachyptila,* which contains symbiotic chemoautotrophic bacteria, sulphide and oxygen bind (reversibly) at different parts of the pigment (Arp & Childress, 1981; Powell & Somero, 1983; Childress *et al.*, 1984; Childress & Fisher, 1992). This allows sulphide uptake to be increased and the chemical auto-oxidation of sulphide to be reduced. The active transport of sulphide to sites of bacterial sulphide 'oxidation' occurs with subsequent energy provision to the host. In addition, the potential for sulphide poisoning in the animal tissue is minimized. The ability to bind sulphide to respiratory pigments has also been described in other vestimentiferan worms including: *Tevnia jerichonana, Escarpia spicata, Lamellibrachia barhami* and two unidentified hydrocarbon seep species *Lamellibrachia* sp. and an *Escarpia* -like species (Childress & Fisher, 1992). It has therefore been suggested that the binding of sulphide by extra-cellular haemoglobin may be a characteristic of this phylum (Vestimentifera have recently been separated from the Pognophora). The haemoglobin sulphide-binding site in *Lamellibrachia* has been proposed as a cysteine residue not involved in intrachain disulphide bridges (Suzuki *et al.*, 1990). A sulphide-binding protein has also been described in the haemolymph in the gill filaments of the hydrothermal vent bivalve *Calyptogena magnifica.* (Arp *et al.*, 1984). More recently, Kraus *et al.* (1996) proposed that intracellular gill haemoglobin may be important in the delivery of sulphide in bivalves containing chemoautotrophic bacterial symbionts.

Far less however, has been established on the effect of sulphide on other respiratory pigments. A small number of marine invertebrates which contain non-haemoglobin respiratory pigments have been investigated in relation to sulphide exposure. The hemerythrin of the priapulid worm *Halicryptus spinulosus* blackens during prolonged exposure to sulphide (Oeschger &

Storey, 1990) although the mechanism remains unclear. The haemocyanin of the Baltic brackish water isopod *Saduria entomon* continues to function during exposure to sulphide (Hagerman & Vismann, 1993), although the synthesis of the respiratory pigment may be inhibited. In addition, the presence of sulphide in the haemolymph of the hydrothermal vent crab *Bythograea thermydron.* (Vetter *et al.*, 1987), the isopod *Saduria entomon* (Vismann, 1991) and the burrowing thalassinidean mud-shrimp *Calocaris macandreae* (see section 3.3.2) has been established during experimental sulphide exposure. Free sulphide, at low micromolar concentrations, was also detected in the haemolymph of freshly caught specimens of the mud-shrimps *Callianassa subterranea* and*C. macandreae* (see section 2.3.5). Any interaction between sulphide and the respiratory pigment may reduce the amount of oxygen available for sulphide 'oxidation' (Hagerman & Vismann, 1993). This mechanism is strongly oxygen dependent (see section 4.4.2) and therefore the functioning of haemocyanin during sulphide 'oxidation' must be of considerable importance.

6.1.3. The effect of thiosulphate on respiratory pigments

Thiosulphate, a sulphide 'oxidation' product (see Chapter 3), has been found to accumulate in the haemolymph of a small number of crustaceans during experimental exposure to sulphide. These include the hydrothermal vent crab *Bythograea thermydron* , the non-vent species *Pachygrapsus crassipes* (Vetter *et al.*, 1987), the Baltic brackish water isopod *Saduria entomon* (Vismann, 1991, 1992) and the thalassinidean mud-shrimp *Calocaris macandreae* (see section 4.3.2). In addition, significant concentrations of thiosulphate have also been detected in the haemolymph of freshly caught specimens of *Bythograea thermydron* (Gorodezky, 1994) and in the mud-shrimps *Callianassa subterranea* and *C. macandreae* (see section 2.2.5). Investigating the possible effects of thiosulphate in haemolymph of the hydrothermal vent crab *Bythograea thermydron*, the brachyuran crabs *Cancer antennarius* and *C. anthonyi* and in

the thalassinidean ghost shrimp *Neotrypaea* (as *Callianassa) californiensis.* Sanders and Childress (1992) found no effect on the oxygen affinity of the haemocyanin of the brachyuran crabs and of the ghost shrimp. In contrast, however, these authors appear to demonstrate that the oxygen affinity of haemocyanin of the vent crab was significantly increased by the addition of 1.5mM thiosulphate. Haemocyanin cooperativity was unaltered in all the species investigated. The increase in oxygen affinity of the haemocyanin of *B. thermydron* in the presence of thiosulphate was of a similar magnitude to that observed when 1.5 mM lactate was added. L-lactate is one of a number of organic molecules and inorganic ions known to modulate haemocyanin-oxygen binding (Mangum, 1983a; 1983b; 1983c; Bridges & Morris, 1986; Lallier & Truchot 1989a; 1989b; Morris, 1990; Sanders & Morris, 1992). It therefore appears that the oxygen affinity of the haemocyanin of *Bythograea thermydron* is increased by thiosulphate and this may be an adaptive response to the high environmental sulphidic and hypoxic conditions to which it may be exposed.

In this study the effects of sulphide on the oxygen transporting properties of three benthic species of decapod crustaceans, which are likely to be exposed to different ambient sulphide concentrations, were evaluated. The species studied were the permanent burrow-dwelling mud-shrimp *Calocaris macandreae*, the burrow-dwelling Norway lobster *Nephrops norvegicus* and the common shore crab *Carcinus maenas* which also bury in soft sediments.

6.2. Materials and methods

6.2.1. Collection and maintenance of animals

Calocaris macandreae were collected as described in section 3.2.1. *Nephrops norvegicus* were collected by beam trawling in the Clyde Sea south of Little (Wee) Cumbrae Island from a depth of approximately 80 m. Specimens of *Carcinus maenas* were collected from the intertidal region on the Isle of Cumbrae (Great Cumbrae). All three species were subsequently maintained in marine aquaria at the University of Glasgow (see section 3.2.1.).

6.2.2. Collection of haemolymph samples

Approximately 50 μl of haemolymph was removed from 5 quiescent specimens of *Calocaris macandreae*. A narrow gauge needle (25 G) was inserted through the pericardium of each mud-shrimp and the haemolymph withdrawn using a 1 ml syringe. The haemolymph samples were then pooled in a 1.5 ml Eppendorf tube maintained on ice, mixed thoroughly (Miximatic, Jencons [Scientific] Limited) and centrifuged at 11500 g for 5 minutes (Microcentaur, MSE) to remove the cellular debris. The supernatant was then removed and pipetted into a 1.5 ml Eppendorf tube maintained on ice. Pooled haemolymph samples were also obtained from two specimens of both *Nephrops norvegicus* and *Carcinus maenas* as described above, except that the haemolymph was withdrawn through an arthrodial membrane at the base of one of the pleopods.

6.2.3. The effect of sulphide on decapod crustacean haemocyanin

100 μl of haemolymph from the pools of samples were diluted in a 1.5 ml cuvette with 1000 μl of distilled water. The absorbance spectrum (between 300 and 400 nm) of the haemocyanins were measured (Philips PU8700 spectrophotometer). The function of the respiratory pigment was then confirmed by carefully bubbling N_2 through the haemolymph in the cuvette for three minutes to form deoxyhaemocyanin. The sample was then re-scanned to

confirm the removal of the 335 nm peak indicating the formation of deoxyhaemocyanin. Air was subsequently bubbled slowly through the cuvette to oxygenate the haemocyanin sample using a 20 ml syringe attached to a narrow gauge needle. The cuvette was then re-scanned to confirm the presence of the 335 nm peak which indicated the re-formation of oxyhaemocyanin. The blood samples were then fixed with monobromobimane for the subsequent accurate determination of sulphide concentration by HPLC (see section 2.2.1b.). The continuing function of the respiratory pigment was re-assessed as described above. 100 μl of 1 % KCN, which converts haemocyanin into the reduced form by displacing oxygen (Cook, 1927), was then pipetted into the cuvette and allowed to stand for three minutes before being re-scanned. The haemolymph in the cuvette was bubbled with air again in an attempt to oxygenate the haemocyanin, as described above, and finally the absorbance determined at wavelengths between 300 nm and 400 nm.

6.2.4. Oxygen transporting properties of the haemolymph from *Calocaris macandreae* and *Nephrops norvegicus*

Pooled haemolymph samples from 25 specimens of *Calocaris macandreae* and 5 specimens of *Nephrops norvegicus* were obtained as described above (6.2.2). For each species, the pooled samples were then thoroughly mixed (Miximatic, Jencons [Scientific] Limited). No attempt was made to determine the *in vivo* pH values for either species, although the pH values of the pooled haemolymph samples were determined (Radiometer BMS II Mk2 haemolymph microsystem coupled to a Corning ion analyser 255). The pooled samples were then divided into 100 μl sub-samples and stored in 1.5 ml Eppendorf tubes at -20 °C until required. Although rapidly freezing of haemocyanin at -70 °C may significantly effect oxygen transport properties, in particular cooperativity, (Morris, 1988); slow and careful freezing to - 20 °C apparently does not (pers. comm. C.R. Bridges). The oxygen affinity and cooperativity of the

haemocyanin from the mud-shrimp *Calocaris macandreae* has been found to be unaffected by freezing in this manner (pers. comm. A.C. Taylor).

6.2.4a. Haemolymph ionic composition

The concentrations of sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}) and chloride ions (Cl^-) in the haemolymph pools were also determined:- (Ca^{2+} & Mg^{2+}, Atomic absorption spectrophotometry, A.A.S. Philips PU9200; Na^+ & K^+, Flame photometry, Corning FS 410; Cl^-, electrochemical titration, (Chloride titrator, C.L. Jenway PCLM3). Physiological salines were prepared from these ion concentrations and the pH adjusted to the original value determined from the pooled haemolymph samples.

6.2.4b. Construction of oxygen binding curves

Oxygen dissociation curves were determined for the haemolymph from both *Calocaris macandreae* and *Nephrops norvegicus* using a diffusion chamber system (Sick & Gersonde, 1967; Anderson, 1989; Astall, 1993) across the pH range of 7.2 to 8.0 (Figure 6.1). The relative oxygen saturation of a 3.5 μl haemolymph smear, placed on a microscope slide in a diffusion chamber, was determined. Changes in absorbance of oxygenated haemocyanin (at 335 nm) were measured using a fibre optic pulsed light source from an Oriel Scientific 3090 spectrometer (Zainal *et al.*, 1992). The haemolymph sample was then successively equilibrated to several oxygen tensions using two precision gas mixing pumps, placed in series, (H. Wösthoff, 2M301) supplied nitrogen, carbon dioxide and air (BOC).

The relative saturation (S) of the haemocyanin was determined at several oxygen tensions while the pH was controlled by the Pco_2 of the gas mixture. Concurrently, the remainder of the 100 μl sample was tonometered against the same gas mixture in a Radiometer BMS II Mk2 haemolymph microsystem coupled to a Corning ion analyser 255 enabling the pH of the haemolymph to be

accurately measured at the half saturation point (P_{50}). The temperature throughout this apparatus (Figure 6.1) was controlled by a circulating water bath system at 10 ± 1 °C.

6.2.4c. Calculation of oxygen affinity and cooperativity

Values for the P_{50} (the Po_2 at which the haemocyanin is 50 % saturated) were calculated as described below. Initially, the saturation of haemocyanin was expressed as a function of Po_2 at each of the pH values. Hill plots (log S/1-S, where S = % saturation, against log Po_2) were constructed at each pH and then used to calculate the P_{50}. This was determined from the x intercept of the Hill plot (logS/S-1 = 0). The gradient of these plots, between 25 % - 75 % saturation was used to estimate the cooperativity (n_{50}) of the haemocyanin. A BASIC computer program was used to calculate the log P_{50} and n_{50} values. Log P_{50} was then plotted against pH for each of the blood samples and regression lines fitted to the data to obtain the value of the slope, which is a measure of the size of the Bohr effect.

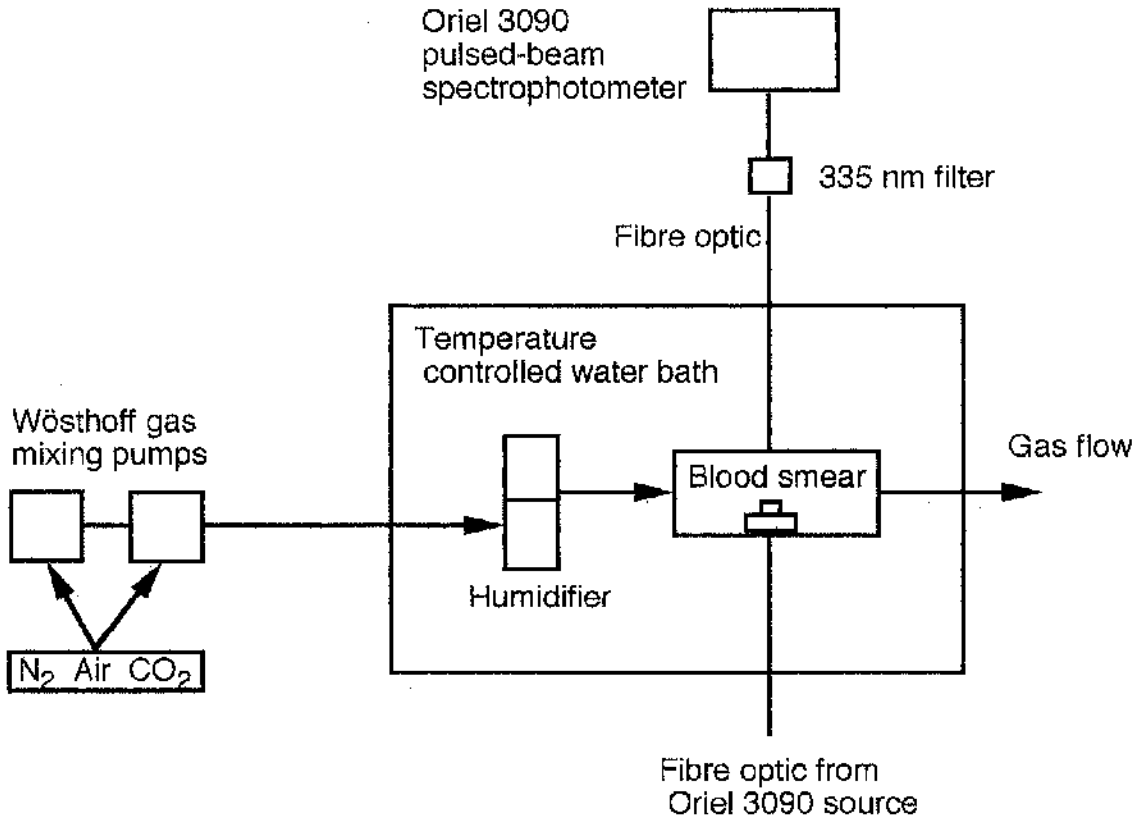

Figure 6.1 Diagram of the diffusion chamber used during the present study (Sick & Gersonde, 1969; Anderson, 1989). The haemolymph smear is supported on a glass slide. Gas mixtures of known composition are produced by the Wösthoff pumps, humidified and passed over the haemolymph smear. The absorbance of the haemolymph smear at 335 nm is measured by an Oriel 3090 pulsed beam spectrophotometer. Temperature is accurately controlled by a water bath at 10 ± 1°C.

6.2.5. The effect of thiosulphate on the oxygen affinity and cooperativity of haemolymph from *Calocaris macandreae* and *Nephrops norvegicus*.

Oxygen dissociation curves constructed for *Calocaris macandreae* and *Nephrops norvegicus*, as described above, were then used to determine any possible changes in oxygen affinity due the thiosulphate. This involved three different treatment groups : a). 100 μl untreated haemolymph (Control 1), b). 90 μl haemolymph + 10 μl saline (Control 2) pH adjusted to 7.8, c). 90 μl haemolymph + 10 μl saline containing 1.5 mM thiosulphate (Treatment), pH adjusted to 7.8. The data from the three different solutions were then compared to ascertain the effect of thiosulphate on haemocyanin-oxygen binding affinity (log P_{50}), Bohr shift (ϕ) and cooperativity (n_{50}).

6.3. Results

6.3.1. The effect of sulphide on decapod crustacean haemocyanin

The addition of 20 mM sulphide (final concentration nominally 1 mM) to haemocyanin appeared to have no effect on the respiratory pigment of *Calocaris macandreae*, (Figure 6.2). Despite the presence of sulphide the haemocyanin remained functional since it was possible to form deoxyhaemocyanin using nitrogen and subsequently oxyhaemocyanin by bubbling air through the sample. In contrast, the use of KCN induced the formation of deoxyhaemocyanin which could not be subsequently oxygenated (Figure 6.2). Very similar effects were seen with the haemolymph of both *Nephrops norvegicus* and *Carcinus maenas*.

In addition, a rapid and significant reduction (ANOVA + Tukey test; $p < 0.05$) in the concentration of sulphide was seen in the sample cuvettes indicating a large sulphide oxidizing capacity of the haemolymph from *Calocaris macandreae*, *Nephrops norvegicus* and *Carcinus maenas* (Table 6.1). Much lower sulphide oxidation rates were seen in the control cuvette containing distilled water.

Control samples of *Calocaris macandreae* haemolymph obtained during January (when the pooled haemolymph samples described above were also prepared) and fixed in the laboratory of the RV Aora were used to determine the concentrations of thiols in similar freshly caught specimens (see section 2.3.5). The concentration of thiosulphate and sulphide were found to be very low (2.3 ± 1.15 μM & 0.44 ± 0.57 μM) respectively ($n = 15$). Sulphite was not detected in the control haemolymph samples. The concentration of L-lactate was also determined (see section 4.2.2b.) in similar control animals (0.44 ± 0.18 mM, $n = 12$) although 48 hours was allowed after capture to enable the mud-shrimps to recover from the stress induced during trawling.

Table 6.1 The concentration of sulphide, thiosulphate and sulphite (mean ± SD mM, n = 3) in 1.5 ml cuvettes (see methods). Details of the associated sulphide oxidation rate (μmol. min^{-1}) and statistics used (ANOVA + Tukey test, a/b/c = significantly different, $p < 0.05$) are also included.

Species	Sulphide (μM)	Oxidation rate (μmol. min^{-1})	Significant difference (a/b/c)
Calocaris macandreae	350 ± 26	-0.651	c
Nephrops norvegicus	307 ± 40	-0.700	c
Carcinus maenas	451 ± 44	-0.535	b
Control cuvette	891 ± 7	-0.010	a

6.3.2. Oxygen transporting properties of the haemolymph of *Calocaris macandreae* and *Nephrops norvegicus*

The concentration of the major ions present in the pooled haemolymph samples of the mud-shrimp *Calocaris macandreae* and the Norway lobster *Nephrops norvegicus* are given below in Table 6.2. These values were used to prepare the physiological salines.

Table 6.2. The concentration of the major ions (mM) in the mud-shrimp *Calocaris macandreae* and the Norway lobster *Nephrops norvegicus* determined from pooled frozen haemolymph samples.

Species	Na^{2+}	Cl^{2-}	K^{+}	Mg^{2+}	Ca^{2+}
Calocaris macandreae	623	503	11.9	40.5	10.6
Nephrops norvegicus	536	602	14.6	54.4	9.8
Sea water, salinity = 34.3‰	470	548	10.0	53.6	10.2

The relationships between pH and oxygen affinity (Log P_{50}) and cooperativity (n_{50}) of the haemolymph from the mud-shrimp *Calocaris macandreae* and the Norway lobster *Nephrops norvegicus* are shown in Figure 6.3 and Figure 6.5 and summarised in Table 6.3.

Table 6.3 The relationship between haemocyanin-oxygen affinity (log P_{50}) and pH for the mud-shrimp *Calocaris macandreae* and the Norway lobster *Nephrops norvegicus* at 10 °C (Figure 6.3 & 6.5). The regression equations are presented in the form Log P_{50} = m (pH) + c. The coefficient of determination (r^2) is also given. Both relationships were significant (ANOVA, $p < 0.001$).

Species	m	c	r^2
Calocaris macandreae	-0.639	5.702	0.967
Nephrops norvegicus	-0.991	8.672	0.822

The haemocyanin of *Calocaris macandreae* was also found to have a high oxygen affinity (P_{50}), 5.22 Torr at pH 7.8 (Figure 6.3). The haemocyanin-oxygen affinity (P_{50}) of *Nephrops norvegicus* was found to be lower, 8.75 at pH 7.8 (Figure 6.5). The cooperativity (n_{50}) of *Calocaris macandreae* (2.37 ± 0.27) and *Nephrops norvegicus* (2.86 ± 0.78) also remained independent of pH (Figure 6.4 & 6.6). A significant (ANOVA, $p < 0.001$) Bohr shift ($\phi = \Delta \log P_{50} / \Delta$ pH) was seen in both species, at 10 °C (Table 6.2., Figure 6.3 & 6.5), although ϕ was found to be larger in the haemolymph of *Nephrops norvegicus*.

6.3.3. The effect of thiosulphate on the oxygen affinity and cooperativity of haemocyanin from *Calocaris macandreae* and *Nephrops norvegicus*

The addition of control salines (containing no thiosulphate) to the haemolymph of *Calocaris macandreae* and *Nephrops norvegicus* (Figure 6.3 & 6.5) did not significantly affect the size of the Bohr shift (ANCOVA, $p > 0.999$ and $p > 0.800$, respectively) or the oxygen affinity of the haemocyanin (ANCOVA, $p > 0.147$ and $p > 0.658$, respectively). The addition of thiosulphate, using the prepared salines to the haemolymph of *Calocaris macandreae* and *Nephrops norvegicus*, resulted in significant increases (ANCOVA, $p < 0.001$) in the oxygen affinity of the haemocyanin of both species (Figure 6.3 & Figure 6.5), although the size of the Bohr shifts were not significantly affected (ANCOVA, $p > 0.1$). The addition of the control salines had no significant effect (ANOVA, $p > 0.1$) on the cooperativity of the haemocyanin of *Calocaris macandreae* and *Nephrops norvegicus*, at 10°C (Figure 6.4 & 6.6). The saline containing thiosulphate was also found to have no significant affect (ANOVA, $p > 0.05$) on the cooperativity of the haemocyanin of *Calocaris macandreae*, although a significant decrease (ANOVA + Tukey, $p < 0.001$) in the cooperativity of the haemocyanin of *Nephrops norvegicus*, however, was seen (Figure 6.6).

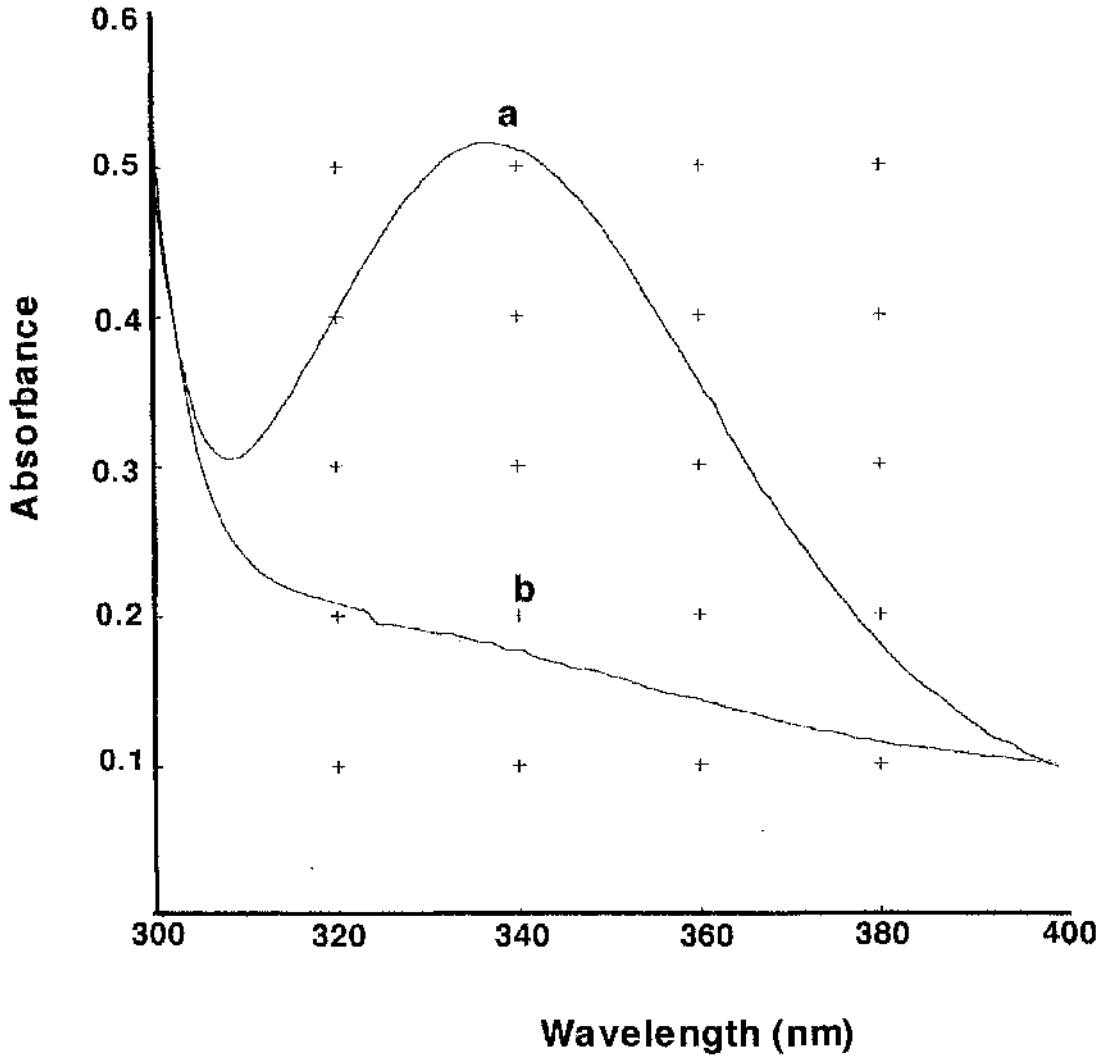

Figure 6.2 Characteristic absorbance spectra for the haemocyanin of the mud-shrimp *Calocaris macandreae*, the Norway lobster *Nephrops norvegicus* and the shore crab *Carcinus maenas*: **a)** oxyhaemocyanin oxygenated with air and after the addition of sulphide, **b)** haemocyanin deoxygenated with nitrogen, deoxygenation with KCN and also after the addition of KCN and oxygenation with air.

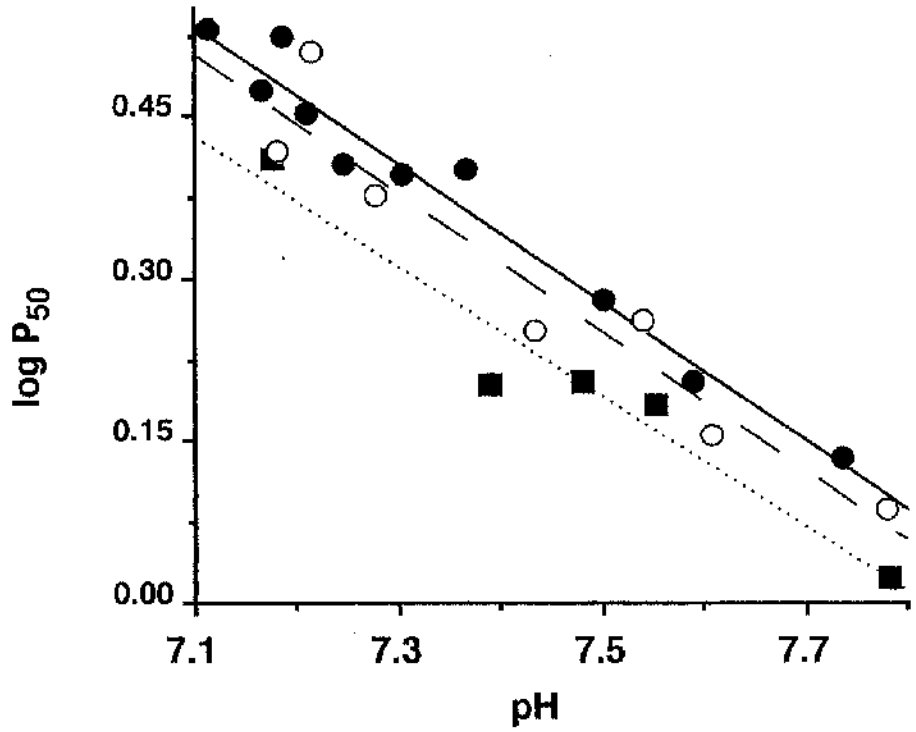

Figure 6.3 The effect, at 10 °C, of thiosulphate ($S_2O_3^{2-}$) on the haemocyanin oxygen affinity of *Calocaris macandreae*. The gradients of the regression lines fitted to the data of untreated haemocyanin (closed circles) and haemocyanin with saline (open circles) and haemocyanin with saline and 1.5 mM thiosulphate (closed squares) were not significantly different from each other (ANCOVA, $p > 0.623$); the elevations between the untreated haemolymph and haemolymph with the added saline control were also not significantly different (ANCOVA, $p > 0.0.146$); however, the elevation of the thiosulphate-treated haemolymph was significantly different (ANCOVA, $p < 0.001$) from the untreated and the saline-treated haemolymph.

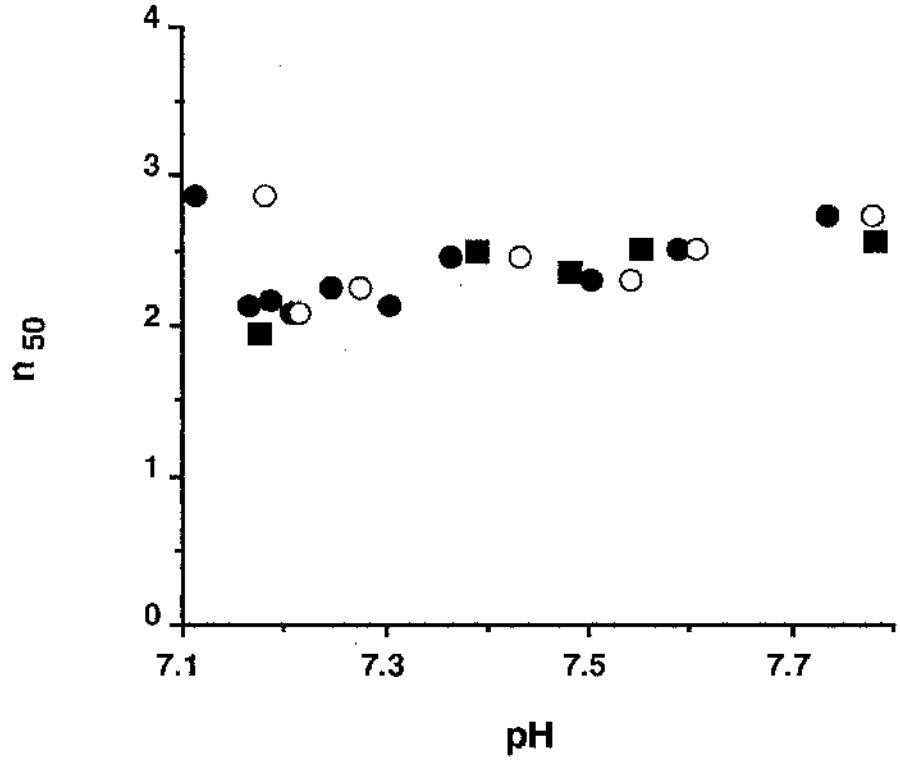

Figure 6.4 The effect, at 10 °C, of thiosulphate ($S_2O_3^{2-}$) on the cooperativity of the haemocyanin of *Calocaris macandreae*: Untreated haemocyanin (closed circles), haemocyanin + saline (open circles), haemocyanin + saline containing thiosulphate (closed squares).

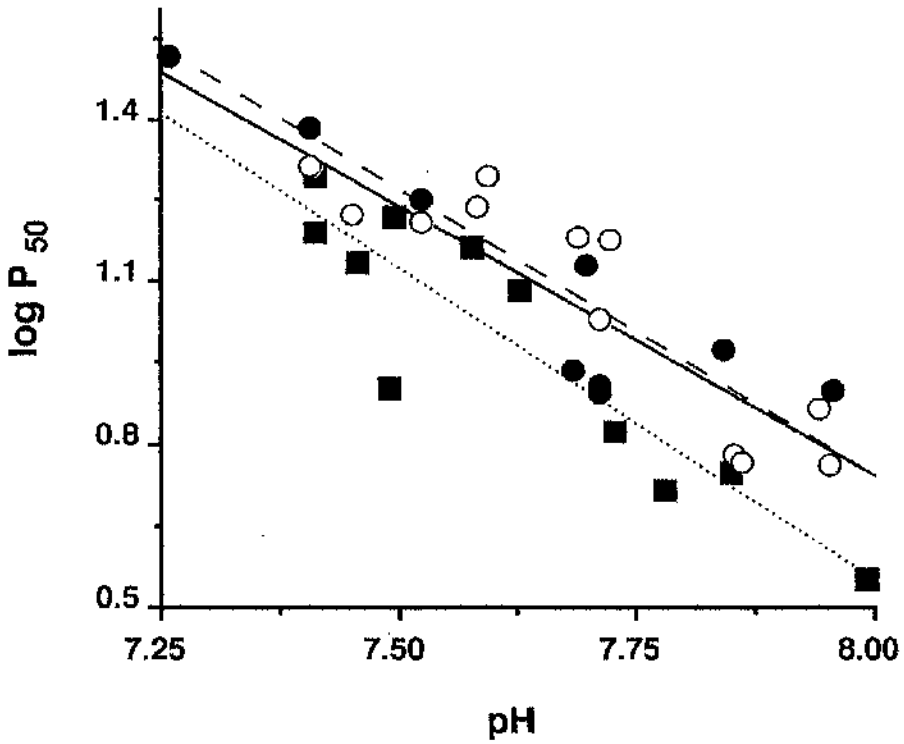

Figure 6.5 The effect, at 10 °C, of thiosulphate ($S_2O_3^{2-}$) on the haemocyanin oxygen affinity of *Nephrops norvegicus*. The gradients of the regression lines fitted to the data of untreated haemocyanin (closed circles) and haemocyanin with saline (open circles) and haemocyanin with saline and 1.5 mM thiosulphate (closed squares) were not significantly different from each other (ANCOVA, $p > 0.531$); the elevations between the untreated haemolymph and the saline and haemolymph were also not significantly different (ANCOVA, $p > 0.657$); however, the elevation of the thiosulphate-treated haemolymph was significantly different (ANCOVA, $p < 0.001$) from the untreated and the saline treated haemolymph.

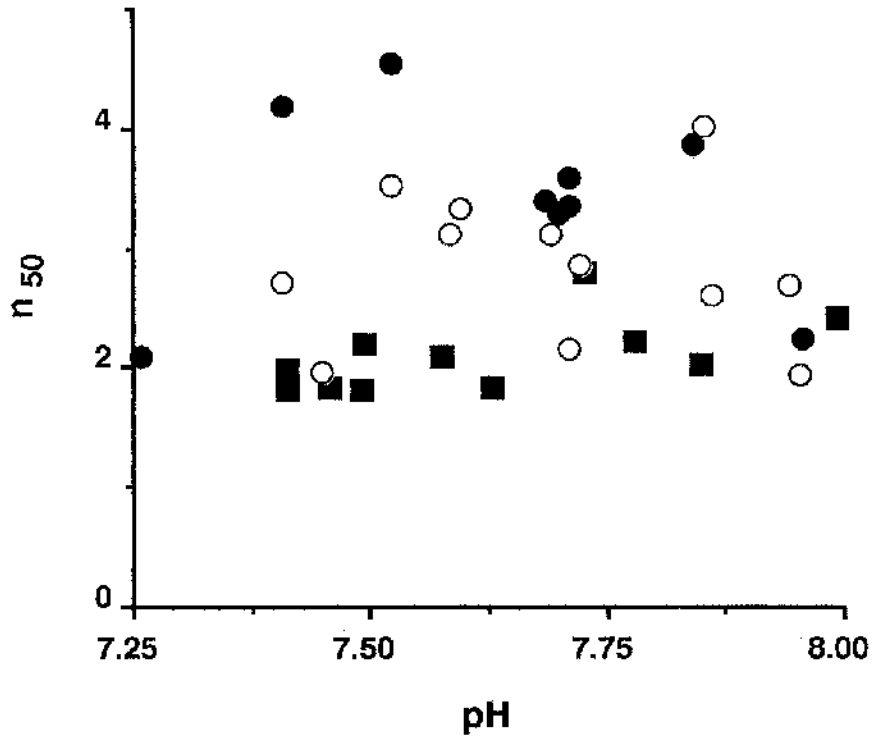

Figure 6.6 The effect, at 10 °C, of thiosulphate ($S_2O_3^{2-}$) on the cooperativity of the haemocyanin of *Nephrops norvegicus*. Untreated haemocyanin (closed circles), haemocyanin + saline (open circles), haemocyanin + saline containing thiosulphate (closed squares).

6.4. Discussion

6.4.1. Sulphide and haemocyanin

Sulphide has been shown to be present in the haemolymph of freshly-caught specimens of the mud-shrimps *Calocaris macandreae* and *Callianassa subterranea* at low micromolar concentrations (see section 2.2.5.). In addition, during experimental exposure to sulphide, it has also been shown to accumulate in the haemolymph of *Calocaris macandreae* (see section 3.3.2.), in the haemolymph of the hydrothermal vent crab *Bythograea thermydron* (Vetter *et al.*, 1987), and in the haemolymph of the Baltic brackish water isopod *Saduria entomon* (Vismann, 1991). Accumulation of sulphide could potentially affect the haemocyanin oxygen transport in these animals. The *in vitro* study of the haemocyanin from the three benthic crustaceans investigated, *Calocaris macandreae*, *Nephrops norvegicus* and *Carcinus maenas*, showed that the haemocyanin function was apparently not affected by sulphide because oxygen remained able to bind reversibly to the haemocyanins. Hagerman & Vismann (1993) investigating the effect of sulphide on the haemocyanin of the Baltic brackish water isopod *Saduria entomon*, established that haemocyanin also remained functional when exposed to sulphide. The synthesis of haemocyanin, however, was apparently inhibited by sulphide. This is in contrast to the result of an earlier study on *Saduria entomon* and other benthic crustaceans which, in response to hypoxia (without the presence of sulphide), may synthesize haemocyanin to facilitate oxygen transport (Hagerman & Oksama, 1985).

The 'oxidation' of sulphide to form metabolites, such as thiosulphate and sulphite, requires oxygen and apparently occurs within mitochondria (Völkel & Grieshaber, 1996). In the case of crustaceans, the transport of oxygen to sites of 'oxidation' must therefore be primarily mediated by the haemolymph, either in solution or bound to the haemocyanin or respiratory pigment. The haemolymph of *Bythograea thermydron* may be insensitive to sulphide and does not apparently contain a sulphide-binding protein involved in active transport

(Childress *et al.*, 1987). The transport of sulphide in physical solution in the haemolymph has therefore been proposed in the hydrothermal vent crab *Bythograea thermydron* (Childress *et al.*, 1987). This is in contrast to other hydrothermal vent species such as the haemoglobin-containing vestimentiferan worm *Riftia pachyptila.* (Arp *et al.*, 1987) and other members of the Vestimentifera (Somero *et al.*, 1989) where sulphide may be reversibly bound to a non-haem site on the haemoglobin molecule. Interestingly, the cytoplasmic haemoglobin within the gills of the clam *Solemya reidi*, which contains intracellular sulphide-oxidizing bacteria remained functional in the presence of sulphide. Despite this, haemoglobin deoxygenation was significantly reduced and incomplete when sulphide was present. This suggests that sulphide may directly influence the rate of haemoglobin deoxygenation and may therefore prolong the intracellular oxygen store when sulphide levels increase and/or oxygen becomes limited (Kraus *et al.*, 1996). Although the haemocyanins of the three decapod species studied here were found to be insensitive in the presence of sulphide, there may be sulphide-mediated changes in the reaction kinetics of these haemocyanins which may affect oxygen transport in the haemolymph.

In this study, the haemocyanin from *Calocaris macandreae*, *Nephrops norvegicus* and *Carcinus maenas* was also seen to oxidize sulphide strongly *in vitro*. This may be important in terms of sulphide 'oxidation'. The details of this mechanism and of any possible 'oxidation' metabolites produced remain to be established. In contrast, the haemolymph of *Saduria entomon* apparently does not oxidize sulphide (Vismann, 1991). A number of haemoglobin-containing animals from sulphide-rich marine habitats have, however, been shown to possess non-haemoglobin haem compounds which can oxidize sulphide. These include haematin (a brown oxidized or ferric compound not associated with protein) found in the coelomic fluid of the echiuran worm *Urechis caupo* (Powell & Arp, 1989) and the polychaete worms *Hediste* (as *Nereis*) *diversicolor* and *Neanthes* (as *Nereis*) *virens* (Vismann, 1990). In

addition, similar haematin compounds, capable of sulphide oxidation have been observed in the haemolymph from the gill tissues of some haemoglobin-containing bivalves. These bivalve species include *Calyptogena magnifica*, *Lucinoma annulata* (Powell & Arp, 1989) and *Scapharca inaequivalvis* (Vismann, 1993). Importantly, the haematin was found to be present *in vivo* in these species and it has therefore been suggested that these pigments catalyse the oxidation of sulphide and may therefore protect the animals from sulphide poisoning. Although similar mechanisms have been suggested in the polychaete worm *Arenicola marina* and other annelids (Patel & Spencer, 1969; Mangum, 1976; Wells & Parkhurst, 1980) their presence *in vivo* has been questioned (Powell & Arp, 1989). Haematins result from the breakdown of haemoglobin during stress induced by handling, or poor condition, and may not be naturally present. No evidence of the formation of additional pigments was seen in the haemolymph of the decapod crustaceans investigated in this study. The *in vitro* oxidation and removal of sulphide in the haemolymph of these species has been established, although this aspect clearly requires further work.

6.4.2. Oxygen transporting properties of haemolymph

Comparison of data between species is notoriously difficult due to the need to make such comparisons under similar experimental conditions and pH values. Despite this, the haemocyanin oxygen affinities of crustaceans that may regularly be exposed to hypoxia, such as burrow-dwellers and hydrothermal vent crabs, appear to be consistently higher than those of decapods from normoxic environments (Childress & Fisher, 1992; Astall, 1993). The values for the Bohr shift (ϕ = -0.639), oxygen affinity (P_{50} = 5.22 at pH 7.8) and cooperativity (n_{50} = 2.4 ± 0.27) for *Calocaris macandreae* and (ϕ = -0.991, P_{50} = 8,75 at pH 7.8, cooperativity = 2.8 ± 0.78) for *Nephrops norvegicus* determined in this study were found to be very similar to those estimated by other workers (Bridges 1986; Anderson, 1989). These values indicate that the haemocyanin of these

species has a high oxygen affinity. In the case of *Calocaris macandreae* these may be adaptations to the often severely hypoxic burrow environment. Haemocyanin having a high oxygen may also be significant during exposure to hypoxic and sulphidic conditions in that oxygen is required, in addition to normal aerobic metabolic processes, to 'oxidize' sulphide (see Chapter 4).

A few haemocyanins from crustaceans from the sulphidic and hypoxic environment of hydrothermal vents have also been extensively studied. Arp and Childress (1981) showed that the haemocyanin from the hydrothermal vent crab *Bythograea thermydron* had a moderate oxygen affinity and Bohr shift (P_{50} = 5.8 Torr at pH 7.55 and 5 °C, ϕ = -0.34, respectively), although there is some suggestion that there may be genetic differences in the haemocyanin of this species of crab from different vent sites (Sanders, 1989). The haemocyanin from another vent crab *Cyanograea praedator* (Childress & Fisher, 1992) has also been found to show a large Bohr shift (ϕ = -0.94 to -1.30). In addition, the haemocyanin of the hydrothermal vent shrimp *Alvinocaris lusca* has a high oxygen affinity (P_{50} = 1.3 to 2.8 Torr at pH 7.95) and a moderate Bohr effect (-0.77 at 5 °C). The haemocyanin oxygen affinities of *Bythograea thermydron*, *Cyanograea praedator* and *Alvinocaris lusca* also show some degree of insensitivity to temperature (Childress & Fisher, 1992). High oxygen affinities, substantial Bohr effects and thermal insensitivity appear to be typical features of hydrothermal vent crustacean haemocyanins. It has therefore been suggested that these properties may be adaptations to the widely varying conditions, of Po_2, temperature and sulphide, that may be encountered at hydrothermal vents. As similar trends in oxygen affinity and Bohr shift have been established in mud-shrimps in this study and by others (Anderson, 1989; Astall, 1993) these adaptations may be also be significant in the potentially sulphidic burrow environment of mud-shrimps.

6.4.3. The effect of thiosulphate on the oxygen affinity and cooperativity of decapod crustacean haemocyanin

Thiosulphate has been detected at micromolar concentrations in the haemolymph of mud-shrimps experimentally exposed to sulphide and also in freshly caught animals (see Chapter 2). Interestingly, in this study, thiosulphate has been shown to have a significant effect (increase) on the haemocyanin oxygen-binding affinity of both *Calocaris macandreae* and *Nephrops norvegicus*. In many studies, however, haemolymph samples are often dialysed against a physiological saline to reduce the concentrations of small molecular weight modulators of oxygen affinity, such as L-lactate and urate, prior to the determination of oxygen affinity. Since only a very small volume of blood was available from each mud-shrimp (*Calocaris macandreae*) the pooled samples were not dialysed. The concentrations of thiosulphate and sulphide in the control haemolymph pools, taken from freshly caught animals, were found to be less than 3 μM and 0.5 μM respectively. In addition, L-lactate concentrations in the haemolymph were found to be low (< 0.5 mM). Due to the low concentrations found in control haemolymph samples of these metabolites any effect on oxygen affinity is likely to be small.

Sanders & Childress (1992) also established that thiosulphate had a similar effect on the haemocyanin oxygen-binding affinity of the haemocyanin from the hydrothermal vent crab *Bythograea thermydron*, although this was seen only at higher physiological pHs. They also described an increase in oxygen affinity by L-lactate, as has been established in the haemocyanins from a large number of other decapod crustaceans (Mangum,1983a; 1983b; Bridges & Morris, 1986; Morris, 1990; Sanders & Morris, 1992). Sanders and Childress (1992) suggest that the increase in oxygen affinity, due to thiosulphate and L-lactate, may counter the Bohr shift when pH decreases enabling the haemocyanin from *Bythograea thermydron* to be functional during exposure to warmer hypoxic and sulphidic water. Kraus *et al.* (1996) suggest that the mechanism for

thiosulphate modulation of haemocyanin oxygen affinity may be similar to that of other inorganic anions such as Cl^- (Mangum & Burnett, 1986). The oxygen affinities of haemocyanin from two other decapod crustacean species, *Cancer anthonyi* and *C. antennarius*, and the thalassinidean *Neotrypaea* (as *Callianassa*) *californiensis*, however, were found not to be effected by thiosulphate (Sanders & Childress, 1992). As has been established in this study (*Calocaris macandreae* and *Nephrops norvegicus*) and for the hydrothermal vent crab *Bythograea thermydron*, thiosulphate is apparently able to modulate the oxygen affinity of some crustacean haemocyanins. The inter-specific differences seen in these studies may be a reflection of the environmental differences in ambient sulphide concentrations. In the case of the mud-shrimp *Calocaris macandreae*, this may be an adaptation to the potentially sulphidic and hypoxic burrow environment (see section 2.3.2) where oxygen partial pressures may be low. An increase in the oxygen affinity by thiosulphate would increase the availability of oxygen for sulphide 'oxidation'. An interesting analogy is L-lactate which is also known to modulate (increase) the oxygen affinity of haemocyanins (Bridges & Morris, 1986). Although this mechanism has been interpreted as having physiological relevance during functional anaerobiosis, it is also thought that the increase in oxygen affinity of haemocyanin during hypoxic conditions may be important to maintain oxygen uptake at respiratory surfaces. A paradox exists here because L-lactate is produced during anaerobiosis in response to low oxygen tensions below the critical Po_2. In terms of exposure to sulphide, however, an increase in haemocyanin oxygen-affinity may be important, in that oxygen is required to 'oxidize' sulphide which may also occur during anaerobiosis. The presence of L-lactate, in the haemolymph of the thalassinideans *Neotrypaea* (as *Callianassa*) *californiensis* and *Calocaris macandreae* (Mangum, 1983b; Anderson, 1989) has, however, been found to have no effect on the oxygen-affinity of the haemocyanin.

6.4.4. Summary of Chapter 6

The main points of Chapter 6 are summarized below:

a). The addition of sulphide appeared to have no effect on haemocyanin-oxygen binding of the mud-shrimp *Calocaris macandreae*, the Norway lobster *Nephrops norvegicus* and the shore crab *Carcinus maenas*. It appears that the haemocyanin in these species remains functional in the presence of sulphide.

b). The haemocyanin of all three species of decapod crustaceans investigated rapidly oxidized sulphide suggesting that an auxiliary sulphide 'oxidation' mechanism may exist in the haemolymph of decapod crustaceans. This remains undescribed.

c). The addition of thiosulphate apparently increased the haemocyanin oxygen affinity of the mud shrimp *Calocaris macandreae* and the Norway lobster *Nephrops norvegicus*. This modulation of oxygen affinity by thiosulphate may therefore increase the oxygen available for sulphide 'oxidation'.

7. Summary, conclusions and direction of future work

Mud-shrimps (Crustacea, Decapoda, Thalassinidea) construct complex burrows in reduced and anoxic soft sediments (Dworschak, 1983; Nash *et al.*, 1984; Atkinson & Taylor, 1988; Nickell & Atkinson, 1995; Ziebis *et al.*, 1996). The conditions within the burrows of many mud-shrimp species have been found to be hypoxic and hypercapnic. (Anderson *et al.*, 1989, Foster & Graf, 1992, 1995; Astall *et al.*, 1997; Ziebis *et al.*, 1996). In recent years it has been established that mud-shrimps, unusually for decapod crustaceans, are very tolerant of hypoxia and anoxia and possess a range of adaptive mechanisms which enable them to survive these conditions (Atkinson & Taylor, 1988; Anderson *et al.*, 1991, 1994; Paterson & Thorne, 1995; Astall *et al.*, 1997). The reduced sediments which mud-shrimps inhabit may also contain significant concentrations of sulphide (a naturally occurring toxin) in the interstitial water. Mud-shrimps may therefore be exposed to potentially toxic concentrations of ambient sulphide in their environment.

Two species of mud-shrimp were investigated during the course of this project, *Calocaris macandreae* and *Callianassa subterranea,* which inhabit sublittoral marine sediments on the west coast of Scotland. Field measurements taken throughout the study of sulphide in the burrow water and interstitial water indicate that mud-shrimps may be exposed to significant concentrations of ambient sulphide in their burrows throughout the year (see Chapter 2). Direct evidence has shown that the mud-shrimp *C. subterranea* may be exposed to potentially toxic levels of sulphide in the burrow water (37 ± 44 μM, range 0 - 206 μM). In addition, sulphide, thiosulphate and sulphite were also found in the haemolymph and tissues of freshly caught specimens of mud-shrimps (*C. macandreae* and *C. subterranea*) indirectly indicating that they may be exposed to ambient sulphide. The concentrations of sulphide found in the burrow water and thiol concentrations within the mud-shrimps also showed seasonal variations (see Chapter 2) which may be correlated with the input of organic material to the

sediment.

The evidence from this study indicates that sulphide is an important ecological factor in relation to thalassinidean mud-shrimps. The wider ecological effects of sulphide in the benthic marine environment have, however, received relatively little attention to date (see section 2.4.3.). How sulphide influences the distribution of benthic marine invertebrates and community structure may be an important area of future research. More detailed surveys, of areas such as Loch Sween, relating the spatial and temporal variation in sulphide concentrations to the distribution of benthic marine invertebrates and also to community structure need to be carried out.

Laboratory investigations during this study have established that mud-shrimps tolerate elevated levels of ambient sulphide in their environment. Three species of mud-shrimp, *Calocaris macandreae, Callianassa subterranea* and *Jaxea nocturna* were found to have considerable ability to survive exposure to low millimolar concentrations of sulphide which was apparently correlated with the use of anaerobic metabolism (see Chapter 3). In addition, the irrigation behaviour of the mud-shrimp *C. macandreae* was apparently unaffected in the presence of micromolar concentrations (360 ± 320 μM) of sulphide (see Chapter 2).

Mudshrimps appear to be physiologically adapted to tolerate elevated levels of sulphide which they may encounter in their natural habitat. A number of mechanisms acting in an integrated manner, allow the oxidation of sulphide in a possible 'detoxification' strategy. The sulphide 'oxidation' mechanism in the mud-shrimp *Calocaris macandreae* is apparently oxygen-dependent and appears to allow the maintenance of aerobic metabolism, at low micromolar concentrations of sulphide, presumably preventing the inhibition of mitochondrial cytochrome c oxidase by sulphide poisoning. Sulphide diffusing into the body tissues is oxidized primarily to non-toxic thiosulphate, in the hepatopancreas and muscle tissues, and accumulates rapidly in the tissues and

haemolymph even during brief exposures to low micromolar concentrations of sulphide (see Chapter 3). Sulphite also appears as a minor and intermediary sulphide 'oxidation' product in the haemolymph and tissues of *C. macandreae*.

The production of thiosulphate is energetically more efficient than sulphite or sulphate synthesis since 1 mole of sulphide is removed for every 1.5 moles of oxygen whereas the oxidation of sulphide to sulphite and sulphate requires three or four moles of oxygen, respectively. This may be important in the hypoxic environment where oxygen supply could be limited and is required for sulphide 'oxidation' in addition to aerobic metabolism. These mechanisms are apparently similar to those reported in a small number of other benthic marine invertebrates which include a variety of 'worms', bivalves and crustaceans and may therefore be widespread amongst different invertebrates (Anderson *et al.*, 1987, 1990; Vismann, 1991b; Völkel & Grieshaber, 1992, 1994, 1996; Arp *et al.*, 1992, Eaton & Arp, 1993, Hagerman & Vismann, 1993). These adaptations may therefore represent a common feature of many marine invertebrates exposed to significant concentrations of sulphide in their environment. Further research into a wider range of these animals needs to be conducted to confirm this hypothesis. Preliminary experiments during the course of this study have established that another crustacean, the isopod *Natatolana borealis,* may also 'oxidize' sulphide to form thiosulphate in a possible sulphide detoxification mechanism. *Natatolana borealis* inhabits U shaped burrows in marine sediments and therefore may also be exposed to sulphide in its burrow and during burrowing activities in the sediment. Interestingly, these animals may be encountered in fish carcasses which they burrow into en masse to feed (Moore & Taylor, 1995). During this process they may also be exposed to sulphide which raises the possibility of sulphidic exposure in micro-habitats in decaying organic material and other areas such as underneath intertidal boulders and decaying macroalgae

A number of sulphide-tolerant animals have been reported to form symbiotic associations with chemoautotrophic bacteria capable of sulphide

'oxidation'. The symbionts, using energy from sulphide oxidation, have a dual function as they fix carbon dioxide into organic compounds for translocation to the host thereby detoxifying sulphide (Felbeck, 1983, 1981; Anderson *et al.*, 1987). No evidence of chemoautotrophic bacterial symbionts could be found in the hepatopancreas and gill filaments of *Calocaris macandreae* and *Callianassa subterranea*, although the possibility of such bacteria in the gut remains to be investigated. In addition, sulphur amino acid metabolism seems not to be directly implicated in sulphide 'oxidation'. It is therefore proposed that the 'oxidation' of sulphide is facilitated by the animal's tissues. Similar mechanisms have been reported in a small number of other-sulphide tolerant invertebrates which inhabit marine sediments and hydrothermal vent sites (Vetter *et al.*, 1987; Vismann, 1991b; Völkel & Grieshaber, 1992, 1994, 1996; Arp *et al.*, 1992, Eaton & Arp, 1993, Hagerman & Vismann, 1993).

The 'detoxification' mechanism (the oxidation of sulphide to form detoxification metabolites such as thiosulphate) is important since aerobic metabolism can be maintained despite the presence of sulphide (see Chapter 4). Aerobic metabolism appears to increase during exposure to sulphide, when oxygen is available, and can be maintained even under severe hypoxic and sulphidic conditions. The oxygen consumption of the mud-shrimp *Calocaris macandreae* was seen to increase during exposure to sulphide concentrations of less than 100 μM sulphide. A similar increase was recorded in the oxygen consumption of a single specimen of *Calocaris macandreae* specimen during exposure to sulphide and hypoxia in the calo-respirometer (see Chapter 5). The sulphide-stimulated increases in oxygen consumption could be interpreted as the additional oxygen required to produce the 'oxidation' metabolites thiosulphate and sulphite, in addition to maintaining normal aerobic metabolism although other factors, such as activity, may contribute to the increase in oxygen consumption. At higher sulphide concentrations, however, oxygen consumption declined and may indicate that oxygen provision within the tissues becomes

limiting as the amount of oxygen required to oxidize sulphide to thiosulphate and sulphite increases, although there was considerable variation between individuals in their rates of oxygen consumption when exposed to high sulphide concentrations.

During exposure to an external concentration of 99.4 μM sulphide under near normoxic conditions the mean Mo_2 of the mud-shrimp *Calocaris macandreae* (n = 16) was 0.631 ± 0.804 $\mu mol.O_2.g^{-1}.h^{-1}$. In contrast, control mud-shrimps under normoxic conditions (n = 8) not exposed to sulphide consumed 0.444 ± 0.082 $\mu mol.O_2.g^{-1}.h^{-1}$. The increase in oxygen consumption during exposure to sulphidic conditions may represent, at least in part, the requirement for oxygen to oxidize sulphide (see above). At a sulphide concentration of 99.4 μM this represents 29.6 % of the total amount of oxygen consumed. To oxidize a concentration of 200 μM would therefore require 0.818 $\mu mol.O_2.g^{-1}.h^{-1}$ if the animal were to remain totally aerobic, approximately 50% for metabolic processes and 50% for sulphide 'oxidation' At this concentration, however, the total oxygen consumption of the mud-shrimps was reduced to 0.185 $\mu mol.O_2.\ g^{-1}.h^{-1}$ which is similar to the maximum increase in oxygen consumption seen during exposure to sulphide. This may indicate that the sulphide 'oxidation' mechanism becomes saturated and may also be limited by oxygen availability. When this occurs, at sulphide concentrations in excess of a critical transition point, the mud-shrimps resort to anaerobic metabolism to survive. Sulphide therefore appears to have a concentration-dependent capacity to either stimulate or inhibit aerobic metabolism in mud-shrimps which is related to oxygen availability.

Calo-respirometric studies of the mud-shrimp *Calocaris macandreae* have suggested that the mud-shrimps may also show metabolic depression during periods of hypoxia, anoxia and sulphidic conditions (see Chapter 5). This reduction in metabolism, however, seems limited in comparison to the large metabolic depressions reported in some bivalves and polychaetes in response to

periods of hypoxia and anoxia (Pamatmat, 1979,1980,1883; Oeschger, 1990, 1992; Guppy *et al.*, 1994; Fritzsche, 1995). Aerobic and anaerobic metabolism appear to operate concurrently during hypoxia with an increasing trend towards anaerobiosis as oxygen tension declines. Presumably this may also occur during exposure to sulphide when the oxidation mechanism becomes saturated above an ambient sulphide/oxygen threshold.

Clearly, the functional characteristics of the respiratory pigment may also be important during exposure to sulphide when oxygen is required for 'detoxification purposes' (see Chapter 6). Haemocyanin function was found to be unaffected by sulphide in mud-shrimp *Calocaris macandreae*, the Norway lobster *Nephrops norvegicus* and the shore crab *Carcinus maenas*. The apparent insensitivity of these haemocyanins to the presence of sulphide may reflect the requirement for oxygen in aerobic metabolic processes and sulphide 'oxidation'. In addition, the haemocyanin of all three species of decapod crustaceans rapidly oxidized sulphide suggesting that an auxiliary sulphide 'detoxification' mechanism may exist in the haemolymph of these animals. The addition of thiosulphate apparently increased the oxygen affinity of the haemocyanin of mud-shrimp *Calocaris macandreae* and the Norway lobster *Nephrops norvegicus*. This modulation of oxygen affinity by thiosulphate may be a physiological adaptation to increase the amount of oxygen available for sulphide oxidation to produce the metabolites thiosulphate and sulphite during severely hypoxic and sulphidic conditions (Sanders & Childress, 1992).

Mudshrimps therefore appear to be physiologically adapted to tolerate the elevated levels of sulphide which they may encounter in their natural habitat. A range of ecophysiological mechanisms act in an integrated manner which influence the sulphide 'detoxification' capacity of these animals. The underlying biochemical mechanism of sulphide 'oxidation' in mud-shrimps has not been addressed during the course of this study. The oxidation of sulphide, however, has been shown to occur in the mitochondria in a small number of other marine

invertebrates. The mitochondria of these animals have therefore been suggested as the site of sulphide 'oxidation' (Bagarinao & Vetter, 1990; Oeschger & Vetter, 1992; Oeschger & Vismann, 1994). It seems likely, therefore, that this may also be the case in mud-shrimps and would be an interesting avenue for further investigation. The mitochondria of some polychaete worms seem capable of oxidising sulphide and the generating energy (ATP) which may provide an alternative mechanism for ATP generation during sulphide exposure (Völkel *et al.* 1995, 1996). Whether this is a common feature in benthic marine invertebrates remains to be elucidated.

Indirect evidence suggests that the underlying biochemical mechanism of mitochondrial sulphide 'oxidation' is enzymatic ('sulphide oxidase') (Völkel & Grieshaber, 1996). Does an underlying and unifying alternative respiratory enzyme exist which allows protection during sulphide exposure ? An alternative terminal respiratory enzyme which is not poisoned and generates energy in the from of ATP has also been suggested (Völkel *et al.* 1995, 1996). The need for direct evidence to clearly establish the existence of such an enzyme (which may be a unifying and underlying 'oxidation' mechanism in all sulphide tolerant animals) is highly likely to be at the forefront of any future work on sulphide and its effects on eukaryotic organisms.

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