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http://dx.doi.org/10.24382/3759
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ECO-PHYSIOLOGY OF THE ENDOSYMBIONT-BEARING LUCINID BIVALVE, CODAKIA ORBICULATA

P. A. G. BARNES

Ph. D. 1993

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Signed. Penelope A. J. Burns

Date. December 20th, 1993

ECO-PHYSIOLOGY OF THE ENDOSYMBIONT-BEARING LUCINID BIVALVE, *CODAKIA ORBICULATA*

by

PENELOPE ANNE GEE BARNES

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

DOCTOR OF PHILOSOPHY

Department of Biological Sciences
Faculty of Science

In collaboration with
Plymouth Marine Laboratory and
Bermuda Biological Station for Research, Inc.

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ECO-PHYSIOLOGY OF THE ENDOSYMBIONT-BEARING LUCINID BIVALVE, *CODAKIA ORBICULATA*

Penelope Anne Gee Barnes

ABSTRACT

The lucinid bivalve *Codakia orbiculata*, whose gills contain sulphur-oxidizing symbiotic bacteria, occurs in high densities (500/m^2) in the rhizosphere of shallow-water *Thalassia testudinum* sediments in Bermuda. Both sulphide and thiosulphate stimulate aerobic respiration in the isolated bacterial symbionts of *C. orbiculata*. Sulphide and thiosulphate stimulate anaerobic $^{14}CO_2$ fixation in bacteria isolated from sulphur-starved bivalves. Interstitial water sulphide concentrations in the bivalves' habitat reach 300 µM, and sulphate-reduction rates are high, but thiosulphate concentrations are low (0.66-32.27 µM). Thiosulphate supplied to the symbionts *in vivo* must be produced by sulphide oxidation, possibly by the host bivalve. Isolated symbionts also respire aerobically and fix $^{14}CO_2$ in the absence of exogenous reduced sulphur, suggesting utilization of intracellular elemental sulphur stores.

Codakia orbiculata symbiotic bacteria are able to respire nitrate. Nitrate concentrations in the interstitial water of *C. orbiculata* habitat can reach 36 µM. Thiosulphate stimulates nitrate respiration in the intact symbiosis, incubated in oxic and anoxic conditions, and in anoxic incubations of isolated symbionts. Intracellular elemental sulphur is also used by the bacteria as a substrate in nitrate respiration. Nitrate respiration in the absence of exogenous nitrate suggests that the symbionts may have a limited ability to store nitrate.

There is no direct evidence that sulphide stimulates nitrate respiration in either the isolated symbionts or the intact symbiosis, incubated in anoxic conditions. Nitrite respiration in the symbionts is stimulated by sulphide (only), however. Because nitrate respiration was measured by nitrite accumulation, complete denitrification would explain the apparent failure of sulphide to stimulate nitrate respiration. High nitrate respiration rates in the intact symbiosis, incubated with sulphide in oxic conditions, may be in response to thiosulphate, supplied to the bacterial symbionts after host oxidation of sulphide. Nitrite respiration in the intact symbiosis, even when incubated in oxic conditions, demonstrates that the symbionts have access to some sulphide *in vivo*, however, and that host sulphide-oxidation may not be 100% efficient.

Nitrate and nitrite respiration in the intact symbiosis, even when incubated in oxic conditions, suggests that the bacteria may be exposed to low oxygen levels *in vivo* and may require the ability to utilize an alternate electron acceptor. Like some free-living bacteria, *Codakia orbiculata* bacterial symbionts may co-respire, or alternately respire, oxygen and nitrate. The *Thalassia testudinum* sediments in Bermuda may be ideal for this bacteria-bivalve symbiosis due to the availability of oxygen, nitrate and sulphide.

DEDICATION

This thesis is dedicated to my mother,

Marian Annie Gee (1913-1992) and

to my father, Thomas Henry Gee.

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ACKNOWLEDGEMENTS

This study was undertaken to determine the habitat characteristics of bacteria-bivalve sulphur-oxidizing symbioses and to investigate sulphur substrate, and electron acceptor utilization in the endosymbiotic bacteria. This study was made possible through financial assistance from the University of Victoria (Canada), the Hawaiian Malacological Society, the Roger Thayer Stone Fellowship, the Canadian Associates of the Bermuda Biological Station for Research, Inc., and the Graduate Intern Program at the Bermuda Biological Station for Research, Inc..

I am grateful to Dr. Brian Bayne for suggesting that I complete my Ph.D. with the University of Plymouth and for his assistance throughout this process. Dr. Malcolm Jones has taken care of administrative issues so that, despite conducting my research in Bermuda, my tenure at the University of Plymouth has been problem-free and enjoyable. I am endebted to both Dr. Bayne and Dr. Jones for valuable comments on the thesis. Dr. Russ Vetter provided hours of helpful discussion and, during his tenure at Scripps Institute of Oceanography, generously gave me unlimited access to his specialized HPLC system for analysis of thiols. I am also endebted to Scripps Institute of Oceanography for providing me with laboratory space and use of equipment at no charge. Mr. Stewart Niven provided valuable advice on HPLC-analysis of elemental sulphur. Dr. Barbara Javor shared her expertise in microbial techniques and instructed me in the use of $^{14}CO_2$. She also

critically reviewed the physiology chapter of this thesis, for which I am extremely grateful. Dr. Colleen Cavanaugh provided helpful advice on the physiology section of this thesis. Dr. Fred Lipshultz allowed me to steadily move equipment from his laboratory into mine, worked with me on a chemiluminescent technique for analysis of nitrate and provided much helpful and stimulating discussion on all aspects of this thesis. Dr. Clay Cook assisted with the $^{35}SO_4^{2-}$ experiments and provided encouragement and support during my time at BBSR. Dr. Susan Cook, in her role as Director of Education at BBSR, provided unwavering interest and enthusiasm for my research and assisted in my receiving, and maintaining, a BBSR Graduate Internship. Mr. Bruce Dilke provided invaluable assistance with statistical analyses and advice on production of figures for this thesis. Mr. Thad Murdoch generously produced the computer-assisted figures in Chapter 1. Ms. Liz Pedro assisted me with the field work and routine laboratory tasks with unfailing enthusiasm. Ms. Sean Rôche, Mr. Bob Bradford and Mr. Dwight Smith all volunteered their assistance in collection of field samples. Mr. T.H. Gee provided valuable assistance in entering HPLC data onto the computer. Finally, my greatest acknowledgment goes to Mr. Harry Barnes, who volunteered his time, his support and his understanding for the duration of this thesis.

AUTHOR'S DECLARATION

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

This study was financed with the aid of grants from the University of Victoria (Canada), the Hawaiian Malacological Society, the Roger Thayer Stone Fellowship Fund, the Canadian Associates of the Bermuda Biological Station for Research, Inc. and the Bermuda Biological Station for Research, Inc.. This study was carried out in collaboration with Plymouth Marine Laboratory and the Bermuda Biological Station for Research Inc..

Advanced study undertaken included a post-graduate course in molecular biology at Stanford University (USA).

Relevant scientific seminars were presented at Scripps Institute of Oceanography, Bermuda Biological Station for Research, Inc. and the University of Plymouth. Abstracts were submitted and accepted for the International Symbiosis Conference (1991), the 27th European Marine Biology Symposium (1992) and the Annual Meeting of the American Society of Zoologists (1992). External institutions were visited for consultation purposes, including Scripps Institute of Oceanography, the Marine Biological Association of the U.K.

and Harvard University. Papers for publication are in preparation based on the chapters of this thesis.

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Date. December 20th 1993

CHAPTER 1

SYMBIOSES WITH CHEMOAUTOTROPHIC BACTERIA IN THE BIVALVE FAMILY LUCINIDAE - A REVIEW

1.1. INTRODUCTION

Bacteria-invertebrate chemoautrophic symbiosis was first described for the giant tube-worm *Riftia pachyptila*, found at hydrothermal vents (Cavanaugh *et al.*, 1981; Felbeck *et al.*, 1981). Since the original discovery, similar symbioses have been found in a number of marine invertebrate phyla in addition to the Vestimentifera: Pogonophora, Annelida, Nematoda, Platyhelminthes and Mollusca (Giere, 1981; Ott *et al.*, 1982; Southward, 1982; Cavanaugh, 1983; Felbeck, 1983; Berg and Alatalo, 1984; Fiala-Medioni, 1984; Fisher and Hand, 1984; Giere, 1985; Dando *et al.*, 1985; Reid and Brand, 1986; Southward, 1986). Among the bivalve molluscs, evidence of chemoautotrophic symbioses has been found in all members of the families Lucinidae (Berg and Alatalo, 1984; Fiala-Medioni, 1984; Fisher and Hand, 1984; Dando *et al.*, 1985; Giere, 1985; Reid and Brand, 1986; Southward, 1986), Solemyidae (Cavanaugh, 1983; Felbeck, 1983) and Vesicomyidae (Cavanaugh, 1983; Fiala-Medioni, 1984; Vetter, 1985) examined. In addition, some members of the bivalve families Mytilidae (Cavanaugh, 1983; LePennec and Hily, 1984), Thyasiridae (Dando and Southward, 1986; Reid and Brand, 1986; Southward, 1986) and Mactridae (Bouvy *et al.*, 1989) have been found to harbour chemoautotrophic symbionts. Without exception, chemoautotrophic bacteria are always found in the gill tissues of these bivalves.

Chemoautotrophic bacteria living in symbioses with invertebrates utilize the energy released upon oxidation of a reduced sulphur compound to fix carbon dioxide into more

complex organic molecules which, either through translocation or through digestion of symbionts, become available to the host (Felbeck, 1985; Fisher and Childress, 1986; Distel and Felbeck, 1987). Symbioses with sulphide-oxidizing bacteria are complex because sulphide is toxic to aerobic respiration; even at low micromolar concentrations, sulphide inhibits cytochrome c oxidase, catalase and other heme-containing enzymes (National Research Council, 1979). A sulphide concentration of <1 μM will typically inhibit cytochrome c oxidase activity by 50% (Nicholls, 1975; Hand and Somero, 1983). Because of this, transport of sulphide to the endosymbionts must utilize mechanisms that avoid poisoning the host cells. In addition, sulphide spontaneously oxidizes in the presence of oxygen (Chen and Morris, 1972) and the need to maintain an adequate supply of both compounds is reflected not just in the physiological adaptations of the symbiosis, but also in habitat and behavioural modifications of the host.

Literature on chemoautotrophic symbioses continues to grow at an astounding rate and it is not surprising that these symbioses have been the subject of several review papers over the last few years. Most of these papers are aimed towards a general review of bacteria-invertebrate symbioses and cannot be expected to cover any one group of invertebrates in detail. Although these reviews are of unquestionable value, the spectrum of research on this subject is now so broad that in order to review the entire subject, it is necessary to summarize and, therefore, to generalize. Generalizations regarding a specific family may be misleading when, upon

closer examination, significant interspecific or intergeneric differences are revealed.

For general background information on chemoautotrophic and methanotrophic symbioses in marine invertebrates, the reader is referred to Fisher (1990). Fisher's review focuses largely on methods used to demonstrate the symbioses and briefly reviews the literature on symbioses in the major invertebrate groups in which they occur. Somero, Childress and Anderson (1989) reviewed the transport, metabolism and detoxification of hydrogen sulphide in animals from sulphide-rich marine environments and briefly reviewed the thyasirid and lucinid bivalves in the section on exploitation of reduced sulphur compounds by intact symbioses. Felbeck, Childress and Somero (1983) reviewed biochemical interactions between bivalves and sulphide-oxidizing bacteria. While providing an excellent overview of the subject, the review focuses on the vent bivalves, *Calyptogena sp.*, and the now much-studied *Solemya reidi*. Only a small amount of information was available on lucinids at that time and the subject, in general, has advanced considerably. Cavanaugh's (1985) review focuses on *Riftia pachyptila* and *Solemya velum* and does not include the lucinids. Southward's 1987 review on the contribution of symbiotic chemoautotrophs to the nutrition of benthic invertebrates and Fiala-Medioni and Felbeck's 1990 review on bacterial symbiosis in bivalve molluscs provide a comprehensive list of lucinids which have been examined for the presence of bacterial symbionts and enzymes associated with autotrophy. Reid (1990) and Reid and Brand (1986)

reviewed the evolutionary implications of sulphide-oxidizing symbioses on the functional morphology of lucinids and of bivalves, respectively. Allen (1985) also discussed this in his manuscript on form and evolution in the recent Bivalvia. Finally, Southward (1989), in his brief review on animal communities fuelled by chemosynthesis, stated that the superfamily Lucinaceae are the most interesting group of bivalves from the aspect of chemoautotrophic symbiosis. The author has found this to be the case.

The purpose of this review is to focus on chemoautotrophic symbioses within the bivalve family Lucinidae. Significant variation that exists within the family is noted and generalizations made only where adequate data allows. In those areas where research on lucinids is lacking or where further examples are required, reference is made to other bacteria-bivalve symbioses. The review is divided into sections as follows: general morphology, evidence for chemoautotrophic symbioses, gill structure and cellular components, host bivalve nutrition, habitat and, lastly, mechanisms for transport and detoxification of reduced sulphur compounds and the forms of reduced sulphur used as energy sources. This review, while obviously covering subject areas beyond the scope of the research conducted for this dissertation, assists in placing the research presented in the following chapters into context and aids in understanding the hypotheses put forward.

1.2. GENERAL MORPHOLOGY OF THE FAMILY LUCINIDAE

The family Lucinidae, belonging to the subclass Lamellibranchia and superfamily Lucinacea, display many distinctive morphological characteristics. Among others, these include a long, vermiform foot which can expand to greater than six times the length of the shell (Allen, 1953). The lucinids lack an inhalant siphon but the foot is used to form an anterior inhalant tube, which is mucus-lined (Allen, 1953; Dando *et al.*, 1985, 1986a; Reid and Brand, 1986) (Fig. 1). The Lucinidae have a posterior inhalent aperture but differ from other families of the superfamily Lucinaceae in possessing an exhalant siphon. The siphon is formed by the inner muscular lobe of the mantle and is highly extensible; when retracted, it is turned inside out and lies in the suprabranchial cavity (Allen, 1958). Although the mantle is generally not fused to any great extent posteriorly, Reid and Brand (1986) note an exception in *Parvilucina tenuisculpta* whose mantle edges are fused posteriorly to form an elastic ribbon with complex musculature. The postero-dorsal descending gill filaments are connected to this muscular organ and the authors suggest it may have a bellows-like effect on the gills, causing flushing of the suprabranchial chamber via the exhalant siphon.

The presence of folds in the mantle tissue ("mantle gills") has been noted in some species of lucinid, although it appears they are not present in all lucinids, as suggested by Allen (1958) (Reid and Brand, 1986). These "gills" are composed of vascularized folds which increase the pallial

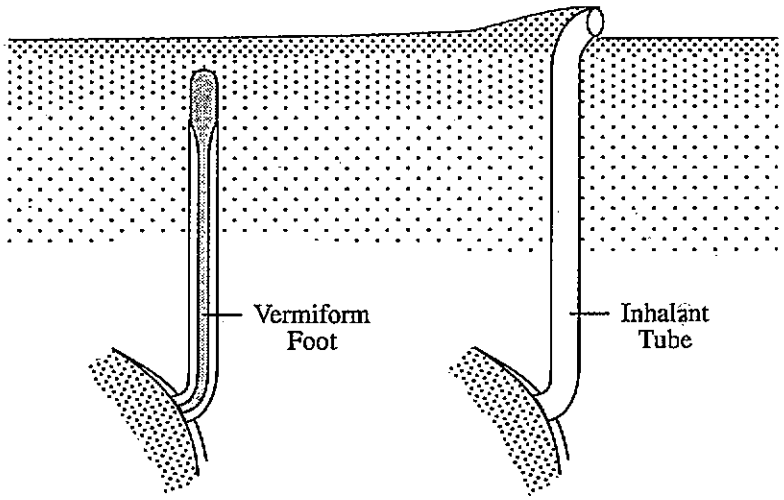

Figure 1. Formation of the inhalant tube in the family Lucinidae (modified from Allen, 1958)

surface and they may serve a respiratory function (Pelseneer, 1911).

Cavanaugh (1985) lists three features typical of bivalves with symbionts: a short and simple gut, reduced palps and thick gills. The family Lucinidae possesses all three features and in all members of the family which have been examined to date, there has been evidence that the bivalves participate in a symbiosis with chemoautotrophic bacteria. The simplified gut of the family Lucinidae has been well documented. Purchon (1958) noted that the stomach of *Lucinoma borealis* was extremely simple in form, with an unusually small number of very simple, digestive diverticula. This simplified structure has been found in all other members of the family examined (Allen, 1958; Herry and Le Pennec, 1987; Reid, 1990) and the gut is so reduced in some species that the original gastric form is barely recognizable (Reid and Brand, 1986). Although the guts are small, they appear to be functional (Allen, 1958); for example, the gut of *Lucinella divaricata* was found to be always full of organic particles, mainly diatoms (Herry and Le Pennec, 1987). Although Allen (1958) suggested the form of the lucinid gut may represent a modification for the acceptance of large particles, Reid and Brand (1986) noticed that *P. tenuisculpta* rejected particles longer than 75 µm and, therefore, found no evidence for gut regression representing an adaptive trend toward a more macrophagous habit. It is now generally accepted that gut simplification, along with other morphological modifications, are more likely correlates of the symbiosis (Reid and Brand, 1986).

Members of the family Lucinidae have also been noted for substantial reductions in the size of the labial palps (Allen, 1958). These palps may be reduced to the point which they are relatively ineffectual for sorting food particles (Reid, 1990). It should be noted, however, that reduction of the labial palps may not, as suggested by Cavanaugh (1985), be characteristic of bivalves with symbionts. Herry *et al.* (1987) note that *Bathymodiolus thermophilus*, a deep-sea hydrothermal vent bivalve with chemoautotrophic bacterial symbionts, has well-developed palps (Hily *et al.*, 1986). Perhaps the statement that reduced palps are limited to bivalves with symbionts is more appropriate.

Lucinid gills consist of one demibranch only (the outer demibranch has been lost) and are flat, homorhabdic and very large, extending well below the limit of the visceral mass (Allen, 1958). Large, thick ctenidia have been described for virtually all lucinid species that have been examined to date. In those cases where thick gills have not been noted, this appears to be a simple omission rather than a contradiction. The gills of *Lucinoma aequizonata, Lucinoma borealis* and *Myrtea spinifera* comprise approximately 35%, 28% and 23%, respectively, of the wet weight of the soft clam tissues (Dando *et al.*, 1985, 1986a; Distel and Felbeck, 1987). This can be compared to 10.0 $\pm$ 2% for the bivalve *Mytilus edulis*, whose gills do not harbour bacterial symbionts (Distel and Felbeck, 1987). The lucinid gill structure and modifications advantageous to housing endosymbiotic bacteria are discussed in detail in section 1.4 of this chapter.

It has been suggested that sulphide-oxidizing symbioses were responsible for the emergence of the Lucinacea and that modification of the gills, siphon, gut and feeding appendages were a series of paedomorphic events (Reid, 1990). In addition, sulphide-oxidizing symbioses may be the most radical evolutionary factor affecting feeding and ventilatory behaviour, as well as hemoglobin adaptation (Reid and Brand, 1986)(see section 1.7).

1.3. EVIDENCE FOR CHEMOAUTOTROPHIC BACTERIAL SYMBIOSIS IN THE FAMILY LUCINIDAE

Symbioses between invertebrates and chemoautotrophic bacteria can be demonstrated in a number of ways. Although the anatomy of the host may suggest that nutrition is obtained in methods alternate to heterotrophy, due to reduced or absent gut or reduced feeding appendages, these observations provide no evidence that chemoautotrophic symbioses are an alternate method. In addition, it cannot be assumed that the specificity of habitat (eg: high sulphide sediments) observed in some chemoautotrophic symbioses is proof of this type of symbiosis. It has been demonstrated, for example, that several bivalve species which do not possess bacterial endosymbionts inhabit high sulphide sediments (Reid and Brand, 1986) and lucinid bivalves with endosymbionts are found in a variety of habitats (Dando *et al.*, 1986b).

Initial investigations into these symbioses virtually always consist of demonstrating the presence of bacteria living in host tissues, usually using electron microscopy to illustrate the features of the prokaryotic cells such as the distinctive cell wall of Gram-negative bacteria. All bivalves belonging to the family Lucinidae, examined to date, have been shown to possess endosymbiotic bacteria. This suggests chemoautotrophic symbioses may be inherent in this family (Fisher, 1990). Table 1 lists the species of lucinids which have been examined for the presence of bacterial endosymbionts using electron microscopy. In section 1.4, the structure and

Table 1. Lucinid bivalves with chemoautotrophic symbiotic bacteria.

Species	Reference
Linga pensylvanica (L.)	1
Codakia orbicularis (L.)	1,4
Lucinoma annulata (Reeve)	2,10
Lucina floridana (Conrad)	3,10
Myrtea spinifera (Montagu)	5,9
Anodontia philippiana Reeve	6
Lucina multilineata Tuomey & Holmes	6
Lucina radians (Conrad)	6
Lucina (=Codakia) costata (d'Orbigny)	6
Lucinoma borealis (L.)	7,9
Parvilucina tenuisculpta (Carpenter)	8
Lucinoma aequizonata (Stearns)	10
Lucinella divaricata (L.)	11,12,13
Loripes lucinalis Turton	13,14
Codakia (=Ctena) orbiculata (Montagu)	15

1 Berg *et al.*, 1983
2 Cavanaugh, 1983
3 Fisher and Hand, 1984
4 Berg and Alatalo, 1984
5 Dando *et al.*, 1985
6 Giere, 1985
7 Dando *et al.*, 1986a
8 Reid and Brand, 1986
9 Southward, 1986
10 Distel and Felbeck, 1987
11 Herry and Le Pennec, 1987
12 Le Pennec *et al.*, 1987
13 Le Pennec *et al.*, 1988
14 Herry *et al.*, 1989
15 Barnes and Brand, unpubl. data.

cellular composition of the gill are examined in detail, focusing on the position of the bacterial symbionts.

Obviously, the presence of bacteria in host invertebrate tissues does not reveal if the symbionts are chemoautotrophic. Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBPC/O, EC4.1.1.39), which catalyses the first reaction in the Calvin-Benson cycle, is only found in autotrophic organisms, carbon monoxide-oxidizing bacteria and some methylotrophic bacteria (low activities) (Tabita, 1988; Fisher, 1990). When the possibility of photoautotrophy can be eliminated (as for deep-sea or sediment-dwelling organisms), substantial activity of this enzyme is indicative of chemoautotrophy (Fisher, 1990). Activity levels must be relatively high to eliminate the possibility that bacteria found externally on the tissues are responsible. All lucinid species tested have been found to have high activity levels of RuBPC/O in the gill tissues and are listed in Table 2. Phosphoribulokinase is another enzyme unique to the Calvin-Benson cycle and is, therefore, also effective in demonstrating chemoautotrophy. Activity of this enzyme has been demonstrated in the gill tissues of *Lucina floridana* and *Lucinoma borealis* (Fisher and Hand, 1984; Dando *et al.*, 1986a).

Since the isotopic fractionation of carbon between an animal and its food source is relatively small, the stable carbon isotope composition of animal tissues can be used to examine trophic strategies (Rau, 1982). Fisher (1990) cautions, however, that stable carbon isotope ratios are suggestive, rather than demonstrative, of a chemoautotrophic

Table 2. Ribulose-1,5-bisphosphate carboxylase-oxygenase activity in lucinid bivalve gill tissues.

Species	Reference
Lucinoma annulata	1
Parvilucina tenuisculpta	1
Codakia orbicularis	2,3
Linga pennsylvanica	2,3
Lucina floridana	4
Lucina nassula (Conrad)	3
Codakia (=Ctena) orbiculata	2,6
Codakia (=Lucina) costata	6
Anodontia philippiana	6
Lucina radians	6
Parvilucina multilineata	6
Myrtea spinifera	5,8
Lucinoma borealis	7,8,10
Lucinella divaricata	9,10
Lucinoma aequizonata	11
Loripes lucinalis	10,12

1 Felbeck *et al.*, 1981
2 Berg *et al.*, 1983
3 Berg and Alatalo, 1984
4 Fisher and Hand, 1984
5 Dando *et al.*, 1985
6 Schweimanns and Felbeck, 1985
7 Dando *et al.*, 1986a
8 Spiro *et al.*, 1986
9 Herry and Le Pennec, 1987
10 Diouris *et al.*, 1988
11 Distel and Felbeck, 1988
12 Herry *et al.*, 1989

symbiosis and that the stable carbon isotope composition of the carbon source (ie: variation within a species may reflect habitat diversity), the sources of isotopic fractionation, and the question of carbon limitation must be considered. These considerations are discussed at length in that review and will not be examined further here. In addition, demonstration of a chemoautotrophic carbon source is not evidence of symbiosis since organisms may be feeding on that carbon source rather than receiving carbon products through a symbiotic relationship (Fisher *et al.*, 1988). Stable carbon isotope ratios should be used, therefore, in conjunction with visual and enzymatic evidence of endosymbionts. Keeping these cautions in mind, however, stable carbon isotope ratios reported throughout the literature can be used to indicate a chemoautotrophic carbon source and, in conjunction with additional evidence, chemoautotrophic symbioses.

Because of the distinctive "isotopic signatures" of chemoautotrophic organisms, transfer of carbon from symbiont to host can be inferred by comparing symbiont-containing tissues (or isolated symbionts) and symbiont-free tissues from a particular host. Once again, care must be used in interpreting these data quantitatively due to possible input from free-living chemoautotrophs and dissolved organic carbon derived from these bacteria (Fisher, 1990).

The stable carbon isotope composition of a sample is usually expressed relative to the PeeDee belemnite (PDB) standard; a negative number reflects a sample enriched in ^{12}C relative to the PDB standard (Rundel *et al.*, 1988). Due to

significant fractionation of stable carbon isotopes during fixation of inorganic carbon into cellular organic carbon, free-living sulphide-oxidizing bacteria have been found to preferentially incorporate isotopically light carbon (^{12}C) during chemoautotrophic growth (Ruby *et al.*, 1987). Hence, invertebrates whose major source of carbon are sulphide-oxidizing bacterial symbionts have distinctive stable carbon isotope ratios (-23‰ to -47‰). Stable carbon isotope ratios for lucinid bivalves are listed in Table 3. Cary *et al.* (1989) reported that the stable carbon isotope ratios of the symbiotic bacteria in *Lucinoma aequizonata* were significantly lighter than host tissue and suggest heterotrophic contribution to the clam's nutrition. This is also true for *Lucinoma borealis*, *Myrtea spinifera* and, to a lesser extent, *Codakia oribicularis* (Spiro *et al.*, 1986). Although for the latter three species, the stable carbon isotope ratios were measured on gill tissues, rather than isolated bacterial symbionts, so the difference in the ratios was not as pronounced as for *Lucinoma aequizonata*. The gutless symbiont-containing bivalve *Solemya reidi* and non-symbiont-containing bivalves have stable carbon isotope ratios of -30‰ and approximately -17.8‰ respectively, suggesting that for the lucinid bivalves discussed above, at least 50% of the body carbon is derived from bacterial autotrophy.

Although by far the most data are available on stable carbon isotope ratios, stable nitrogen isotope ratios have also been used in investigations into chemoautotrophic symbioses; the ratios reported for chemoautotrophic symbioses

Table 3. $^{13}C/^{12}C$ ratios in the tissues of bivalve molluscs belonging to the family Lucinidae.

Species	Tissues	$^{12}C/^{13}C$ (‰)	Ref.
Codakia orbicularis	gill	-23.9,-28.3	1
	body	-23.2,-28.1	1
Lucinoma borealis	gills	-28.1 to -29.0	2
	rest (gut removed)	-24.1 to -25.9	2
Myrtea spinifera	gills	-24.2	2
	rest (gut removed)	-23.4	2
Lucina nassula	whole body	-23.0	2
Pseudomiltha sp.	gills	-32.5 to -37.7	3
Lucinoma aequizonata	bacteria	-34.0 ± 0.8	4
	other	-29.0 ± 0.7	4

1 Berg and Alatalo, 1984
2 Spiro *et al.*, 1986
3 Brooks *et al.*, 1987
4 Cary *et al.*, 1989

are considerably lower than typical values for marine biological samples (Fisher, 1990). Negative stable isotope ratio values, such as those of the lucinid *Pseudomiltha sp.*, suggest that the symbionts fix nitrogen (Brooks *et al.*, 1987). Stable sulphur isotopes are of little value in demonstrating chemoautotrophic symbioses since some apo-symbiotic animals are also able to oxidize sulphide (Somero *et al.*, 1989) and the isotopic sulphur composition of apo-symbiotic animals from high sulphide environments may reflect, therefore, the $\delta^{34}S$ of the sulphide from that environment (Spies and DesMarais, 1983; R. Vetter, pers. comm.).

Enzymes associated with sulphur metabolism, when found along with activity of RuBPC/O, can be considered good evidence for chemoautotrophic symbioses fuelled by sulphur. Tables 4a(i) and 4a(ii) list lucinid bivalve species for which sulphur enzyme activity has been measured in the gill tissues and other body tissues, respectively. The possible role of sulphur enzymes in detoxification of sulphide is discussed in section 1.7. Sulphur enzymes commonly measured include adenosine-5'-phosphosulphate reductase (APS reductase, EC 1.8.99.2), sulphate adenylyltransferase (ADP)(ADP sulphurylase, EC 2.7.7.5), sulphate adenylyltransferase (ATP sulphurylase, EC 2.7.7.4) and thiosulphate sulphurtransferase (rhodanese, EC 2.8.8.1). APS reductase catalyzes the reaction of AMP and sulphite to produce adenosine phosphosulphate in some autotrophic sulphur bacteria (Trüper and Peck, 1970) and can catalyze the reverse reaction in some sulphate reducing bacteria (Peck *et al.*, 1965). Although Fisher (1990) suggests

Table 4a. Enzymes of sulphur metabolism in lucinid bivalves.

(i). Gill tissue

Species	Enzyme	Reference
Lucinoma annulata	ATP-sulphurylase	1
	rhodanese	1
Parvilucina tenuisculpta	ATP-sulphurylase	1
Codakia orbicularis	ATP sulphurylase	2
	rhodanese	2
Lucina floridana	APS reductase	3
	ATP sulphurylase	3
Myrtea spinifera	APS reductase	4
	ATP sulphurylase	4
	ADP sulphurylase	4
Lucinoma borealis	APS reductase	5,7
	ATP sulphurylase	7
	rhodanese	7
Lucinella divaricata	APS reductase	6,7
	ATP sulphurylase	7
	rhodanese	7
Loripes lucinalis	APS reductase	7,8
	ATP sulphurylase	7,8
	rhodanese	7,8

(ii). Non-gill tissues.

Species	Tissue	Enzyme	Reference
Myrtea spinifera	foot	sulphate	4
		adenylyl transferase	4
Myrtea spinifera	mantle	sulphate	4
		adenylyl transferase	4
Loripes lucinalis	body (no gill)	ATP sulphurylase	8
	body (no gill)	rhodanese	8

1 Felbeck *et al.*, 1981
2 Berg *et al.*, 1983
3 Fisher and Hand, 1984
4 Dando *et al.*, 1985
5 Dando *et al.*, 1986a
6 Herry and Le Pennec, 1987
7 Diouris *et al.*, 1988
8 Herry *et al.*, 1989

that APS reductase has only rarely been demonstrated in chemoautotrophic symbioses, it was reported for eight of the eleven lucinids which have been examined for sulphur enzymes and it was not tested for in the other three (Table 4a(i)). The absence of APS reductase is not, however, evidence against chemoautotrophy since sulphite oxidation is catalyzed by other enzymes in other chemoautotrophs (Charles and Suzuki, 1966; Fisher, 1990). ADP sulphurylase converts the APS formed to sulphate and ADP (Gottschalk, 1986). ATP sulphurylase catalyzes the reversible reaction of adenosine-phosphosulphate and pyrophosphate to yield ATP and sulphate. Although ATP sulphurylase is found in a variety of organisms (Peck, 1974) it has only been found in high activities in chemoautotrophic sulphur bacteria and chemoautotrophic symbioses. In the latter, it has been demonstrated in all symbioses tested and only in symbiont-containing tissues (Fisher, 1990). Hence, while not diagnostic, it is certainly a good indicator of chemoautotrophic symbioses. Rhodanese can catalyze a variety of reactions and is found in a number of different organisms at relatively high activities (Fisher, 1990). Rhodanese activity cannot, therefore, be considered as diagnostic of sulphur-oxidation (Cavanaugh, 1985).

Sulphide oxidase is a catalyst which may be responsible for the first step in the oxidation of hydrogen sulphide in a number of animals. The presence of this catalyst has been demonstrated in symbiotic and aposymbiotic tissues of organisms with chemoautotrophic symbionts and in the tissues of apo-symbiotic animals from sulphide-rich environments

(Powell and Somero, 1985; Powell and Somero, 1986; Brooks *et al.*, 1987). Sulphide oxidase cannot be considered indicative of chemoautotrophic symbioses, therefore. The kinetics and pathway of this enzyme are unknown, currently. The benzyl viologen (B.V.) assay used to measure sulphide oxidase activity also measures non-enzymatic sulphide oxidation (Powell and Arp, 1989), emphasizing the importance of adequate controls when attempting to determine enzymatic activity. It has been suggested that B.V. assays conducted at very high levels of sulphide (eg: 1 mM) measure mostly non-enzymatic oxidation since these levels are physiologically unrealistic and can inhibit enzymatic oxidation (Wilmot and Vetter, 1992). Sulphide oxidase data available on lucinids are presented and discussed in section 1.7.

Elemental sulphur can be an intermediate product in the oxidation of sulphide by chemoautotrophic bacteria and can also result from the anaerobic oxidation of thiosulphate (Schedel and Trüper, 1980). It is non-toxic, can be stored within the bacterial cells, and then oxidized later, in the absence of external forms of reduced sulphur, to sulphite or sulphate to produce additional energy (Vetter, 1985; Cary *et al.*, 1989). The oxidation of sulphide to elemental sulphur has a free energy change of -210 kJ/mole, while the further oxidation of elemental sulphur to sulphate has a free energy change of -496 kJ/mole (Table 4b). Observations suggest that a store of elemental sulphur can be consumed within a few days in well-oxygenated conditions (Dando *et al.*, 1985; Vetter, 1985).

Table 4b. Standard free energy changes of reactions involving oxidation of sulphur compounds (from Jannasch, 1984).

Reaction	G°'(kJ/mol)
$H_2S + 1/2O_2 = S^\circ + H_2O$	-210
$HS^- + 2O_2 = SO_4^{2-} + H^+$	-716
$S^\circ + 1.5O_2 + H_2O = H_2SO_4$	-496
$S_2O_3^{2-} + 2O_2 + H_2O = 2SO_4^{2-} + 2H^+$	-936

The presence of elemental sulphur, contained in small vesicles formed by invagination of the cytoplasmic membrane of the bacterial symbionts, has been demonstrated for numerous species of lucinid bivalve and is discussed further in this chapter, section 1.4. Apo-symbiotic marine organisms do contain sulphur but at relatively low levels: 0.45 to 2.8% dry weight (d.w.) total sulphur (this is probably largely organic sulphur) (Kaplan *et al.*, 1963). Vetter (1985) noted that the foot tissue of *Lucinoma aequizonata* had comparable levels to those of apo-symbiotic marine organisms, 1.4% d.w., and demonstrated that the gills of this lucinid bivalve contained elemental sulphur in much higher levels (21-26% d.w.). Elemental sulphur in the gill tissues can be suggestive, therefore, of sulphide-oxidizing chemoautotrophs. The elemental sulphur appeared to be present in liquid crystal form in globules, similar to that observed for free-living sulphur bacteria (Lawry *et al.*, 1981; Schedel and Trüper, 1980; Vetter, 1985). X-ray diffraction analysis of gill tissues revealed stored globular elemental sulphur (SGES) in

Lucina floridana and *Parvilucina tenuisculpta* (Fisher and Hand, 1984; Reid and Brand, 1986). SGES content of the gills has been measured for *Myrtea spinifera* (3.3 - 25.0 µgatoms S/gwetwgt), *Lucinoma borealis* (9.4 - 68.1 µgatoms S/gwetwgt), *L. aequizonata* (18-144 µgatoms S/gwetwgt) and *Pseudomiltha sp.* (6.25 - 256.25 µgatoms/gwetwgt) (Vetter, 1985; Dando *et al.*, 1985, 1986a; Brooks *et al.*, 1987). SGES levels are thus highly variable and appear to be correlated to availability of reduced sulphur forms. When whole gills of *Myrtea spinifera* were supplied with $Na_2{}^{35}S$, a small quantity was converted to SGES (Dando *et al.*, 1985).

Significant activity of the enzymes of nitrogen metabolism (nitrate reductase (EC 1.6.6.1) and nitrite reductase (EC 1.7.7.1)) are indicative of chemoautotrophy since the ability to assimilate inorganic nitrogen sources is restricted to autotrophic organisms (where the possibility of photoautotrophy can be eliminated). The enzymes catalyze the reduction of nitrate to nitrite and the formation of ammonia from nitrite respectively. Nitrate reductase has been demonstrated in *Parvilucina tenuisculpta* and *Codakia orbicularis* (Felbeck *et al.*, 1981; Berg and Alatalo, 1984). Fisher and Hand (1984) demonstrated nitrite reductase in *Lucina floridana*. It is possible, however, that these enzymes have a role in dissimilatory, not assimilatory, nitrogen metabolism. Hentschel *et al.* (1993) demonstrated the presence of respiratory nitrate reductase in *Lucinoma aequizonata*, noting higher activity in animals maintained anoxically.

Nitrogen assimilation in *L. aequizonata* has been suggested, based on incorporation of ^{15}N-ammonia and ^{15}N-nitrate into acid insoluble compounds and by the presence of GS-GOGAT pathway enzymes in the symbionts (U. Hentschel, pers. comm.). The enzyme responsible for nitrogen fixation, nitrogenase (EC 1.18.6.1), has not yet been demonstrated for chemoautotrophic symbioses.

The results of physiological research, discussed in detail later in this review (section 1.7), can also provide evidence of chemoautotrophic symbioses. For example, an increase in carbon dioxide fixation rate in whole animals or tissues in response to specific reduced sulphur substrates, provides additional evidence of chemoautotrophy (Fisher and Hand, 1984; Dando *et al.*, 1985, 1986a) and demonstration of translocation of fixed carbon from bacteria to host is indicative of the symbiosis (Herry *et al.*, 1989 for *Loripes lucinalis*). Net uptake of inorganic carbon in the presence of reduced sulphur compounds (Anderson *et al.*, 1987 for *Solemya reidi*) or recording growth of host organisms in response to reduced sulphur compounds (Cary *et al.*, 1989 for *Lucinoma aequizonata*) also demonstrate functioning chemautotrophic symbioses.

1.4. STRUCTURE AND CELLULAR COMPOSITION OF THE GILL

The structure and cellular composition of lucinid gills and the location of the bacterial symbionts within the gills have been extensively studied. While generalizations for the family Lucinidae can be made for gill structure, there appear to be some differences in the cellular composition, which may represent intergeneric or interspecific variation. The spatial arrangement of the endosymbionts in the host gill tissues, in conjunction with physiological mechanisms, determines bacterial access to oxygen (or possibly other electron acceptors) and sulphide, in addition to host cell exposure to the potentially toxic effects of sulphide. The position of the bacteria is, therefore, integral to understanding the transport of reduced sulphur compounds, detoxification mechanisms and energy exploitation in the symbiosis. For this reason, the cellular structure of lucinid gills is reviewed here in some detail. The functions of the various cell types are discussed only briefly, since cell function, gill structure and cellular composition are related to the physiological mechanisms of chemoautotrophic symbioses in section 1.7.

1.4.1. Gill structure

The gills of lucinid bivalves consist of a single demibranch (Allen, 1958). The outer portion of the gill, referred to by Distel and Felbeck (1987) as the "ctenidial filament zone", is typical of eulamellibranchs and consists of a parallel array of ciliated filaments which are joined

together by interfilamentar junctions (Allen, 1958). This forms a pair of grid-like lamellae which are joined together at the bottom and numerous interlamellar junctions extending the length of the lamellae (dorsoventrally), form the interlamellar space into vertical water tubes (Dando *et al.*, 1985; Distel and Felbeck, 1987) (Fig. 2). These vertical tubes, again characteristic of eulamellibranchs, open into a continuous space, between the gill and visceral mass at the upper end of the gill, which extends posteriorly to the base of the exhalant siphon (the suprabranchial chamber in Reid and Brand (1986) or the dorsal space in Dando *et al.* (1985)) (Purchon, 1968; Barnes, 1980). The efferent and afferent blood vessels are carried in the ascending and descending lamellae (Allen, 1958) and the blood sinuses in lucinid ctenidia are enlarged (Reid, 1990). The lucinids appear to utilize regular cilial sorting and transport of food particles and the demibranchs have thin ciliated food grooves at the ventral margins (Allen, 1958; Dando *et al.*, 1985; Reid and Brand, 1986; Herry *et al.*, 1989).

Closer examination of the subfilamentar tissues below the ciliated surface of the gill reveals that lucinid gills are unusual for eulamellibranchs. The subfilamentar tissue is exceptionally thick and gives the gills their distinctive dark colour (Allen, 1958; Distel & Felbeck, 1987). It is this tissue which contains the bacterial symbionts. The lucinid gill may represent a compromise between filter feeding (ciliated zone) and symbiosis (thick subfilamentar zone with bacteriocytes) (Reid and Brand, 1986). Distel and Felbeck

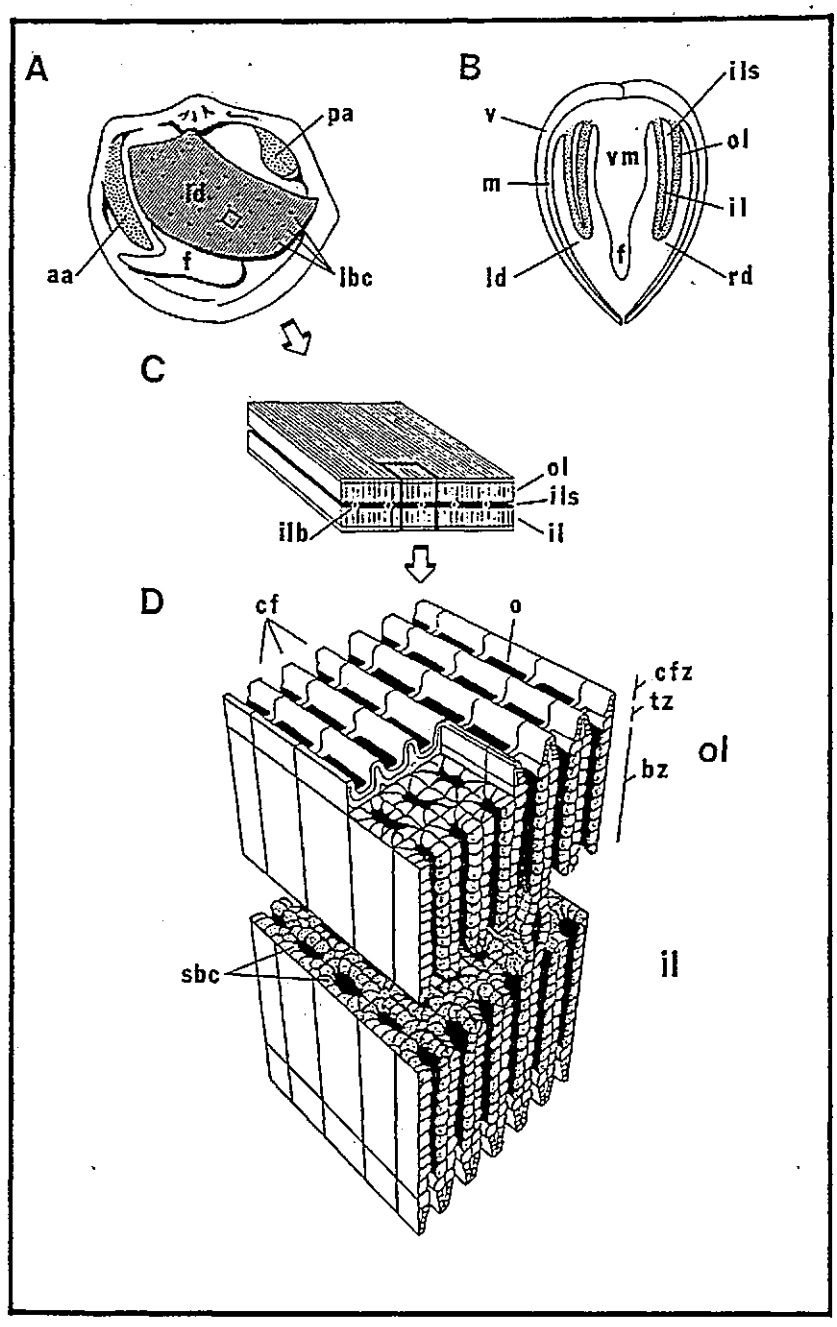

Figure 2. Structure of the gills in *Lucinoma aequizonata*, identical to that in *Codakia orbiculata*. (A) Clam with left valve removed. (B) Transverse section through entire clam at median point of anterior-posterior axis. (C) Enlargement of block of gill tissue removed from region indicated by box in (A). (D) Enlargement of block of gill tissue removed from region indicated by box in (C). aa: anterior adductor; bz: bacteriocyte zone; cf: ctenidial filaments; cfz: ctenidial filament zone; f: foot; il: inner lamella; ilb:interlamellar bridge; ils: interlamellar space;lbc: large bacteriocyte channel; ld: left demibranch; m: mantle; o: ostia; ol: outer lamella; pa: posterior adductor; rd: right demibranch; sbc: small bacteriocyte channel; tz: transition zone; v: valve; vm: visceral mass. Figure 2 is from Distel and Felbeck (1987).

(1987) divide the subfilamentar tissue into two distinct zones, based on cellular composition: the "transition zone" and the "bacteriocyte zone". The presence of a transition zone may not be consistant throughout the family and is discussed later in this text. Regardless of the number of zones in the subfilamentar tissue, the three dimensional gill structure described by Distel and Felbeck (1987) appears consistent with other published descriptions for lucinid gills (Fig. 2). The subfilamentar tissue, rather than a simple medial extension of the filament tissue, is organized as an array of cylindrical tubes. The ostia of the ciliated zone (typical of eulamellibranch bivalves) connect to "small bacteriocyte channels" which run continuously through the subfilamentar tissues (the transition zone and the bacteriocyte zone) into the interlamellar space. The small bacteriocyte channels run through the centre of bacteriocyte cylinders, which are arranged in a tightly packed array and extend into the interlamellar space perpendicular to the gill surface. These cylinders are made up largely of bacteriocytes that are organized to form open-centered rings with the inner surfaces of the cylinders (small bacteriocyte channels) lined with thin epithelial cells (microvillar intercalary cells) (see following section) forming a partial barrier between the bacteriocytes and the outside world. The cells of the transition zone are also organized as a ring, lying between the bacteriocyte and ctenidial filament zones. Hence, mantle fluid can pass from the mantle cavity through the interlamellar space and into the suprabranchial cavity. The

outer sides of the bacteriocyte cylinders are fused to form a continous sheet of tissue which communicates with the hemocoel; the basal surface of the bacteriocytes, therefore, is in contact with the ctenidial blood sinus.

1.4.2. Gill Cellular Composition

The presence of the ciliated cells in the frontal portion of the gill filaments is constant throughout all members of the family Lucinidae examined to date (Fisher and Hand, 1984; Giere, 1985; Dando *et al.*, 1985; Reid and Brand, 1986; Distel and Felbeck, 1987; Herry and Le Pennec, 1987). These cells never contain bacteria and are typical of the normal bivalve filament in that they contain numerous mitochondria: an observation made for *Parvilucina tenuisculpta*, *Lucina floridana*, *Anodontia philippiana*, *Lucina multilineata*, *Lucina costata* and *Lucina radians* (Fisher and Hand, 1984; Giere, 1985; Reid and Brand, 1986). The well-defined ciliary bands (frontal, latero-frontal and lateral) are described in detail for *Lucinella divaricata* (Herry and Le Pennec, 1987) and a detailed description of the variety of ciliated cells in *Loripes lucinalis* can be found in Herry *et al.* (1989). In the latter, observations of the frontal, prolatero-frontal, eulatero-frontal, prolateral and eulateral cells in the ciliated zone indicated that only the eulateral cells have numerous mitochondria and that the frontal, prolateral-frontal and prolateral cells are covered with microvilli.

The distinct zones present in the lucinid gills clearly indicate different requirements for gas and solute exchange.

Since the ciliated zone of lucinid gills is typical of eulamellibranch bivalves, it suggests that the function may also be the same: to ventilate blood and filter particles. Although these ciliated cells are rich in mitochondria, lucinids are known to be stenohaline osmoconformers (Jackson, 1973), suggesting that the mitochondria-rich epithelium is not required for active, transepithelial movement of ions (Fisher and Hand, 1984). It is likely that maximal oxygen transport occurs in the ctenidial filament zone and Fisher and Hand (1984) suggested that the ciliated gill epithelium may have an oxygen-scavenging role, protecting oxygen-sensitive enzymes found in the deeper bacteriocytes. Since RuBPC/O exhibits both oxygenase and carboxylase activities, due to competition between oxygen and carbon dioxide for the same active site, carbon dioxide fixation will proceed most efficiently when the intracellular ratio of CO_2 and O_2 is high, allowing normal hexose assimilation (Chollet, 1977). Hence, maintaining low oxygen levels in the water passing through the bacteriocyte channels would be advantageous to bacterial RuBPC/O function. A similar control on oxygen levels has been suggested by Distel and Felbeck (1987). Giere (1985) suggests oxygen-scavenging is unlikely to be important in the low oxygen environments in which the organisms are found. In either case, however, the subfilamentar gill tissue will be exposed to lower oxygen levels than are found in the environment. Another possibility is that the mitochondria in the ciliated cells, similar to the host cell mitochondria of *Solemya reidi*, may

oxidize sulphide (see section 1.7) (Powell and Somero, 1986; O'Brien and Vetter, 1990).

Allen (1958) noted the large amount of subfilamentar tissue in lucinid gills and the presence of large cells containing brown pigment granules, which were never found in the ciliated cells. All reports agree that it is the subfilamentar zone which gives the gills their colour. In the subfilamentar zone and immediately below the ciliated zone, Distel and Felbeck (1987) described a cell layer no more than a few cells thick which they term the "transition zone". Cells in this zone never contain bacteria or pigment granules. They are also refered to as storage epithelium, due to the presence of numerous glycogen and mucopolysaccharide granules, by Reid and Brand (1986) or intermediary cells by Herry *et al.* (1989) (the latter not to be confused with Giere's (1985) "intermediate" cells, which resemble the intercalary cells discussed later in this text). The transition zone is described for *Lucinoma aequizonata*, *Lucinoma annulata*, *Lucina floridana*, *Parvilucina tenuisculpta* and *Loripes lucinalis* (Reid and Brand, 1986; Distel and Felbeck, 1987; Herry *et al.*, 1989). In contrast to the former four species, *L. lucinalis* transition zone cells are densely covered with microvilli on the apical surface. In addition, they are noted to contain numerous mitochondria, an observation omitted for transition zone cells in the other lucinid species (Herry *et al.*, 1989). There is no mention of this "transition zone" in *Anodontia philippiana*, *Lucina multilineata*, *Lucina radians*, and *Codakia (Lucina) costata* (Giere, 1985), *Myrtea spinifera* (Dando *et*

al., 1985), *Lucinoma borealis* (Southward, 1986) nor Fisher and Hand's (1984) description of *L. floridana*. The latter contrasts to Distel and Felbeck's (1987) description of the same bivalve. It is possible that the transition zone has been overlooked in some species or it may be absent, representing an intergeneric and interspecific difference within the family. The only suggested function of the transition zone cells is one of storage (Reid and Brand, 1986).

Mucus-secreting cells were observed by Allen (1958) to usually occur half-way along the filamentar extension in lucinids. Studies since then have noted mucus-producing cells in some, but not all, members of the family Lucinidae. Mucus cells are absent in *Myrtea spinifera* gill tissue and rare in *Lucinoma borealis* and *Loripes lucinalis* gill tissues (Dando *et al.*, 1985; Southward, 1986; Herry *et al.*, 1989). Mucus-producing cells have been found in the transition zone of *Lucinoma aequizonata*, *Lucinoma annulata* and *Lucina floridana*, particularly concentrated at the border facing the ctenidial filaments (Distel and Felbeck, 1987), suggesting a function related to filter feeding.

With the noted exceptions of the transition zone and mucus-secreting cells, most reports on the subfilamentar tissue of lucinids list three basic cell types: bacteriocyte, intercalary and storage cells. All references agree that the predominant cell type in the subfilamentar tissue is the bacteriocyte. Distel and Felbeck (1987) described the tissue in which the bacteriocytes are located, immediately beneath the transition zone, as the "bacteriocyte zone" and noted that

the interlamellar junctions (that form the vertical water tubes between the inner and outer lamellae) are an extension of a double row of bacteriocyte cells across the interlamellar space. Southward (1986) noted for *Lucinoma borealis*, however, that all internal parts of the filaments, interfilamentar bridges and interlamellar bridges are covered with thick brown epidermis made up of intercalary cells and storage cells, as well as bacteriocytes. The bacteriocytes vary in size between species, with diameters ranging from 6-60 µm (Fisher and Hand, 1984; Giere, 1985; Dando *et al.*, 1985; Southward, 1986; Distel and Felbeck, 1987; Le Pennec *et al.*, 1988; Herry *et al.*, 1989). In some species these cells are reported as microvillous (*Myrtea spinifera*, *Lucinoma borealis*, *Lucinella divaricata*, *Loripes lucinalis*, *Parvilucina tenuisculpta*, *Anodontia philippiana*, *Codakia costata*, *Lucina multilineata* and *Lucina radians*) while the bacteriocytes of other species (*Lucinoma annulata*, *Lucinoma aequizonata* and *Lucina floridana*) apparently lack microvilli (Dando *et al.*, 1985; Giere, 1985; Reid and Brand, 1986; Southward, 1986; Distel and Felbeck, 1987; Herry and Le Pennec, 1987; Herry *et al.*, 1989). The bacteriocytes are typically large but vary in shape from roughly cubical with a wide base and a narrower apical region (*M. spinifera*, *L. borealis*) to the reverse, with the longest side adjacent to the ciliated zone of the filament, becoming shorter towards the mid-lateral region (*L. lucinalis*) (Dando *et al.*, 1985; Southward, 1986; Herry *et al.*, 1989). The basal surfaces of the bacteriocytes are in contact with the ctenidial blood sinuses (Dando *et al.*, 1986a; Distel and

Felbeck, 1987; Southward, 1987). The basal membranes of the bacteriocytes in *L. divaricata* and *L. lucinalis* are convoluted, suggestive of exchange between the bacteriocytes and the blood sinus (Herry and Le Pennec, 1987; Herry *et al.*, 1989).

In the bacteriocytes of most lucinids examined to date, there are few mitochondria (Fisher and Hand, 1984; Dando *et al.*, 1985; Giere, 1985; Vetter, 1985; Southward, 1986). In contrast to the observations on *Lucina floridana* by Fisher and Hand (1984) and Vetter (1985), those of Distel and Felbeck (1987) indicate the presence of numerous mitochondria in the bacteriocytes of this species. Endoplasmic reticulum is scarce or absent (Vetter, 1985; Southward, 1986) but there are lots of glycogen particles (Southward, 1986; Distel and Felbeck, 1987; Herry *et al.*, 1989). The nuclei of the bacteriocytes are usually observed to be located in the inner ("central" or "basal") parts of the cell (Giere, 1985; Distel and Felbeck, 1987; Herry and Le Pennec, 1987; Le Pennec *et al.*, 1988; Herry *et al.*, 1989). The bacteriocytes contain numerous vacuoles which contain from one to two (Dando *et al.*, 1985) to tens of bacteria (Distel and Felbeck, 1987). It has been observed for several lucinid species that the bacteria tend to be located towards the distal (external) part of the bacteriocyte, near the bacteriocyte channels and the inhalant water flow (Giere, 1985).

All lucinid endosymbiotic bacteria examined to date appear to be Gram-negative, with a distinctive, multi-layered cell wall (Brock and Madigan, 1988). The bacteria range in

size, commonly exhibiting size variation within one animal. For example, the bacteria of *Parvilucina tenuisculpta* range from 1-5 µm across (Reid and Brand, 1986) and those of *Myrtea spinifera*, 0.4 - 2.5 µm in diameter (Dando *et al.*, 1985). In all members of the family Lucinidae examined to date, the bacteria fit within a 0.4-5.0 µm diameter size range (Southward, 1986). The bacteria are usually spherical or rod shaped, often with both shapes found in one animal (Cavanaugh, 1983; Fisher and Hand, 1984; Giere, 1985; Vetter, 1985; Southward, 1986; Herry and Le Pennec, 1987; Distel and Felbeck, 1987; Herry *et al.*, 1989). The presence of small vesicles formed by invagination of the cytoplasmic membrane have been noted in all lucinid bacterial symbionts whose structure has been closely examined (Dando *et al.*, 1985; Giere, 1985; Southward, 1986; Herry and Le Pennec, 1987; Herry *et al.*, 1989). These vesicles contain globular elemental sulphur (Fisher and Hand, 1984; Vetter, 1985; Dando *et al.*, 1985; Reid and Brand, 1986; Southward, 1986; Distel and Felbeck, 1987), which gives the bacteria, and sometimes the gill itself, a pale yellow colour. The bacterial cytoplasm contains numerous electron-dense "granules" or particles (Dando *et al.*, 1985; Giere, 1985; Southward, 1986; Herry and Le Pennec, 1987). Giere (1985) suggests these "granules" may be "carboxysomes" due to size, shape and enzyme tests (Schweimanns and Felbeck, 1985). This suggestion has not been made by other researchers, however, and Dando *et al.*, (1985) note that they did not see carboxysomes in *M. spinifera.* Electron-dense particles in *Lucinoma borealis* and *M. spinifera*

symbionts may be clumped ribosomes or glycogen particles (Dando *et al.*, 1985; Southward, 1986). In addition, large electron-dense granules, often associated with cytoplasmic membrane and especially around sulphur vesicles, have been observed in *L. borealis*, *M. spinifera* and *Lucinella divaricata* and may be polyphosphate granules (Dando *et al.*, 1985; Southward, 1986; Herry and Le Pennec, 1987).

The bacteria are usually reported as the most numerous inclusion in the bacteriocytes, but the presence of numerous pigmented granules in these cells is typical of lucinid gills. The pigment granules found in lucinid gill bacteriocytes, probably responsible for the colouration of lucinid gills (Allen, 1958), are generally reported as brown and yellow (Fisher and Hand, 1984; Dando *et al.*, 1985; Reid and Brand, 1986; Southward, 1986; Distel and Felbeck, 1987). The "dense globules" found in the bacteriocytes of *Loripes lucinalis* and *Lucinella divaricata* (Le Pennec *et al.*, 1988; Herry *et al.*, 1989) are probably pigment granules. The colours of lucinid gills fall within the range from dark brown to creamy-beige described for *Lucinoma aequizonata*, *Lucinoma annulata*, *Lucina floridana*, *Lucinoma borealis* and *L. lucinalis* (Southward, 1986; Distel and Felbeck, 1987; Herry *et al.*, 1989), yellow and dark brown described for *Parvilucina tenuisculpta* (Reid and Brand, 1986), dark red for *Myrtea spinifera* (Dando *et al.*, 1985), "nearly black" for *Lucina pectinata* (Kraus *et al.*, 1990) and, generally, "dark" for numerous species belonging to the genera *Lucina*, *Parvilucina* and *Codakia* (Jackson, 1973; Felbeck *et al.*, 1981; Berg and Alatalo, 1984; Schweimanns and

Felbeck, 1985). Most reports indicate the granules resemble lysosomal structures; they are electron-dense, often contain the remains of bacteria and some reports note they contain dense aggregates of membrane or membrane whorls, resembling myelin figures typically seen in lysosomal residual bodies (Giere, 1985; Southward, 1986; Distel and Felbeck, 1987; Le Pennec *et al.*, 1988). The role of the granules in bacterial lysis and nutrition of the host bivalve is discussed further in section 1.5. Reid and Brand (1986) analyzed the elemental composition of the granules in *P. tenuisculpta* and suggested the granules may be nephrolith-like bioaccumulation granules that have sequestered iron, nickel and chromium from the water column or from the blood. Fisher and Hand (1984) detected the presence of iron and suggested an alternate function for the granules: that they may contain respiratory pigments (myoglobin, hemoglobin) or iron-containing cytochromes. That the pigment granules may contain hemoglobin has also been suggested by Read (1962) and Jackson (1973) but there is no evidence for this and observations on *M. spinifera* suggest it may be located elsewhere in the cell (Dando *et al.*, 1985). The granules do appear to contain hematin, however, which can oxidize sulphide (Powell and Arp, 1989). The potential role of the granules in sulphide-oxidation is discussed in section 1.7. Giere (1985) suggests, however, that the iron content of the granules could relate to lysosomal function rather than playing a role in oxygen transfer; the granules could accumulate metabolic end-products, acting similarly to "cytosomes" or "spherites" often found in molluscs (Preto,

1979). It is possible that these two suggestions are both applicable: if the granules digest bacteria, the pigments in the granules (which oxidize sulphide non-enzymatically) could be a result of heme compounds present in the bacteria (R. Vetter, pers. comm).

In addition to the bacteriocytes, the subfilamentar gill tissue contains intercalary cells in virtually all species of lucinids examined to date. The intercalary cells have no bacteria but contain more mitochondria than the bacteriocytes (Dando *et al.*, 1985; Southward, 1986). The intercalary cells may contain few (*Lucinoma borealis*) or many pigment granules (*Lucinoma annulata*, *L. aequizonata*, *Lucina floridana*) (Southward, 1986; Distel and Felbeck, 1987). Intercalary cells have surface microvilli and alternate with the bacteriocytes. From a narrow base, the intercalary cells spread at the surface to form a thin layer that covers, or partially covers, the neighbouring bacteriocytes with the microvillous distal surface of the cell (Fisher and Hand, 1984; Dando *et al.*, 1985; Reid and Brand, 1986; Southward, 1986; Distel and Felbeck, 1987; Herry and Le Pennec, 1987; Herry *et al.*, 1989). Giere (1985) noted that bacteriocytes alternated regularly with "normal" or "intermediate" cells which fit the description of intercalary cells (microvillous surface, numerous mitochondria). However, Giere's (1985) interpretation of positioning of the intercalary cells and bacteriocytes, in different species of lucinid, varies from that described above. He suggested that the interpretation of the bacteriocytes covered by an external layer of bacteria-

free cells, which are interpreted here to be intercalary cells, is a result of examination of latero-longitudinal sections as opposed to cross sections of the gill filaments. These conflicting observations could represent true interspecific differences or they may represent fixation artifacts which affect interpretation of cell positioning. If the difference is truly interspecific variation, then Giere's interpretation can be considered to be at one end of the range (no overlap) and the interpretation of Distel and Felbeck (1987), who described the intercalary cells as a thin layer of epithelial cells lining the central channel of the bacteriocyte cylinders, can be considered to be at the other end of the range (complete overlap). The description of the "normal" or "intermediate" cells (intercalary cells) given by Giere (1985) does, however, somewhat resemble the description of storage cells given for other species of lucinids (see later in text). Herry *et al.* (1987) even reports that storage cells have a microvillar surface. Hence, it is possible that Giere's "normal" or "intermediate" cells may actually consist of two cell types.

The hypothesized function of the intercalary cells varies: they may divide to produce new bacteriocytes or have a transport role linked to bacterial metabolism (Reid and Brand, 1986). The former role is supported by Herry *et al.* (1989) but Distel and Felbeck (1987) suggest the intercalary cells may be involved in the elimination of waste products of bacterial lysis. They observed pigment granules in intercalary cells, often at the margin of, or protruding into,

the lumen of the bacteriocyte channel and suggest these structures may be excreted either exocytotically or by sloughing off of the entire cell. They observed no evidence of endocytosis of bacteria or other particulate materials from the external environment. This suggestion agrees with the possible function of the pigment granules suggested for the family Thyasiridae by Allen (1958). The common observation of abundant mitochondria in these cells not only suggests an oxygen-scavenging role but also the alternate function of host mitochondrial oxidation of sulphide (Reid, 1990). This suggestion is discussed at length in section 1.7 of this chapter.

Storage cells have been found in the subfilamentar tissues of most lucinids examined to date. The location of these cells is variable, however. The storage cells in *Parvilucina tenuisculpta* contain numerous glycogen and mucopolysaccharide granules and were found only in the transition zone; they did not appear to be distributed among the bacteriocytes (Reid and Brand, 1986). Distel and Felbeck (1987) and Herry *et al.* (1989) also note the presence of a transition zone but do not comment on a potential storage function for these cells. Remaining reports do not limit storage cells to a specific "zone" but instead, locate them among the bacteriocytes. *Myrtea spinifera* storage cells contain large globular inclusions of electron-dense homogeneous material which may be protein (Dando *et al.*, 1985). Similar inclusions, which disappeared when the animal was starved, were observed for the storage cells of *Lucinoma*

borealis (Southward, 1986). Storage cells have been found among the bacteriocytes of *Loripes lucinalis* and *Lucinella divaricata* and those of the latter have microvilli at the apical pole (Herry and Le Pennec, 1987; Herry *et al.*, 1989). This is the only report of storage cells with microvilli, unless the "normal" or "intermediate" cells described by Giere (1985) are considered storage cells. Certainly the presence of spherical globules in those cells seems consistent with the description of storage cells given above. Storage cells have not been described for *Lucinoma annulata*, *Lucinoma aequizonata* or *Lucina floridana* (Distel and Felbeck, 1987).

1.5. HOST NUTRITION

Stable carbon isotope ratios measured on lucinid tissues suggest that the host bivalves obtain a significant portion of carbon from chemoautotrophy (Spiro *et al.*, 1986; Brooks *et al.*, 1987; Cary *et al.*, 1989) but they do not provide evidence regarding the method by which the host obtains carbon from the bacterial symbionts. It is possible that the lucinid bivalves, which possess a functional gut, may digest chemoautotrophic bacteria from their environment. They may also obtain carbon through phagocytosis of bacterial symbionts, as opposed to translocation of carbon. Distel and Felbeck (1988) found compounds suitable for translocation produced by the symbiotic bacteria of *Lucinoma aequizonata* but did not demonstrate translocation. When they examined the release of carbon dioxide fixation products from purified bacterial isolates, they found that the symbionts never released more than 5% of the carbon they fixed into the incubation medium (incubations from 7.5 to 60 min) and suggest that the 5% is the result of lysis or leakage from bacterial cells. They do not rule out the possibility of translocation of soluble metabolites, however, since it may be possible that host factors (compounds produced by the hosts) are required to induce release of translocation products from symbiont to host. This has been indicated for mollusc-algal symbioses (Muscatine, 1967; Gallop, 1974). Qualitative information on translocation of fixed carbon in the symbiotic *Loripes lucinalis* was provided by pulse-chase tissue autoradiography (Herry *et al.*, 1989). Translocation and incorporation of labelled metabolites into

either metabolically active or structural tissues was observed. Labelled compounds appeared first in the gill, as might be expected if the autotrophic symbionts are fixing carbon, followed by an increase of fixed carbon in symbiont-free tissues. Studies on the gutless solemyid bivalve, *Solemya reidi*, demonstrated that the symbiont can translocate about 40% of the carbon they fix (in the form of soluble organic compounds) to their host (Fisher and Childress, 1986). No comparable study has been carried out for the lucinid bivalves.

Evidence of translocation of carbon products from symbiont to host does not preclude digestion of symbionts as a supplementary means of nutrition. The host may receive nutrients and energy by harvesting symbiont cells (Distel and Felbeck, 1987). This mechanism would be as effective in providing for the host's metabolic needs as translocation of soluble metabolites (Distel and Felbeck, 1988). The digestion of symbiotic bacteria by host bacteriocytes has been suggested from examination of electron micrographs of the gill cells. The cellular mechanisms (lysosomes) and evidence for lysis of symbiont cells (lysosomal residual bodies) are clearly present in the bacteriocyte tissues (Distel and Felbeck, 1987). In *Lucinoma aequizonata*, *Lucinoma annulata* and *Lucina floridana*, the prominence of pigment granules in the bacteriocytes, which have an abundance of membrane whorls resembling myelin figures typically seen in lysosomal residual bodies, suggests that bacteria are continually phagocytized in bacteriocytes (Holtzman, 1976; Vetter, 1985; Distel and Felbeck, 1987). The

presence of many membrane-bound phagosomes filled with granular, electron-dense material which may contain remains of bacteria have been observed in *Myrtea spinifera* and *Lucinoma borealis*, although examples of bacteria being lysed inside their vacuoles were rare (Southward, 1986). Progressive degradation of the bacteria, followed by total lysis, has been observed for *Lucinella divaricata* (Le Pennec *et al.*, 1988a,b). On the basis of the iron content of the pigment granules, Giere (1985) suggested they may have a lysosomal function, acting as sinks for metal-containing substances. Golgi apparatus has also been observed with the lysosomes and presumably produces primary lysosomes containing enzymes associated with degradation of bacterial cells (Vetter, 1985). The presence of enzymatic equipment necessary for bacterial lysis has yet to be proven, however (Herry *et al.*, 1989).

Pigment granules have been observed in intercalary cells, at the margin or protruding into the lumen of the bacteriocyte channel, suggesting the structure may be excreted exocytotically or by sloughing off the entire cell (Allen, 1958; Distel and Felbeck, 1987). This observation led to the suggestion that the intercalary cells may be involved in elimination of waste from bacterial lysis (Distel and Felbeck, 1987). Also, the central blood space of the subfilamentar region of *Myrtea spinifera* was observed to contain hemocytes, including phagocytes carrying granular material similar to the brown granules in the bacteriocytes, which may be waste material (Dando *et al.*, 1985). Similar observations have been made for *Lucinella divaricata* and *Loripes lucinalis* (Herry and

Le Pennec, 1987; Herry *et al.*, 1989) and in the former species these hemocytes have been observed being extruded from the gill epithelium (Le Pennec *et al.*, 1988). The bacteriocytes in some species have been observed to have a highly convoluted basal membrane, suggesting this may be related to transport of material from cell to vascular lumen (Herry and Le Pennec, 1987; Le Pennec *et al.*, 1988). Further circumstantial evidence for digestion of the prokaryotes, at least under adverse conditions, is provided by Dando *et al.* (1986a) who found no enzyme activity and no bacteria in gills of *L. borealis* kept for twelve months in oxygenated sediment. Although it has been suggested that phagocytosis could be used to control bacterial numbers (Distel and Felbeck, 1987), it has been observed that the granules get bigger, suggesting ingestion of bacteria, when the bivalves are starved (Southward, 1986).

Fisher (1990) points out that if a host does not assimilate digested symbionts and receives only translocated organic carbon from its symbionts, then it must obtain other essential compounds (amino acids, fatty acids) by alternate means. The symbiotic solemyid bivalve *Solemya reidi* is capable of moderate uptake rates of amino acids (Felbeck, 1983; Lee *et al.*, 1992). The lateral epidermis, an important site of dissolved organic compound uptake in apo-symbiotic bivalves (Wright *et al.*, 1984), may serve a similar function when this tissue houses bacteria (Southward, 1986). Giere (1985), interpreting that the bacteriocytes are not separated from the external environment by intercalary cells, determined that the bacteriocytes and intermediate cells are open for uptake of

dissolved compounds from external water with bacteria being ideally located for further transfer of their metabolic products into neighbouring cells or into the vascular system. In contrast, for those species where the bacteriocytes are separated from the environment by intercalary cells, it has been suggested that the bacteria do not function directly in the uptake of dissolved organic material from the environment but they may function in maintaining a concentration gradient favourable to the transport of solute into the cells by utilizing the solute (Distel and Felbeck, 1987). Certainly the microvillar surface of the bacteriocytes or the intercalary cells would indicate they may function in the uptake of dissolved organic compounds, as observed for microvillous cells in other bivalves (Wright *et al.*, 1984). In addition to direct uptake of dissolved organic material, the symbionts may be able to recycle ammonia[1] produced as waste by host animals or utilize the ammonia which is abundant in the organically-enriched sediments in which many symbiotic bivalves are found (Southward, 1987). Nitrogen assimilation in *Lucinoma aequizonata* based on incorporation of ^{15}N-ammonia and ^{15}N-nitrate into acid insoluble compounds and by the presence of GS-GOGAT pathway enzymes in the symbionts has been suggested (U. Hentschel, pers. comm.).

[1] "Ammonia" is used throughout the text to denote ΣNH_3 : NH_3 and the NH_4^+ radical (ammonium). Ammonium is the most abundant form in interstitial water.

Finally, unlike the symbiotic solemyid bivalves, all lucinids examined have functional, but reduced, guts. The small guts of *Lucina floridana*, *Lucinella divaricata* and *Lucinoma borealis* have been noted to contain detritus or diatom remains (Fisher and Hand, 1984; Dando *et al.*, 1986a; Herry and LePennec, 1987). Despite finding the stomachs of four specimens of *Lucinoma aequizonata* empty upon collection, Cary *et al.* (1989) report maintaining this bivalve alive for up to one year without a source of reduced sulphur, indicating that the role of heterotrophy may be significant. As discussed in section 1.2, stable carbon isotope ratios indicate a partial (<50%) contribution of heterotrophy to lucinid nutrition.

1.6. HABITAT

Lucinid bivalves are found in tropical, temperate and sub-Arctic marine environments at depths ranging from the low intertidal to 600 m and in habitats including clean coral sands and low sulphide sediments, sewage outfalls, mangrove swamps, and especially seagrass beds (Allen, 1958; Jackson, 1973; Young and Young, 1982; Fisher and Hand, 1984; Dando *et al.*, 1985; Schweimanns and Felbeck, 1985; Cary *et al.*, 1989; Herry *et al.*, 1989). One species may be found in more than one type of habitat: *Loripes lucinalis* has been found in high-sulphide reducing sediments near a sewage outfall in France and in sulphide-free sandy sediments in Portland Harbour, U.K. (Herry *et al.*, 1989; Dando and O'Hara, 1990; Southward and Southward, 1990; P. Barnes, pers. obs.). Lucinids are often found in exceptionally high densities; for example, *Lucinoma borealis* has been reported reaching densities of 1500/m^2 in seagrass beds on the west coast of Brittany, which compares with a density of 1-10/m^2 for apo-symbiotic bivalves in the same area (Monnat, 1970).

Allen (1958) noted that lucinids were found with a marked lack of co-occurring infauna and suggested this may be due to the presence of hydrogen sulphide in some of the habitats (noted by the strong smell). A similar observation regarding the family was made by Bretsky (1976). Allen (1958) suggested a tolerance to hydrogen sulphide throughout the family, attributed to the presence of the inhalant tube connecting to the sediment-surface seawater. Although many lucinid species are found in high sulphide sediments, there are notable

exceptions. Several species are found in the Caribbean in "clean coral sands" (Allen, 1958) and, recently, *Lucinoma borealis*, *Myrtea spinifera*, *Loripes lucinalis* and *Lucinoma aequizonata* have all been reported from sediments where free sulphide is <1 µM (Dando *et al.*, 1985, 1986a; Cary *et al.*, 1989; Dando and O'Hara, 1990). The presence of sulphur-oxidizing symbioses in low sulphide environments suggests a much greater habitat range for chemoautotrophic symbioses than previously expected. Also, the interstitial sulphide concentrations reported for "high sulphide" habitats may not be representative of levels to which the bivalve is directly exposed since measuring sulphide levels in the immediate vicinity of the animal, or in its inhalant tube, can be problematical. In fact, behavioural modifications in both high and low sulphide sediments, as discussed in the following text, may decrease or increase dissolved sulphide in the animal's immediate environment. Interstitial sulphide levels represent the "raw material" which the bacteria-invertebrate symbiosis has "to work with". Analysis of mantle fluids may be the best indicator of levels to which the organism is directly exposed.

In the lucinid habitats which are sulphide-rich, the hydrogen sulphide is produced biologically, unlike the geothermally-produced hydrogen sulphide at the hydrothermal vents, a well-studied habitat of invertebrates containing bacterial symbionts. Hydrogen sulphide accumulates in marine sediments where the amount of organic deposition is greater than oxygen available for degradation. Anoxic conditions arise

and decomposition is then accomplished by the activity of bacteria utilizing inorganic compounds other than oxygen, such as nitrate, nitrite and sulphate, as electron acceptors (Fenchel and Riedl, 1970). Because sulphate is readily available in seawater and because of the large populations of sulphate-reducing bacteria, hydrogen sulphide is often the major inorganic constituent in the sediment interstitial water (Jørgensen and Fenchel, 1974). Inshore areas such as seagrass beds and mangrove swamps, common habitats for lucinids, receive large amounts of self-generated plant material; seagrass beds, in particular, are known to stabilize sediments, reduce water movements, and trap detritus and organic debris from outside the bed (Fenchel, 1970; Jackson, 1972). Sulphide levels in seagrass bed interstitial water can reach 2.5 mM (Fisher and Hand, 1984).

Access to both sulphide and oxygen seems to be a requirement of sulphide-oxidizing bacteria-invertebrate symbioses (Fisher, 1990). Bivalves appear to be particularly good hosts since they can position themselves at the sulphide-oxygen interface and they have the ability to control the supply of oxygen and reduced sulphur, possibly through spatial and temporal partitioning as a result of behavioural mechanisms (Reid and Brand, 1986). These mechanisms may vary between families and environments (Fisher, 1990). The following text discusses the behavioural mechanisms used by bivalves in regulating sulphide and oxygen levels. The roles of both physiological and behavioural mechanisms in detoxifying sulphide and transporting reduced sulphur forms

are discussed, in detail, in section 1.7. Behavioural adaptations have been clearly demonstrated for some of the non-lucinid symbiont-containing bivalves. The vent clam, *Calyptogena magnifica*, lives in crevices and "bridges the interface" by extending its foot into sulphide-rich upwelling water while accessing oxygenated seawater with its extended siphon (Arp *et al.*, 1984) (Fig. 3(a)). The symbiont-containing *Solemya velum* lives in Y-shaped burrows and obtains oxygen by moving up in the burrow or by ventilating the upper burrow. Sulphide may be obtained in the deep stem of the Y-shaped burrow (Doeller, 1984) (Fig. 3(b)). *Solemya reidi* lives in U-shaped burrows and alternates between oxygen and sulphide uptake by ventilating the burrow through pallial ciliary activity to obtain oxygen and by ceasing ventilation while remaining open to the absorption of sulphide that accumulates in the burrow in the absence of aeration (McMahon and Reid, 1984) (Fig. 3 (c)).

Lucinids burrow readily and, although lacking an inhalent siphon, maintain access to sediment-surface seawater through a mucus-lined inhalent tube constructed using the vermiform tip of the foot. They have a posterior exhalent siphon that can be extended out the posterior end of the shell and upward to the sediment surface. Lucinids lack a posterior inhalent siphon but do have a small inhalent aperture (Allen, 1958). The depth to which they burrow varies greatly and appears to be related to clam size (due to the length of the foot) (Allen, 1953) and type of substrate (P. Barnes, pers. obs.).

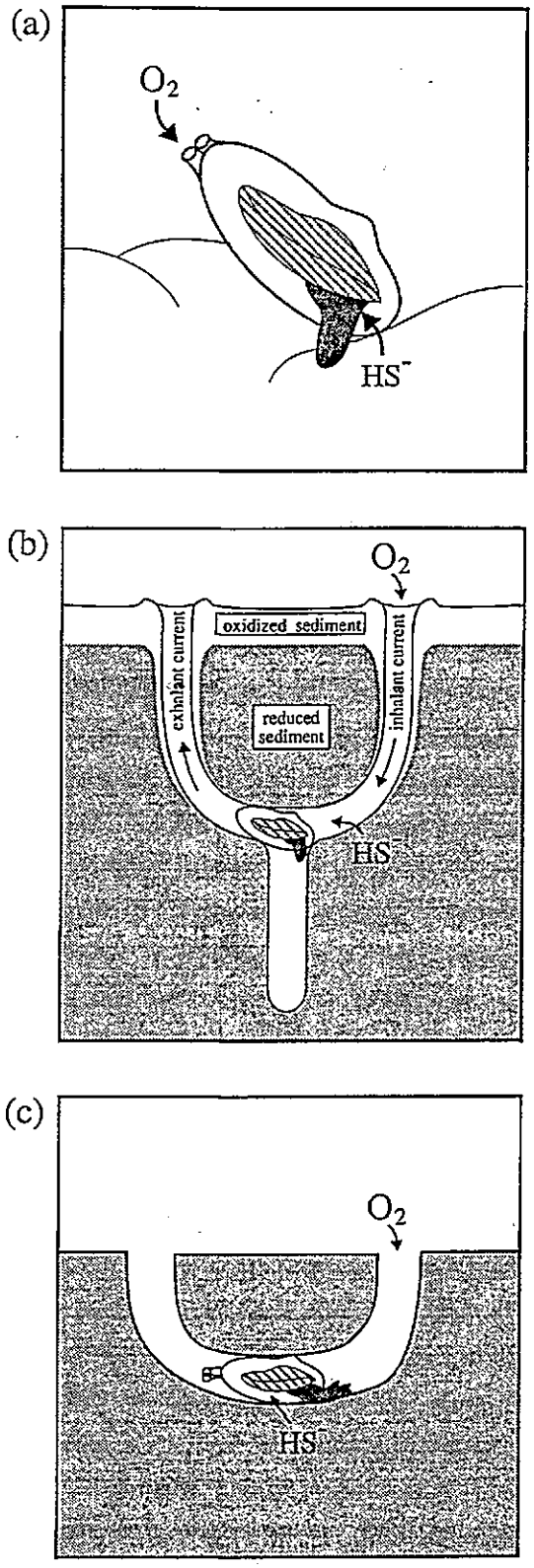

Figure 3. Distribution of bacteria-bivalve symbioses in relation to oxygen and dissolved sulphide. 3(a). Diagram of *Calyptogena magnifica*, the vent clam, located at the sulphide-oxygen interface. (Figure modified from Reid and Brand (1986)). 3(b). Drawing of *Solemya velum* Y shaped burrow. (Figure modified from Fisher (1990)). 3(c). Drawing of *Solemya reidi* U shaped burrow. (Figure modified from Reid and Brand (1986)).

Lucinids, found in habitats where dissolved sulphide accumulates, may obtain sulphide from the water entering the posterior inhalant aperture. In addition, cessation of ventilation would allow sulphide to accumulate and enter the mantle cavity through the anterior gape, unimpeded by an inhalant siphon (Reid, 1990). In *Parvilucina tenuisculpta* there is a posterior fusion of the gills with the mantle edge and it has been suggested that contraction of the mantle edge would create a bellows-like action with the gills, bringing sulphide from the external environment into the suprabranchial chamber and into contact with the bacteriocytes while limiting exposure of the ciliated frontal cells (Reid and Brand, 1986). The seagrass bed dweller, *Lucina floridana*, does not appear to construct long enough inhalant tubes to access the oxygen in sediment-surface seawater but typically is found close to the seagrass roots (Fisher and Hand, 1984). Since it has been suggested that oxygen is released from the roots of some marine angiosperms (Armstrong, 1970), the bivalves may be obtaining their oxygen through this route (Fisher and Hand, 1984). Although this requires further study, the fact that *L. floridana* was not found in areas devoid of seagrasses nor at sediment depths below the rhizosphere (20 cm) (Fisher and Hand, 1984) suggests an association with the seagrass sediments.

Unlike members of the bivalve family Solemyidae, which have only been reported from sulphide-rich habitats such as sewer outfall, pulp mill effluent and wood fibre bed sediments and seagrass beds (Reid, 1980; Felbeck, 1981; Cavanaugh, 1983;

Shepard, 1986), bivalves belonging to the family Lucinidae also occur in low-sulphide sediments (Allen, 1958; Dando *et al.*, 1985; Cary *et al.*, 1989). Low sulphide levels occur in sediments with low organic input or in sediments with high organic input where uptake of free-living bacteria may help to maintain low levels (Berner, 1985). In addition, high levels of dissolved iron available for forming iron sulphides will limit the levels of dissolved sulphide (Dando *et al.*, 1986a). The effect of sulphide utilization by the sulphide-oxidizing symbioses may also be a factor in maintaining low environmental levels. It has also been suggested that organically enriched sediments that are well-mixed by bioturbation can maintain a redox condition limiting the accumulation of sulphide (Cary *et al.*, 1989). Sulphate reduction measurements are required to help determine sulphide availability and the reason(s) for low sulphide in sediments.

The sulphide concentrations in the habitat of *Myrtea spinifera* were found to be <1.0 μM but since their gills contained SGES (Dando *et al.*, 1985), indicative of an active sulphur metabolism, the bivalves obviously had access to some form(s) of reduced sulphur. Allen (1958) noted that several lucinid species were found in "clean coral sands" and, more recently, two species of European lucinid (*M. spinifera* and *Lucinoma borealis*) and one species of North American lucinid (*Lucinoma aequizonata*) have been found in sediments with barely detectable levels of dissolved sulphide (<1 μM) and other reduced sulphur forms such as thiosulphate (<10 μM) in the interstitial water (Dando *et al.*, 1985, 1986; Cary *et al.*,

1989). It has been estimated that reduced sulphide concentration would need to be greater than 10 µM to support bivalve symbioses; at lower levels, the bivalves would have to pump an impossibly large volume of interstitial water to extract sufficient dissolved sulphide or thiosulphate to obtain a substantial part of their nutrition from the prokaryotes (Dando *et al.*, 1985). *M. spinifera* may obtain the required reduced sulphur either by raising sulphide production in its immediate vicinity through enrichment of the neighbouring sediment with a deposit of pseudofaeces or by using the oxygen in the inhalant tube to oxidize iron sulphides in the sediment around the tube (Dando *et al.*, 1985). This latter hypothesis has also been suggested for *L. borealis* (Dando *et al.*, 1986a). Pyrites may be oxidized to thiosulphate and tetrathionate in marine sediments (Goldhaber, 1983) and although the chemical oxidation product of ferrous sulphide under marine conditions is elemental sulphur (Nelson *et al.*, 1977), the oxidation mechanisms in the inlet tube are likely to be both chemical and microbial (Dando *et al.*, 1986a). When testing this hypothesis in the laboratory, both thiosulphate and sulphide were produced, probably arising from both chemical and bacterially-mediated reactions (Dando and O'Hara, 1990; Southward and Southward, 1990; Barnes, unpubl. data). This hypothesis is further supported by the presence of rust-coloured iron oxides adhering to the inhalant tube where sediments contain iron and by the observation that freshly collected individuals often have a rust-brown patch on the shell where the inhalant tube adheres (Dando *et al.*, 1985;

P. Barnes, pers. obs.). In the laboratory, these bivalves formed a new inhalant tube every few days (Dando *et al.*, 1985), suggesting the need to access new supplies of sediment-bound sulphides. This method of accessing reduced sulphur compounds has also been suggested for *Loripes lucinalis* and *L. borealis*. *L. lucinalis* is found at Portland Harbour (U.K.) in sand with undetectable levels of sulphide (Southward and Southward, 1990; Barnes, unpubl. data) but is more numerous in patches of sand where there are black accumulations of iron sulphide (Southward and Southward, 1990). Reid and Brand (1986) suggest an additional source of reduced sulphur may be sulphide released, as a result of low pH and anoxia in the gut, from sediments that are ingested into the digestive system. There is currently no evidence in support of this hypothesis, however.

Lucinoma borealis, found in low sulphide sediments and usually below the depth of maximum sulphate reduction, is often located close to decaying organic matter within the sediment (Dando *et al.*, 1986a). This suggests that the organisms may utilize more than one method for obtaining reduced sulphur, including accessing isolated pockets of sulphide when available. This latter method may also apply to the Bermudian lucinid, *Lucina radians* and the California species, *Lucinoma aequizonata*. *L. radians* occurs in low densities, with a patchy distribution, in clean coral sands (Schweimanns and Felbeck, 1985). Bermuda's carbonate sediment interstitial water is low in iron, probably due to lack of continental detrital input (Lyons *et al.*, 1979) and, as a

result, concentrations of sediment-bound sulphides are also likely to be low. It seems probable, therefore, that the patchy distribution of *L. radians* may be related to isolated pockets of organics (P. Barnes, pers. obs.). *L. aequizonata*, found at 500 m depth in the Santa Barbara Basin off California, had blood thiol levels that indicated exposure to significant amounts of sulphide and/or thiosulphate (Cary *et al.*, 1989). However, no sulphide accumulation occurred in the sediments immediately associated with the clam and the oxygen concentration in the overlying seawater was <20 μM (Cary *et al.*, 1989). The suggestions of Dando *et al.* (1985) regarding the biogenic remineralization of accumulated pseudofaeces and oxidation of metal sulphides to utilizable forms of reduced sulphur are, therefore, not applicable (Cary *et al.*, 1989). Sediments did contain randomly dispersed small pockets of black mud, possibly a result of the breakdown of dead organisms, with sulphide levels two orders of magnitude higher than the surrounding sediments (Cary *et al.*, 1989). It seems likely, therefore, that a method of obtaining reduced sulphur, similar to that hypothesized for *L. borealis* and *L. radians*, is utilized. It should be noted, however, that the sediment data in Cary *et al.* (1989) are based on only two cores, with different variables measured on each core.

The vermiform foot of the lucinids may be used in detecting these sulphide pockets and several species have been observed to form channels that penetrate well below their burrow (Stanley, 1970; Dando *et al.*, 1986b; Cary *et al.*, 1989; Southward, 1989) (Fig. 4). If the foot was capable of sulphide

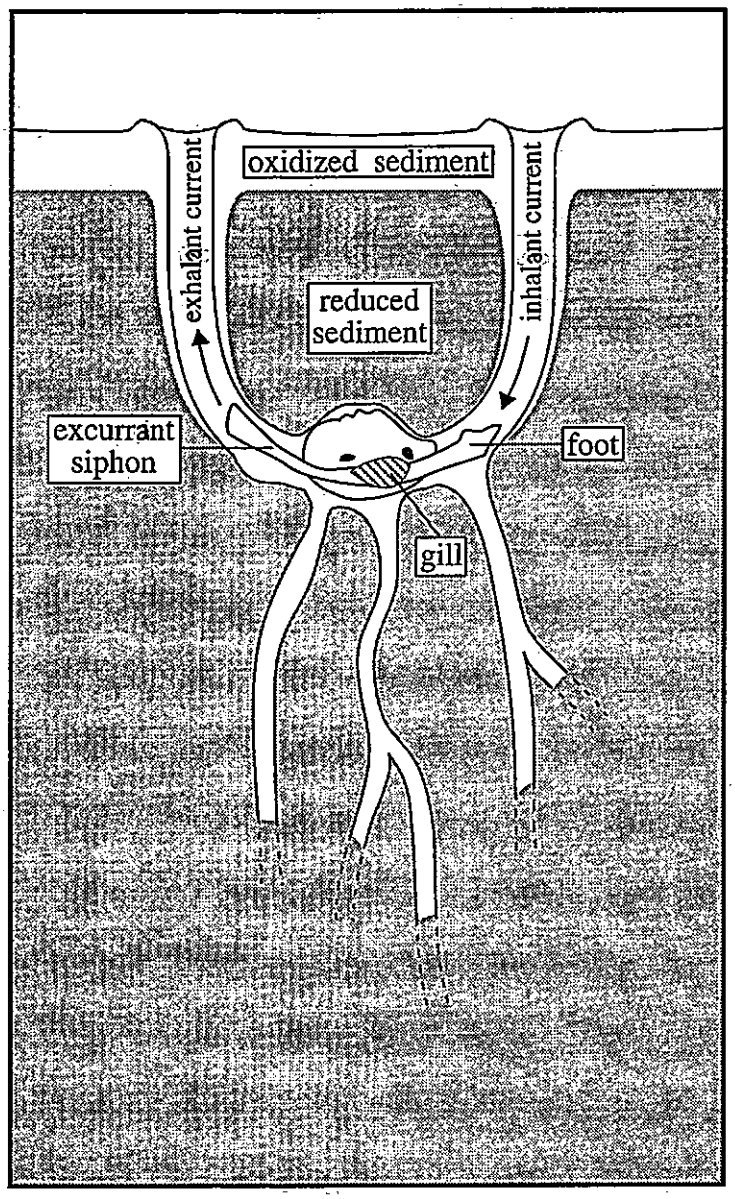

Figure 4. Drawing of lucinid bivalve burrow showing channels excavated using vermiform foot. (Figure modified from Fisher (1990) and Stanley (1970).

uptake, in addition to detection, the situation would be superficially similar to that described for *Calyptogena magnifica* (Arp *et al.*, 1984), except that the lucinids lack a sulphide-binding protein in the blood. Obviously, specific routes of acquisition of sulphide in the lucinids still need to be defined through experimentation (Fisher, 1990).

Discovery of sulphide-oxidizing symbioses in low-sulphide environments, which are widespread in the marine environment (Volkov and Rozanov, 1983), suggests this relationship may be more common than previously thought. Utilization of energy from sediment-bound sulphur would decrease dependency on dissolved sulphide, which could be ephemeral if organic input is seasonal or irregular (Dando *et al.*, 1986a). The ability to exploit low sulphide environments appears to be a result of behavioural adaptations designed to provide adequate reduced sulphur sources; all animals examined, regardless of environmental levels of reduced sulphur compounds, contained globular elemental sulphur in the gills indicative of an active sulphur metabolism. In addition, the same lucinid species can occur in both low and high sulphide sediments (Dando *et al.*, 1986a; Herry *et al.*, 1989), presumably utilizing different behavioural strategies in each habitat. The distribution of some sulphide-oxidizing symbioses in "high" sulphide environments (sewage outfalls, seagrass beds) may be due to the ready availability of sulphide, rather than the high concentrations. Behavioural adaptations, such as burrow formation and ventilation, are utilized to moderate the sulphide levels in the bivalves'

immediate environment (Reid, 1980; Reid and Brand, 1986). In addition, an interstitial water pH of 7.5-8.0 and an internal pH of 7.5 (Wilmot and Vetter, 1992) are likely to moderate the toxic effects of sulphide since, at these pH values, only 3-4% of the sulphide pool exists as the uncharged, freely diffusible H_2S form (Millero *et al.*, 1986).

Lucinids acquire oxygen from the water column by way of the anterior feeding tube (Allen, 1958). In some lucinid habitats, however, oxygen levels in both the sediment-surface seawater and the interstitial water are very low (Cary *et al.*, 1989). In these suboxic environments, it has been suggested that environmental nitrate may be useful as an alternate electron acceptor for the bacterial symbionts (Cary *et al.*, 1989). Nitrate respiration[2] occurs in many free-living, facultatively anaerobic bacteria (Knowles, 1982; Stewart, 1988; Tiedje, 1988). *Lucinoma aequizonata*, found in suboxic sediments, has symbionts that appear capable of nitrate respiration under both anoxic and oxic conditions and which have not yet been found to consume oxygen (Hentschel *et al.*, 1993). In low oxygen environments, available oxygen would be depleted by the host and it is unlikely much oxygen would diffuse into bacteriocytes. The obvious advantage to the symbionts respiring nitrate in low oxygen habitats is lack of competition with the host for oxygen. Nitrate levels in the habitat interstitial water of *L. aequizonata* decreased from

[2] Except where noted otherwise, "nitrate respiration" implies respiratory reduction of nitrate to nitrite.

approximately 30 µM at the sediment surface to <10 µM down to 25 cm (Cary *et al.*, 1989). Ammonia has typically been found in high concentrations (up to 200 µM) in lucinid habitats (Dando *et al.*, 1986b; Cary *et al.*, 1989). These high levels are not unusual for marine sediments (Henrichs and Farrington, 1980). Both ammonia and nitrate may serve as sources of nitrogen for this symbiosis, as suggested for *L. aequizonata* (Henstchel, unpubl. data) and discussed in section 1.4. Southward (1987) suggests that the symbionts may be able to recycle ammonia produced as waste by host animals in addition to utilizing the abundant ammonia in the sediments. Direct uptake of dissolved free amino acids may also be an important source of nitrogen in lucinids, as well as an alternative source of reduced carbon for lucinids in habitats where availability of reduced sulphur is unpredictable (Cary *et al.*, 1989). The uptake of dissolved organic compounds across the lateral epidermis has been demonstrated for apo-symbiotic bivalves (Wright *et al.*, 1984). The symbiont-containing *Solemya reidi* has been shown to be capable of moderate uptake rates of amino acids (Felbeck, 1983; Lee *et al.*, 1992). Levels of dissolved free amino acids reported for lucinid habitats vary from <15 µM (Dando *et al.*,1986b) to >200 µM (Cary *et al.*, 1989). Lower levels may not be representative of the total reserves, however, since most amino acids are probably adsorbed onto sediment particles (Christensen and Blackburn, 1980).

1.7. TRANSPORT, DETOXIFICATION AND METABOLISM OF REDUCED SULPHUR COMPOUNDS

Because sulphide is toxic to aerobic respiration and spontaneously oxidizes in the presence of oxygen, numerous questions arise when considering the complex physiology of the sulphur-oxidizing bacteria-lucinid symbioses. For example:

- which forms of reduced sulphur are utilized by the symbionts?
- how are reduced sulphur compounds transported to the bacteria and how is poisoning of host clam cells (if the compound is sulphide) avoided?
- do host cells have the ability to oxidize reduced sulphur and, if so, can they harness the energy released or is it a simple detoxification mechanism?

In the following section, possible mechanisms used in the detoxification of sulphide and the transport of reduced sulphur forms in bacteria-lucinid and other bacteria-bivalve symbioses are reviewed. In light of these mechanisms, the forms of reduced sulphur utilized by the intact symbioses and the bacterial symbionts are dicussed for the much-studied gutless protobranch bivalve, *Solemya reidi* (Solemyidae) and hypothesized for members of the family Lucinidae.

1.7.1. Detoxification of sulphide and transport of reduced sulphur forms

1.7.1.1. Behavioural Modifications

The role of behavioural modifications in regulating sulphide concentrations in the immediate environment of

lucinids found in high-sulphide sediments may be critical. Like *Solemya reidi*, the lucinids appear to find sulphide concentrations greater than 100 µM deleterious (demonstrated by reduced gill cilial activity) (Dando *et al.*, 1986a; Anderson *et al.*, 1987). For some species, the toxicity level may even be lower; *Lucinoma aequizonata* demonstrated elevated levels of sulphide in the blood only when animals were maintained in aquaria with 30 µM sulphide, suggesting that the sulphide-oxidizing mechanism could not cope with the elevated environmental sulphide, allowing sulphide to accumulate in the blood and causing stress to the animal (Cary *et al.*, 1989). Behavioural mechanisms may be required, therefore, to maintain sulphide in the vicinity of the bivalves at levels with which detoxification mechanisms can cope.

Data on interstitial sulphide levels indicate that sulphide may be present in toxic concentrations in some habitats (Childress and Lowell, 1982; Fisher and Hand, 1984), although sulphide in the immediate vicinity of the animals is difficult to measure. These bivalves all appear to have behavioural adaptations which serve to lower the sulphide levels in their immediate environment. *Solemya velum* lives in a Y-shaped burrow and moves between the sulphide-rich deeper portion of the burrow and the oxygenated upper arms, thus limiting its sulphide exposure (Doeller, 1984). *Solemya reidi* ventilates its burrow with oxygenated sediment-surface seawater, which would reduce sulphide levels to which the animal is exposed (Reid, 1980). Since carbon dioxide fixation ceases when whole bivalves are exposed to greater than 100 µM

sulphide (Anderson *et al.*, 1987), it must be assumed that the animal maintains sulphide levels below this. A similar ventilatory mechanism is suggested for lucinids found in high-sulphide sediments. These bivalves maintain contact with the oxygenated sediment-surface seawater by means of the inlet tube constructed using the foot (Allen, 1958; Reid and Brand, 1986). It is important to remember, when relating reported environmental sulphide levels to the physiological detoxification and transport mechanisms, that as a result of behavioural mechanisms, the sulphide concentrations to which the bivalve is immediately exposed are probably significantly lower than those in the interstitial water. If not for these behavioural adaptations, detoxification mechanisms would be relatively ineffective against such high levels of sulphide.

Lucinids found in low sulphide environments may search out isolated pockets of sulphide (Cary *et al.*, 1989) or, through inlet tube ventilation, oxidize sediment-bound sulphides to sulphide (or thiosulphate) (Dando *et al.*, 1985; Southward, 1990). These organisms, although not surrounded by sulphide-rich sediments, must also have physiological mechanisms for detoxification and transport of sulphide. The combination of behavioural and physiological mechanisms utilized by these organisms should be optimal for maintaining a constant internal environment.

1.7.1.2. Physiological Modifications

The cytochrome oxidases of animals from high-sulphide environments, including organisms participating in sulphur

oxidizing symbioses, are invariably as sensitive to sulphide (even at low μM range) as the cytochrome oxidase of animals from sulphide-free environments (Somero *et al.*, 19891; Hand and Somero, 1983; Powell and Somero, 1983, 1986). Therefore, methods of detoxifying sulphide and protecting aerobic respiration must be utilized in addition to, and sometimes in combination with, mechanisms for transporting reduced sulphur to locations where it can be utilized as an energy source.

In some organisms, sulphide is rapidly and efficiently bound to proteins in the blood (Arp and Childress, 1983; Childress *et al.*, 1984). The presence of sulphide-binding proteins, which have a sufficiently high affinity for sulphide to control the sulphide distribution within an animal's body, are ideal for symbiont-containing invertebrates. In fact, they are found only in symbiont-containing animals where the bacteria serve as a sink. They play an important role in protecting the aerobic respiratory systems of the animals from sulphide poisoning while transporting sulphide, in a chemically stable form, to the sites where energy exploitation takes place (Powell and Somero, 1986; Somero *et al.*, 1989). Such circulating proteins have been found in the phylum Vestimentifera (hemoglobin) and the bivalve family Vesicomyidae (plasma-borne protein) (Arp and Childress, 1983; Arp *et al.*, 1984, 1987). In the blood of the vesicomyid clam, *Calyptogena magnifica*, sulphide binds with a high molecular weight, nonheme serum protein which has a high affinity for sulphide and, therefore, protects cytochrome c oxidase from sulphide inhibition (Powell and Somero, 1986). The blood of *C.*

magnifica is able to concentrate sulphide above external levels (Arp *et al.*, 1984). In addition, the blood of *C. magnifica* has a high concentration of hemoglobin, localized within erythrocytes (Terwilliger, *et al.*, 1983; Arp *et al.*, 1984) which, unlike that of the vestimentiferan worm, *Riftia pachyptila*, does form sulphemoglobin in the presence of sulphide *in vitro* and is then unable to bind oxygen. Since sulphemoglobin is not found *in vivo*, however, it appears that the sulphide-binding protein of *C. magnifica* protects the hemoglobin (Arp *et al.*, 1984).

The exact composition of lucinid blood is apparantly unknown (Giere, 1985) and, apart from the observation that the central blood space contains hemocytes (Dando *et al.*, 1985; Herry and Le Pennec, 1987; Le Pennec *et al.*, 1988; Herry *et al.*, 1989), has been largely undescribed. Dando *et al.* (1985) found no evidence of hemoglobin-containing cells (erythrocytes) in the blood of *Myrtea spinifera* and there has been no evidence reported of hemoglobin in the blood of other lucinid species. Lucinid blood does not contain hemocyanin, which assures blood oxygen transport in *Solemya velum*, and there has been no evidence, to date, of a circulating non-heme serum protein similar to that found in *Calyptogena magnifica* (Arp *et al.*, 1984).

Cytoplasmic hemoglobins (myoglobins) occur at high concentrations in the gills of many bivalves containing bacterial symbionts (Dando *et al.*, 1985; Wittenberg, 1985). The presence of intracellular hemoglobin has been suggested for several lucinid species on the basis of the reddish colour

of the gills (Giere, 1985) and has been confirmed for *Codakia* (*Parvilucina* d'Orbigny) *costata* (Jackson, 1973), *Codakia orbicularis* (Jackson, 1973; Wittenberg, 1985), *Codakia orbiculata* (= *Ctena orbiculata* Montagu) (Jackson, 1973), *Parvilucina tenuisculpta* (Reid and Brand, 1986), *Myrtea spinifera* (Dando *et al.*, 1985), *Lucina pectinata* (= *Phacoides pectinatus* Gmelin) (Read, 1962; Wittenberg, 1985; Kraus and Wittenberg, 1990) and *Lucina floridana* (Wittenberg, 1985). The reported concentrations of hemoglobin range from 1.2 mM (L. *pectinata*) to 0.02 mM (*L. floridana*) per kg tissue (Witenberg, 1985). The lack of red in the gills may not, as suggested for *Lucinoma borealis* (Southward, 1986), be a reliable indicator of the absence of hemoglobin. It has been demonstrated that the lucinid gills change from the characteristic yellow-brown colour, indicative of an ample supply of reduced sulphur forms and globular elemental sulphur stores, to dark red when deprived of sulphide (Vetter, 1985). Hence, colour of the gills may be more a function of the amount of SGES reserves, in combination with pigment granules, than an interspecific difference in hemoglobin.

The oxygen-binding hemoglobin found in lucinids may act as a buffer, protecting the symbionts from excess oxygen which may interfere with their autotrophic processes (Dando *et al.*, 1985). Free-living sulphur-oxidizing bacteria are frequently microaerophilic (Jørgensen, 1982) and the absence of bacteria in the "intermediary" cells or the "transition zone" has been suggested to be the result of oxygen levels too high to allow the presence of symbionts in these cells (Herry *et al.*, 1989).

If the bacterial symbionts are capable of utilizing nitrate as an alternate electron acceptor, as demonstrated for *S. reidi* symbionts (Wilmot and Vetter, 1992; B. Javor, pers. comm.), then, by binding oxygen, the hemoglobin would also be protecting oxygen-sensitive nitrate reductase. The oxygen-binding hemoglobin in chemoautotrophic bivalve gills may be analagous to the leghemoglobin of the *Rhizobium*-legume root symbiosis, acting as an oxygen store but keeping the partial pressure of oxygen low (Wittenberg *et al.*, 1974), helping to control oxygen tension close to the bacteria (Dando *et al.*, 1985). Oxygen-binding hemoglobin would help avoid spontaneous oxidation of sulphide and may act as an oxygen store during periods of oxygen deprivation, as suggested for *M. spinifera* (Dando *et al.*, 1985). The suggestion that the hemoglobin may act to protect the animal cells from excessive depletion of oxygen when the bacteria are actively oxidizing sulphur compounds (Dando *et al.*, 1985) may not be relevant if the symbionts are able to utilize nitrate as an alternate electron acceptor (discussed later in this section). Wittenberg (1985) noted that cytoplasmic hemoglobin is always accompanied by non-hemoglobin iron (in a one-to-one molar equivalence) and suggested that this iron may play a significant, although currently unknown, role in tissue oxygen-transport.

Non-circulating, cytoplasmic hemoglobin which can bind to sulphide, forming ferric hemoglobin sulphide, has been described in the gill tissues of the solemyid bivalves, *Solemya reidi* and *Solemya velum* and the lucinid, *Lucina pectinata* (Doeller *et al.*, 1988; Kraus and Wittenberg, 1990).

These bivalves also have intracellular hemoglobin which binds to oxygen in the presence of sulphide (ie: does not form the physiologically nonfunctional sulphemoglobin) (Carrico *et al.*, 1978; Doeller *et al.*, 1988; Kraus and Wittenberg, 1990). The hemoglobin may act to transport both oxygen and sulphide across cells to the bacterial symbionts, a method of separating sulphide and oxygen *in situ* (Doeller *et al.*, 1988; Kraus and Wittenberg, 1990; D. Kraus, pers. comm.). Kraus and Wittenberg (1990) suggest that, in *L. pectinata*, hydrogen sulphide would be released if ferric hemoglobin sulphide accepts electrons in rapid reaction, liberating the ferrous protein and hydrogen sulphide. The hemoglobin-bound sulphide may also serve as a sulphide store when external sulphide is unavailable or may act to bind sulphide in excess of levels manageable by host oxidation mechanisms. *L. pectinata* is, to date, the only lucinid reported to have sulphide-binding hemoglobin. The hemoglobin of *Myrtea spinifera* has been studied and found to be heterogenous, with a quite high oxygen affinity but with no evidence of sulphide binding. Sulphide was readily bound, however, by two fractions of gill extract (Mr>100,000 and Mr = 29,000) (Dando *et al.*, 1985). The majority of lucinid species remain to be investigated for sulphide-binding hemoglobin.

In *Solemya reidi*, sulphide-reactive hemoglobin is found only in the "bacteria-harbouring domain", where it may aid the sulphide supply to the symbiont while the oxygen-reactive hemoglobins are widely distributed (Kraus and Wittenberg, 1990). The "bacteria-harbouring domain" probably includes the

intercalary cells since the authors distinguish this domain from the "mitochondria-rich ciliated domain" of the gill. Similar to observations on the gills of most lucinid species, the bacteriocytes of *S. reidi* are separated from the external medium by a thin layer of mitochondria-rich intercalary cells. It seems probable, therefore, that the bacteria may be supplied with some sulphide which is transported across the intercalary cells to the bacteriocytes by hemoglobin. Since the hemoglobin is highly localized, this lends support to the hypothesis that it may also act as a short-term sulphide store for the bacteria. Localization of specific hemoglobins within the gill tissues of lucinid bivalves is currently being investigated (D. Kraus, pers. comm.).

The mechanisms discussed above bind sulphide and prevent poisoning of host tissues, while also functioning as transport mechanisms. The location of the bacteria deep within the gill tissues precludes the bacteria playing a direct role in detoxification of sulphide (Distel and Felbeck, 1987) but preliminary enzymatic or non-enzymatic sulphide oxidation may occur in host tissues (Powell and Somero, 1986; Powell and Arp, 1989; Bagarinao, 1992). This would result in detoxification of sulphide and produce a partially oxidized form of reduced sulphur, such as thiosulphate, which might be available to the symbionts.

Sulphide oxidation can be catalyzed by metal ions, heme-associated iron and certain proteins and low molecular weight organic substances (Chen and Morris, 1972; National Research Council, 1989). Non-enzymatic sulphide oxidation has been

suggested for iron-containing pigment granules found in the bacteriocytes of several lucinid species (Allen, 1958; Jackson, 1973; Fisher and Hand, 1984) and has now been determined in electron-dense, brown granules found in the gills of *Solemya reidi* (termed "sulphide-oxidizing bodies"), *Calyptogena magnifica* and *Lucinoma annulata* (Powell and Somero, 1985; Powell and Arp, 1989). The granules in these three species were found to contain hematin but the sources of hematin and the exact composition of the hematin granules have yet to be determined (Powell and Arp, 1989). The ability of free heme compounds (hemoglobin derived hematins = oxidized hemes not associated with proteins) to catalyze sulphide oxidation has been known for many years (Keilin, 1933; Sorbo, 1960). With the exception of sediment-dwelling marine animals (Falk, 1964), however, free heme compounds are not normally present in animal tissues (Mangum and Dales, 1965). These hematin pigments, containing ferric iron, have also been found in the blood of sulphide-tolerant invertebrates and shown to catalyze sulphide oxidation (Patel and Spencer, 1963; Powell and Arp, 1989). In fact, the absence of sulphide-binding hemoglobin in the blood of marine invertebrates exposed to sulphide has been suggested to indicate that heme compounds, such as hematin granules, may be oxidizing sulphide (Vismann, 1991). In the lucinids, the hematin granules are located in bacteriocytes in the gills (Powell and Arp, 1989) and the hemoglobin is cytoplasmic with sulphide-insensitive oxygen-binding components and, in some cases, sulphide-binding

components (Kraus and Wittenberg, 1990; D. Kraus, pers. comm.).

In *Calyptogena magnifica*, *Solemya reidi* and *Lucinoma annulata*, sulphide oxidation activity was found to be proportional to the total heme content of gill homogenates (hematin >80% of total heme content), suggesting that the heme compounds may be a major component of the sulphide oxidation activity (Powell and Arp, 1989). It is critical to note, however, that experiments revealing oxidation of sulphide by these granules have generally been carried out at sulphide concentrations greatly in excess of realistic environmental levels (eg: 1 mM sulphide used by Powell and Arp, 1989). These high levels are physiologically unrealistic and are likely to be inhibitory for enzymatic oxidation (R. Vetter, pers. comm.). The "role" of these hematin pigments *in vivo* is, therefore, questionable. It has been demonstrated for *Solemya reidi* bacterial suspensions, which still contained pigment granules, that aerobic respiration rate is greater than the rate of oxygen consumption due to non-enzymatic oxidation (pigment granules) at sulphide concentrations <150 μM (peaking at approximately 20 μM). At sulphide concentrations greater than 150 μM, however, the oxygen consumption rate of the granules continued to increase linearly, while the aerobic respiration rate decreased and then levelled off (Wilmot and Vetter, 1992). Hence, granular sulphide oxidation may occur at lower sulphide concentrations but its "role" at these concentrations is questionable in light of bacterial and mitochondrial oxidation (see later in

text) at those concentrations. The possible role of hematin granules in oxidizing high sulphide concentrations down to levels that can be rapidly metabolized by other mechanisms, such as bacterial or mitochondrial oxidation, seems doubtful. The pigments alone are unlikely to have sufficient affinity for sulphide to prevent poisoning of cytochrome c oxidase by reducing sulphide to low nanomolar levels (Bagarinao, 1992). Also, the pigment granules are found in the bacteriocytes, eliminating any possible "peripheral defense" strategy for protecting the host cells or the bacteria from high sulphide levels. In addition, the granular shape, in comparison to a diffused distribution, would reduce their effectiveness in oxidizing sulphide (R. Vetter, pers. comm.).

Specific catalysts which oxidize sulphide and which are termed "sulphide oxidases" in the literature, are found in the tissues of symbiont-containing invertebrates (Powell and Somero, 1985). Benzyl viologen (B.V.) assays are usually employed for these determinations but in the absence of adequate controls, or if physiologically unrealistic sulphide concentrations are used, it is difficult to determine if the sulphide oxidation activity measured is the result of enzymatic or non-enzymatic oxidation (see section 1.3). "Sulphide oxidase" enzymes have yet to be purified and characterized and the products of sulphide oxidation catalyzed by these enzymes have yet to be identified. In the benzyl viologen assay, B.V. acts as the electron acceptor but the electron acceptor used in the absence of B.V., critical to maintaining the redox balance of the cell, has yet to be

determined. Since the assay must be carried out under anoxic conditions, it also does not measure mitochondrial oxidation. Regardless, however, evidence from these assays indicates sulphide can be oxidized in the outer few cell layers of tissues in contact with the environment, suggested as a "peripheral defense" strategy against sulphide toxicity (Somero *et al.*, 1989). Sulphide oxidation can also occur in internal organs if sulphide penetrates the body and enters circulation, as demonstrated for the hydrothermal vent crab, *Bythograea thermydron* (Vetter *et al.*, 1987). Once again, where assay conditions are non-physiological (eg: very high sulphide concentrations), sulphide oxidation measured is likely due to non-enzymatic activity. In *S. reidi*, however, it appears that at least some of the sulphide oxidation activity measured is enzymatic (Powell and Somero, 1986).

To date, "sulphide oxidase" activity has been demonstrated in several species of lucinid bivalve. None of the following studies, however, reported the results of boiled controls, the reappearance of the "enzyme" catalyst, the products of sulphide oxidation nor the sulphide concentrations used in the assay. If the latter are physiologically unrealistic, activity measured is most likely a result of non-enzymatic, possibly hematin/pigment granule, oxidation. The average basal activity of "sulphide oxidase" in non-symbiotic animal tissues is 0.25 units/gFW (Powell and Somero, 1985), although presumably this is dependent on the concentration of sulphide used. *Pseudomiltha sp.*, a lucinid found at a deep sea hydrocarbon seep, was found to have 2.03 units/gFW "sulphide

oxidase" activity in the gill tissues; this is significantly lower than activities reported for the gills of other symbiont-containing bivalves (Brooks *et al.*, 1987). In *Lucinoma aequizonata*, "sulphide oxidase" activity was found to be greater in the foot than in the mantle, although no activities or further information are given (J. O'Brien, pers. comm. in Cary *et al.*, 1989). These data were used in support of the hypothesis that the foot scavenges for sulphide. If this hypothesis is correct, then the products of the enzymatic, or non-enzymatic, sulphide oxidation must be a usable form of reduced sulphur such as thiosulphate. Sulphide oxidizing activity in *Lucinoma borealis* and *Loripes lucinalis* was found to be x2-3 greater in the gills than in the foot tissues (Southward, 1990). The higher activity in the gills may be due to the bacteria or to the pigment granules which have been associated with non-enzymatic oxidation and which have not been observed in the foot of lucinids. Foot activity could be a result of non-enzymatic activity not associated with pigment granules or due to enzyme activity not associated with mitochondria, although there is no direct evidence for either.

Sulphide oxidation by mitochondria appears to be a common method used by marine organisms to protect cytochrome c oxidase against sulphide toxicity (Bagarinao, 1992). The oxidation of sulphide by mitochondria may involve a specific sulphide oxidase enzyme or cytochrome c may act as an enzyme (Bagarinao, 1992). Mitochondrial sulphide oxidation has been shown to be linked to ATP synthesis in *Solemya reidi* (Powell

and Somero, 1986; O'Brien and Vetter, 1990). The same has now been demonstrated for the non-symbiotic but sulphide-tolerant teleost, *Fundulus parvipinnis* (Bagarinao and Vetter, 1990). In *Solemya reidi*, however, the ATP yield of mitochondrial sulphide oxidation was well below the potential yield, suggesting that this mechanism may be primarily for detoxification (O'Brien and Vetter, 1990). The mitochondrial sulphide oxidation system described by O'Brien and Vetter (1990) is the only system that seems to explain *S. reidi* physiological data. Whole animal respiration was strongly inhibited when sulphide levels exceeded 100 µM (Anderson *et al.*, 1987) and the bivalves were found to switch to anaerobic metabolism at sulphide concentrations equal to or higher than 250 µm, even when oxygen was present, indicating sulphide-inhibited aerobic respiration (Anderson *et al.*, 1990). This suggests that the sulphide detoxification mechanism does not function above this level and sulphide at concentrations below 100-250 µM must be metabolized rapidly to prevent inhibition of cytochrome c oxidase. "Sulphide oxidase" systems (Powell and Somero, 1985) and hematin oxidation (Powell and Arp, 1989) had K_m's greater than one millimolar and, unlike mitochondria, were not inhibited by high sulphide (O'Brien and Vetter, 1990). In addition, potential electron acceptors, required to maintain the redox balance of the cell, have yet to be identified for additional enzymatic oxidation.

Studies on the mitochondrial sulphide-oxidation systems in *Solemya reidi* and in sulphide-tolerant fish found that maximum activity rates were displayed at less than 50 µM

sulphide and that thiosulphate was the only product (Powell and Somero, 1986; Bagarinao and Vetter, 1990; O'Brien and Vetter, 1990). Thiosulphate is non-toxic and, unlike elemental sulphur, it is soluble (Bagarinao, 1992) and, therefore, a good detoxification product. Mitochondrial oxidation probably explains the accumulations of thiosulphate found in the blood of the symbiont-containing bivalve *S. reidi* (Anderson *et al.*, 1987) and in apo-symbiotic organisms exposed to sulphide (Vetter *et al.*, 1987; Bagarinao and Vetter, 1989). Thiosulphate accumulates in the blood of *S. reidi* only under oxic conditions. Further support that the accumulation of thiosulphate in the blood is indicative of host mitochondrial sulphide oxidation are the low levels of thiosulphate in most sediment interstitial water (Dando *et al.*, 1986a; Cary *et al.*, 1989; Wilmot and Vetter, 1992) combined with the observation that *S. reidi* did not accumulate thiosulphate in the blood above external concentrations (Wilmot and Vetter, 1992). In contrast, sulphide appeared to accumulate in the blood only when detoxification systems were "overloaded" and the animals were in distress (Vetter *et al.*, 1987; Bagarinao and Vetter, 1989; Cary *et al.*, 1989).

Mitochondrial oxidation of sulphide has not been studied for the lucinid bivalves but is suggested by the accumulation of thiosulphate in the blood of *Lucinoma aequizonata* incubated in the presence of 30 µM sulphide (Cary *et al.*, 1989). There is currently no evidence linking mitochondrial oxidation in lucinids to ATP production. ATP production by crude gill extracts of *Lucinoma borealis* in response to sulphide or

sulphite (100 μM) (Dando *et al.*, 1986a) is attributed to bacterial metabolism, in light of the tissue preparation methods. The foot and mantle tissues of *L. borealis* did not produce ATP when exposed to sulphide or sulphite (Dando *et al.*, 1985), indicative of the absence of bacterial symbionts. Once again, since the preparation method was not designed to isolate healthy mitochondria, this result cannot be considered suggestive of an absence of mitochondrial oxidation.

Obviously, the role of non-enzymatic versus enzymatic sulphide oxidation in host tissues (the latter linked, possibly, to ATP synthesis) and utilization of the products of both these processes, needs further research in all bacteria-bivalve symbioses. Whole animal toxicity experiments may be required to elucidate the roles of mitochondrial and non-enzymatic sulphide oxidation. For example, in both *Solemya reidi* and some teleosts, the toxicity level of sulphide for whole animals is the same as for mitochondria, rather than for the pigment granules (Powell and Somero, 1985; Anderson *et al.*, 1987; Bagarinao and Vetter, 1989; Bagarinao, 1992; Wilmot and Vetter, 1992). If whole animals are poisoned at higher sulphide levels than poison mitochondria, then non-mitochondrial sulphide oxidation systems may play a role. While the products of non-enzymatic sulphide oxidation in marine invertebrates are likely to include thiosulphate, in addition to other products such as polysulphides (Vetter *et al.*, 1987; Vismann, 1991), it has yet to be determined if

these products can be further utilized by the bacterial symbionts.

1.7.2. Forms of reduced sulphur used as energy sources

When interpreting the results of experiments designed to identify the sulphur compound(s) used as energy sources by the symbiosis, the biological preparation used (intact symbiosis (whole clam), isolated gills, gill homogenates, isolated bacteriocytes, isolated host cell mitochondria or isolated symbionts) must be carefully considered. For example, if the experimental preparation used consists of isolated bacteriocytes, then ATP synthesis measured in response to sulphide could reflect some mitochondrial, in addition to bacterial, processing (Hand, 1987). In the vent clam, *Calyptogena magnifica*, sulphide-binding proteins present in the blood suggest that sulphide can be delivered to the bacterial symbionts with minimal toxic effects to host cells (Arp and Childress, 1983; Arp *et al.*, 1984) but studies on the specific sulphur substrates used by the bacteria are conflicting (Powell and Somero, 1986; Hand, 1987), probably due to the tissue preparations used. Since the nature of the experimental preparation may determine the systems' response to reduced sulphur compounds, use of the intact symbiosis may be required (as discussed for studies on enzymatic versus non-enzymatic sulphide oxidation) to obtain a realistic picture of the sulphur metabolism systems (Somero *et al.*, 1989). In addition, concentrations of reduced sulphur compounds must be

physiologically realistic (Somero *et al.*, 1989; Bagarinao, 1992).

Since the processes involved in the utilization of sulphur forms can be complex, incorporating the transport and detoxification mechanisms discussed earlier, the following text first summarizes the processes used by *Solemya reidi*, then the potential processes used by the family Lucinidae.

1.7.2.1. *Solemya reidi*

Thiosulphate accumulation in the blood of *Solemya reidi* exposed to sulphide and oxygen (Anderson *et al.*, 1987) suggests both preliminary oxidation by the host (probably host cell mitochondria (Powell and Somero, 1986)) and the low ability of thiosulphate to cross membranes (Holmes and Donaldson, 1969). However, the thiosulphate in blood of freshly collected *S. reidi* quickly disappeared when bivalves were maintained in sulphide-free oxygenated seawater (Wilmot and Vetter, 1992). Combined with the nearly constant concentrations of thiosulphate which occurred in the blood of *S. reidi* in the presence of continually flowing sulphide, this suggests the continual removal of thiosulphate as an energy source for the symbionts (Anderson *et al.*, 1987) and infers the presence of a good active transport mechanism for thiosulphate from the blood to the bacteriocytes (R. Vetter, pers. comm.). In addition to host mitochondrial sulphide oxidation, resulting in thiosulphate in the blood, it is possible that this process occurs in the intercalary and bacteriocyte cells of the gills. The intercalary cells are

rich in mitochondria (Felbeck, 1983; Gustafson and Reid, 1988) which could oxidize sulphide diffusing into these cells. Thiosulphate would then be transported to the bacteria utilizing an active transport mechanism, similar to that suggested for transporting thiosulphate from the blood. Because mitochondrial oxidation in *S. reidi* results in ATP production, the benefits of host oxidation of sulphide are thus two-fold: sulphide is oxidized to a non-toxic form, such as thiosulphate, which still contains energy and can be supplied to the bacterial symbionts and there are energetic benefits for the host. Figure 5 is a diagrammatic representation of the whole animal sulphide metabolism model in *S. reidi*, as summarized by Anderson *et al.* (1987).

While the above hypothesis does not exclude the possibility that the symbionts may also oxidize sulphide, it has been suggested, due to the lack of "sulphide oxidase" activity demonstrated using the B.V. assay, that the bacterial symbionts of *Solemya reidi* do not oxidize sulphide (Powell and Somero, 1985). The cautions inherent in interpreting the results of the benzyl viologen assay, discussed earlier in this text, must be applied (Powell and Somero, 1985). Wilmot and Vetter (1992) addressed this issue and noted that the B.V. assay probably did not measure electron transport chain-linked activity because the high sulphide concentrations used inhibited oxidation. In addition, these studies were conducted on gill tissue homogenates and pigment granules (sulphide-oxidizing bodies) were undoubtedly present in the homogenate. The pigment granules contain hematin and the B.V.

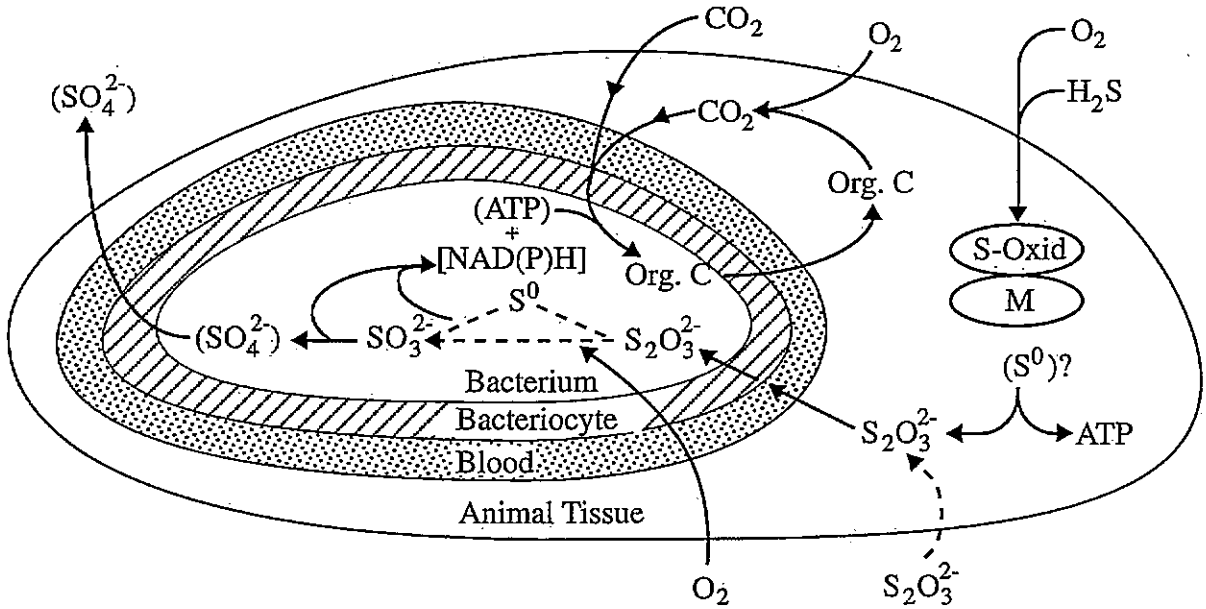

Figure 5. Diagram of the whole animal model for sulphide metabolism in *Solemya reidi* as suggested by Anderson *et al.* (1987), based upon the results of Anderson *et al.* (1987), Felbeck (1983), Fisher and Childress (1986) and Powell and Somero (1985, 1986). Org. C = organic carbon; M = mitochondrion; S-oxid. = sulphur oxidation. (Figure from Anderson *et al.* (1987)).

assay does measure non-enzymatic ferric iron catalysis in the presence of high sulphide (Powell and Arp, 1989). It has recently been demonstrated that the isolated bacteria are capable of sulphide oxidation at sulphide concentrations <150 µM; at sulphide incubation concentrations >150 µM, however, non-enzymatic oxidation (by pigment granules) far exceeded bacterial respiratory oxidation, which was inhibited (Wilmot and Vetter, 1992). In addition, $^{14}CO_2$ fixation in bacteria isolated from *S. reidi* was stimulated by both thiosulphate (1 mM) and sulphide (500 µM) (B. Javor, pers. comm.). Further evidence that the bacterial symbionts may utilize sulphide is provided by the presence of intracellular sulphide-binding hemoglobin in the "bacteria-harbouring domain" of the gills of *S. reidi* (D. Kraus, pers. comm.), suggesting the bacteria may be provided with sulphide as an energy source. The presence of sulphide-binding molecules in the absence of a "sink", such as the bacteria, would be a physiological disadvantage (Bagarinao, 1992). In light of available evidence, it must be assumed that the *S. reidi* bacteria are capable of utilizing, and most likely have access to, both thiosulphate and sulphide. Hence, the whole animal model put forward by Anderson *et al.* (1987) must be modified to include sulphide oxidation by the endosymbionts.

It is important to note that results of experiments utilizing the intact symbiosis or isolated ctenidia can be ambiguous in terms of defining the utilization pathway of sulphur forms. For example, net carbon dioxide uptake has been demonstrated for the *Solemya reidi* intact symbiosis in

the presence of sulphide (<250 μM) or thiosulphate (>250 μM) (Anderson *et al.*, 1987) and in the excised gills of *Solemya velum*, both sulphide (0.2 mM) and thiosulphate (1.0 mM) stimulated carbon dioxide fixation (Cavanaugh, 1981). These data suggest both forms of reduced sulphur can be used to fuel the symbioses but do not reveal if, when the gill or intact symbiosis is supplied with sulphide, the bacteria are utilizing sulphide directly or the oxidation product, thiosulphate. Other evidence which has been put forward as suggestive of the form of sulphur utilized by the bacteria must be examined closely. Isolated gill ctenidia of *S. reidi* (ie: no host blood supply) exposed to oxygen and sulphide accumulated globular elemental sulphur and produced protein. It was suggested that this indicates sulphide and oxygen enter the bacteriocyte directly from seawater (Vetter, 1990) but it is also possible that sulphide may first be oxidized to thiosulphate in the intercalary cells. In addition, the argument of Wilmot and Vetter (1992), that the location of the bacteria within the gill can be used as evidence supporting the oxidation of sulphide by the bacteria, must be questioned. They report that the bacteriocytes are separated from seawater by a thin epithelial cell with few mitochondria and that the bacteria are not packed close to the blood space which contains the mitochondrial product of sulphide oxidation, thiosulphate. In other reports, the intercalary (epithelial) cells contain numerous mitochondria (Felbeck, 1983; Gustafson and Reid, 1988), so oxidation of sulphide by host cells is feasible, and removal of thiosulphate from the blood to fuel

the bacteria (see evidence presented earlier) definitely seems to be occurring.

Stored globular elemental sulphur may be utilized as an energy store by the bacterial symbionts when environmental levels of reduced sulphur are low (Vetter, 1985). This has been observed for free-living sulphur bacteria (Jørgensen, 1982; Nelson *et al.*, 1986). Globular elemental sulphur is deposited by the bacterial symbionts and, for *Solemya reidi*, incomplete oxidation of sulphide due to oxygen limitation may be a factor in its accumulation (Anderson *et al.*, 1987). Average elemental sulphur stores in *S. reidi* may be insignificant, however, to the sulphur and carbon flux maintained by an active clam but might act as a short-term store for the symbionts (Anderson *et al.*, 1987).

When oxidizing reduced sulphur forms, the bacterial symbionts of *Solemya reidi* may use nitrate and nitrite, in addition to oxygen, as electron acceptors. Whole animal experiments on *S. reidi*, conducted at low oxygen levels or at sulphide concentrations above 250 μM, demonstrated a loss of net autotrophy and suggested that the bacterial metabolism was inhibited (Anderson *et al.*, 1987). However, observed inhibition at low oxygen levels may be due to lack of an alternate electron-acceptor. Recent research has demonstrated that the symbionts of *S. reidi* (isolated and *in vivo*) were able to utilize alternate electron acceptors (nitrate, nitrite) in the oxidation of reduced sulphur compounds (Wilmot and Vetter, 1992; B. Javor, pers. comm.). Wilmot and Vetter (1992) noted that thiosulphate accumulation in the blood was

the result of aerobic sulphide oxidation by the animals but that the intact symbiosis exhibited nitrate and nitrite respiration under anoxic, and nitrate respiration under oxic, conditions. Sulphide (150 µM) and thiosulphate (250 µM) may be utilized by isolated symbionts, if the bacteria are maintained anoxically or under low oxygen conditions, in the presence of an alternate electron acceptor (Wilmot and Vetter, 1992).

1.7.2.2. Family Lucinidae

In lucinid habitats, sulphide is the most likely reduced sulphur form present in the interstitial water. Even in the low-sulphide habitats described for some lucinids, sulphide is available through location of isolated pockets of sulphide (Cary *et al.*, 1989) or through oxidation of sediment-bound sulphides by ventilation (Dando and O'Hara, 1990; Southward and Southward, 1990). Mechanisms for oxidation of sulphide to thiosulphate, similar to those described for *Solemya reidi*, may be present in the lucinid bivalves. Accumulation of thiosulphate in the blood of *Lucinoma aequizonata*, incubated in the presence of 30 µM sulphide (Cary *et al.*, 1989), suggests the host partially oxidizes sulphide to thiosulphate. In lucinid habitats studied to date, thiosulphate was present in only very low levels in the interstitial water (Dando *et al.*, 1986a; Cary *et al.*, 1989) so adequate uptake from the environment is unlikely. "Sulphide oxidase" activity, discussed at length earlier in the text, indicated sulphide oxidation in the peripheral tissue layers of the foot, gill

and mantle of some species (Cary *et al.*, 1989; Southward and Southward, 1990). In *L. aequizonata*, it has been suggested that the foot seeks pockets of sulphide which, based on "sulphide oxidase" activity in foot tissues, may be oxidized to thiosulphate and transported in the blood (Cary *et al.*, 1989). Whether enzymatic or non-enzymatic, it is possible that this process may yield thiosulphate which could be made available to the symbionts. There is currently no direct evidence for the products of these processes, however. Although host oxidation is attributed to "sulphide oxidase" activity (Cary *et al.*, 1989), it seems probable that sulphide may also be oxidized by host cell mitochondria and that this process produces the thiosulphate accumulation found in the blood. Host cell mitochondrial oxidation has been demonstrated for *S. reidi* and seems applicable, although has yet to be demonstrated, for the lucinids. This process not only detoxifies sulphide entering the mantle cavity, but the non-toxic and soluble product, thiosulphate, could be transported in the blood. The lucinid mantle is highly vascularized, so transport of thiosulphate in the blood would be facilitated. In lucinid bivalves, the basal portion of the bacteriocytes are in contact with the ctenidial blood sinus (Dando *et al.*, 1986a; Reid and Brand, 1986; Distel and Felbeck, 1987; Southward, 1987). Thiosulphate carried in the blood would, presumably, be transferred to the bacteriocytes using an active transport system. It has been suggested that thiosulphate in the blood of *L. aequizonata* is utilized by

bacteria for metabolic energy and for the production of intracellular globular elemental sulphur (Cary *et al.*, 1989).

The ciliated cells and the intercalary cells of the lucinid gill are, like those of *Solemya reidi*, mitochondria-rich, suggesting that mitochondrial oxidation of sulphide by these cells would be a viable method of sulphide detoxification. Thiosulphate produced in this way would be transported across the cells to the bacteria, presumably using an active transport mechanism similar to that suggested for transfer from the blood. Mitochondrial oxidation of sulphide in the intercalary cells or bacteriocytes may be the primary method used by *Parvilucina tenuisculpta*, where the "bellows action" of the posterior inhalant opening would bring sulphide-rich water directly into contact with the inner sides of the gill lamellae, avoiding the surface ciliated cells (Reid and Brand, 1986). Host cell mitochondrial oxidation of sulphide may explain the arrangement of the intercalary cells, which contain numerous mitochondria (Fisher and Hand, 1984; Dando *et al.*, 1985; Giere, 1985; Southward, 1986), in relation to the bacteriocytes (Reid, 1990). The mantle gills, present in many lucinids and considered to serve a respiratory function (Allen, 1958), have yet to be examined for a role in sulphide uptake or for detoxification mechanisms, although the latter has been suggested by Reid and Brand (1986).

The lucinids do not have a circulating sulphide-binding protein and it has yet to be determined if sulphide is directly utilized by the bacterial symbionts. Low levels of sulphide were found in the blood of *Lucinoma aequizonata*

exposed to elevated external levels of sulphide. Rather than suggesting a transport mechanism, however, this is probably a result of external sulphide levels elevated above the oxidizing capacity of host tissues (Cary *et al.*, 1989). The presence of sulphide-binding cytoplasmic hemoglobin in the gill tissues of *Lucina pectinata* (Kraus and Wittenberg, 1990) is indicative, however, that sulphide enters the gill cells and that not all of the sulphide diffusing into the cells is immediately oxidized to thiosulphate. This hemoglobin, as suggested for *Solemya reidi*, may act as a sulphide store when environmental sulphide levels are low and it may also serve to transport the sulphide across the intercalary cells and within the bacteriocyte to the symbionts. The bacteria tend to be located towards the exterior part of the bacteriocyte, near the bacteriocyte channels and the inhalant water flow and, therefore, near the sulphide source. In those lucinid species where the surface of the bacteriocytes are not covered by extensions of intercalary cells (Giere, 1985), much more sulphide would be likely to reach the bacteria.

The bacterial symbionts of the lucinids, theoretically, have access to thiosulphate (from the blood and possibly across the intercalary cells) and sulphide (across the gill and bound to hemoglobin in some species). Direct evidence for which form of sulphur is utilized or preferred by the symbionts is currently lacking, however. As discussed for *Solemya reidi*, results of experiments determining sulphur forms used by the intact symbiosis or isolated ctenidia can be ambiguous in terms of defining the sulphur forms utilized by

the symbionts. Carbon fixation in whole gills of *Myrtea spinifera* was stimulated by 20-30 µM free sulphide (Dando *et al.*, 1985) but this may have been due to the symbionts oxidizing sulphide or thiosulphate, since the latter is the oxidation product of the host cell mitochondria in the gills (or other oxidation processes). Crude extracts of gill tissues of *Lucinoma borealis* phosphorylated ADP on the addition of sulphite or sulphide (100 µM) but there was no significant ATP production when sodium thiosulphate (up to 1 mM) was used (Dando *et al.*, 1986a). The ATP measured was attributed to bacterial metabolism but unless the extract is completely free of pigment granules or host cell mitochondria, the possibility that sulphide was oxidized to thiosulphate prior to bacterial oxidation, cannot be eliminated. The apparent inability of *L. borealis* symbionts to utilize thiosulphate (up to 1 mM) (Dando *et al.*, 1986a) is unique among the lucinids (Cary *et al.*, 1989; Hentschel *et al.*, 1993) and solemyids (Powell and Somero, 1986; Anderson *et al.*, 1987; Wilmot and Vetter, 1992). In isolated gill tissues of starved and fed *L. borealis*, 100 µM sulphide enhanced bicarbonate fixation and without sulphide, the fixation rate was identical to that found in the gill of an aposymbiotic bivalve (Dando *et al.*, 1986a). The symbionts may be stimulated by sulphide directly or by thiosulphate produced through host cell oxidation. Although sulphide resulted in less stimulation of CO_2 fixation in the gills of fed animals, the total fixation rate was equal to or greater than that by starved gills in the presence of

sulphide, indicating that either the starved gills were in poorer condition or the fed gills were utilizing an alternate energy source, such as SGES.

The presence of SGES in the symbiotic bacteria has been demonstrated in numerous species of lucinid bivalve (Dando *et al.*, 1985, 1986a; Vetter, 1985; Dando and Southward, 1986; Brooks *et al.*, 1987) and appears to be utilized as an energy store in the absence of external sulphide (Vetter, 1985). Cary *et al.* (1989) noted an increase in SGES accumulation in the gills of *Lucinoma aequizonata* in response to increasing external sulphide levels in whole clam incubations, despite the fact that these sulphide levels were deleterious to the bivalves. The bivalves demonstrated decreased growth rates and higher mortality, suggesting the sulphide regulatory mechanisms of the host were overloaded. In light of the randomness and transitory nature of sulphide in the habitat of *L. aequizonata*, Cary *et al.* (1989) suggest the bacterial symbionts use SGES reserves when thiosulphate in the blood is depleted and sulphide is not available.

Similar to *Solemya reidi*, lucinid bivalves may be able to oxidize reduced sulphur forms by utilizing electron acceptors other than oxygen. The structure of the lucinid gill suggests that the surface ciliated cells scavenge oxygen, so that the water passing through the bacteriocyte channels has had a large portion of the oxygen removed (Fisher and Hand, 1984; Distel and Felbeck, 1987). In addition, the gills contain hemoglobin with a high oxygen affinity (Dando *et al.*, 1985; Kraus and Wittenberg, 1990) which may keep partial pressures

of oxygen low (Dando *et al.*, 1985). Low levels of oxygen in the vicinity of the bacteriocytes would be beneficial to chemoautotrophy (Chollet, 1977; Dando et al., 1985), as discussed earlier. These observations, combined with data on the low levels of oxygen in some lucinid habitats, suggest the use of an alternate electron acceptor by the bacteria. Although low in oxygen (<20 μM), the environment of *Lucinoma aequizonata* had 30 μM nitrate present in the sediment-surface seawater, suggesting nitrate as an alternate electron acceptor for the symbionts (Cary *et al.*, 1989). Hentschel *et al.* (1993) was unable to demonstrate oxygen consumption in the symbionts of *L. aequizonata* but determined the presence of respiratory nitrate reductase. Nitrate reductase is oxygen-sensitive and the low oxygen habitat, structure of the gill and oxygen-binding hemoglobin would serve to protect the enzyme from oxygen poisoning. Both isolated gills and isolated bacteria were shown to reduce nitrate to nitrite in the presence of 100 μM thiosulphate, at high rates anoxically and at lower rates under suboxic and oxic conditions. These experiments were not repeated with sulphide.

1.8. RESEARCH OBJECTIVES

Although shallow-water species of lucinid bivalves are commonly found in seagrass bed sediments (Allen, 1958), very little data are available on this habitat in regard to resources available for sulphur-oxidizing symbioses. In Bermuda, *Codakia orbiculata* is found only in shallow-water *Thalassia testudinum* sediments, where it is the most common infaunal species (P. Barnes, pers. obs.). The availability of reduced sulphur forms, nutrients and levels of other variables which may be critical to sulphur-oxidizing symbioses needs to be examined. Knowledge of habitat levels of reduced sulphur and alternate electron acceptors, such as nitrate, are critical to the interpretation of experimental data on respiration and sulphur metabolism in these organisms. In addition, variability within the *Thalassia testudinum* sediment habitat in Bermuda may present the lucinids with physiologically challenging conditions which need to be explored. Research presented in Chapter 2 of this dissertation defines habitat characteristics of the lucinid bivalve *C. orbiculata* and examines both spatial and temporal heterogeneity of critical variables within the habitat. Results of tissue analyses on *C. orbiculata* collected from the habitat (globular elemental sulphur content in the gills, thiosulphate and sulphide in mantle fluid) are related to habitat conditions.

The preferred form of reduced sulphur utilized by the intact symbioses and by the bacterial symbionts in lucinid bivalves is, as evidenced in this review, unknown. Hypotheses

regarding detoxification methods, sulphide transfer and forms of sulphur utilized are based largely on circumstantial evidence and by inference from other, more well-studied, bacteria-bivalve symbioses. Research presented in Chapter 3 of this dissertation was designed to address the following questions on sulphide and thiosulphate metabolism in *Codakia orbiculata*: 1) Can the intact symbiosis survive, using elemental sulphur stores, in the absence of an external source of reduced sulphur? 2) Can the intact symbiosis and the isolated bacterial symbionts utilize both thiosulphate and sulphide?; 3) Can the isolated bacterial symbionts use nitrate and nitrite as electron acceptors; 4) Can the bacterial symbionts use nitrate and nitrite as electron acceptors in vivo with the host under both oxic and anoxic conditions? In Chapter 4, the results of the physiological studies are summarized and discussed in light of habitat characteristics to provide insight into the functioning of these sulphur-oxidizing symbioses *in situ*.

CHAPTER 2

HABITAT CHARACTERISTICS OF THE LUCINID BIVALVE, *CODAKIA ORBICULATA*

2.1. INTRODUCTION

Bacteria-bivalve symbioses dependent on forms of reduced sulphur are found in a wide variety of habitats in most oceans (Reid, 1980; Felbeck *et al.*, 1981; Cavanaugh, 1983; Paull *et al.*, 1984; Brooks *et al.*, 1987). Lucinid bivalves have been found in habitats ranging from deep-sea hydrocarbon seeps to shallow-water sewage outfalls, mangrove sediments and seagrass bed sediments (Fisher and Hand, 1984; Schweimanns and Felbeck, 1985; Herry *et al.*, 1989). These habitats are often sulphide-rich as a result of geothermally- or biologically-produced sulphide.

Lucinids are found also in sediments where dissolved sulphide levels in interstitial waters are low or undetectable (Allen, 1958; Dando *et al.*, 1985; Cary *et al.*, 1989). *Myrtea spinifera*, *Lucinoma borealis*, *Loripes lucinalis* and *Lucinoma aequizonata* have been reported from sediments where dissolved sulphide concentrations in the interstitial waters were <1 µM and levels of other reduced sulphur forms, such as thiosulphate, were <10 µM (Dando *et al.*, 1985, 1986a; Cary *et al.*, 1989; Dando and O'Hara, 1990). The gill tissues of these bivalves were found to contain elemental sulphur, however, suggesting that the endosymbiotic bacteria had access to some form(s) of reduced sulphur (Dando *et al.*, 1985). It has been suggested that iron sulphides in the sediment bordering the the bivalves' inhalant tubes become oxidized and that partial oxidation products may be available as energy sources for the endosymbiotic bacteria (Dando *et al.*, 1985; Dando *et al.*, 1986a). It has been demonstrated, in the laboratory, that

oxygen pumped through a microporous tube embedded in habitat sediment of *L. lucinalis* results in the production of both thiosulphate and sulphide, probably through chemical and bacterially-mediated reactions (Dando and O'Hara, 1990; Southward and Southward, 1990; Barnes, unpubl. data). In addition, it has been hypothesized that some lucinids found in low sulphide sediments may access isolated pockets of sulphide (Schweimanns and Felbeck, 1985; Cary *et al.*, 1989). One species may not be limited to one type of habitat: *L. lucinalis* has been found in both high-sulphide reducing sediments near a sewage outfall in France and in sulphide-free sandy sediments in Portland Harbour, U.K. (Herry *et al.*, 1989; P. Barnes, pers. obs.).

In most lucinid habitats, even when dissolved sulphide levels are low, oxygen levels in the interstitial water are very low (Dando *et al.*, 1986b; Cary *et al.*, 1989). Lucinids require oxygen for respiration and for host sulphide detoxification, as well as sulphide oxidation by the bacterial endosymbionts. The bivalves are able to access oxygen in sediment-surface seawater by way of a mucus-lined inhalant tube constructed using the vermiform tip of the foot (Allen, 1958). The depth to which they burrow varies greatly and appears to be related to clam size (due to the length of the foot and exhalant siphon) (Allen, 1953) and type of substrate (P. Barnes, pers. obs.). It has been suggested that the seagrass bed dweller, *Lucina floridana*, does not construct inhalant tubes which are long enough to access oxygen in sediment-surface seawater but typically is found close to

seagrass roots (Fisher and Hand, 1984). Oxygen, produced photosynthetically, is released from the roots of some marine angiosperms (Armstrong, 1978) and it has been hypothesized that the bivalves may obtain oxygen through this route (Fisher and Hand, 1984).

In environments where both interstitial water and sediment-surface seawater are low in oxygen, nitrate may be utilized by the bacterial symbionts as an alternate electron acceptor (Cary *et al.*, 1989). Nitrate respiration occurs in many free-living, facultatively anaerobic bacteria (Knowles, 1982; Stewart, 1988; Tiedje, 1988) and the endosymbiotic bacteria of *Lucinoma aequizonata* (Hentschel *et al.*, 1993). *Codakia orbiculata* bacterial symbionts are capable of both nitrate and nitrite respiration *in vivo* (Chapter 3). The obvious advantage of nitrate respiration in bacteria-bivalve symbioses is the lack of competition for oxygen with the host clam when external oxygen levels are low. It is also suggested that, even in high oxygen environments, the endosymbionts may be maintained in low oxygen conditions *in vivo* to facilitate chemoautotrophic processes (Hand and Somero, 1984; Dando et al., 1985; Distel and Felbeck, 1987). Hence, nitrate may be an important electron acceptor even when oxygen is not limiting. Nitrate levels in the habitat interstitial water of *L. aequizonata* were 30 µM at the sediment surface and 10 µM at 25 cm sediment depth (Cary *et al.*, 1989). Nitrate availability in other lucinid habitats is unknown.

Investigation of the environments in which bacteria-bivalve symbioses are found is vital in determination of the

energy sources available to the intact symbioses *in situ*. Accurate habitat data on availability of electron acceptors other than oxygen, in conjunction with laboratory respiration experiments, are required to determine the potential for anaerobic respiration in the symbioses *in situ*. The range of habitats in which lucinids are found implies a variety of strategies for obtaining and utilizing reduced sulphur and of detoxification mechanisms. With the exception of the European studies on low sulphide lucinid habitats (Dando *et al.*, 1985; Dando and Southward, 1986; Dando *et al.*, 1986a, 1986b), comprehensive information on habitat conditions is scarce in the literature. Marine sediments are highly variable and both spatial and temporal heterogeneity of energy sources and electron acceptors, critical to the symbioses, must be considered. It must be noted that levels of reduced sulphur species, and other environmental variables, measured in lucinid habitats often represent only average levels in the vicinity of the animals; measuring dissolved sulphide in the immediate vicinity of the animal or in the inhalant tube is problematical. Behavioural and physiological adaptations, as discussed in Chapter 1, may modify conditions in the animal's immediate external environment and internal environment, respectively. Mantle fluid thiol levels may be the best indicator of conditions to which the organism is exposed.

In Bermuda, specimens of *Codakia orbiculata* are found in high densities only in *Thalassia testudinum* bed sediments. Seagrass beds receive large amounts of self-generated plant material and are known to stabilize sediments, reduce seawater

movements, and trap detritus and organic debris from outside the bed (Fenchel, 1970; Jackson, 1972). As a result of anaerobic microbial breakdown of the organic material, dissolved sulphide accumulates in the interstitial water (Fisher and Hand, 1984). Research into carbonate sediment chemistry of tropical and subtropical seagrass beds has focused on nutrient chemistry and the effects of anoxia and high sulphide on seagrass growth (Larkum *et al.*, 1989; Pulich, 1989; Fourqurean *et al.*, 1992) but these sediments have yet to be comprehensively examined as habitat for chemoautrophic symbioses.

The purpose of this study was to describe the lucinid seagrass bed habitat in Bermuda. Special attention was given to quantifying potential energy sources (dissolved sulphide, thiosulphate, elemental sulphur and acid-volatile sulphide (FeS)) and electron acceptors (oxygen, nitrate and nitrite) available for the chemoautotrophic symbioses. Oxygen levels were measured in the sediment-surface water and the level of oxygenation of sediments was inferred from chemical redox sequences. Environmental variables considered pertinent to general sediment chemistry (eg: percent organic content) were also measured. Clam distribution, density, gill elemental sulphur accumulation (indicative of a source of reduced sulphur) (Vetter, 1985; Chapter 3), and mantle fluid thiols were measured and related to environmental variables.

In addition to measuring these variables over a range of sediment depths, data were collected seasonally. Seagrass growth rates were also measured seasonally. Bermuda's inshore

seawater temperature varies typically from 16.5°C in winter to 30.5°C in summer (Morris *et al.*, 1977) and resulting seasonal fluctuations in *Thalassia testudinum* productivity, if substantial, would affect both organic input and oxygen release from the seagrass roots into the sediments (Howes *et al.*, 1981; Smith, R.D. *et al.*, 1984). Because the lucinid seagrass sediment habitat in Bermuda is intertidal (seawater depths ranging from 0.1 to 1.2 m), environmental variables were also measured at tidal extremes to determine any effect(s). *Codakia orbiculata* was not found outside the seagrass sediments. Data were collected from outside the seagrass bed in an effort to determine, by comparison with data from inside the bed, if specific variables affect the distribution of the bivalve. Hence, the characterization of the habitat was divided into 3 separate studies: seasonal, tidal and comparative. Because standing pool data on sulphur species provide only limited insight into the processes occurring, sulphate reduction rates were measured on sediment samples from cores collected in October 1991. These analyses are also presented as a separate study.

The results of the environmental studies are combined to produce a detailed description of the *Codakia orbiculata* habitat. Habitat characteristics are related to the physiology of sulphur-oxidizing symbioses.

2.2. MATERIALS AND METHODS

The following text describes the study site, the general statistical analyses utilized and the determination of the general sampling design. In addition, the specific sampling designs and methods for the collection, processing and analysis of samples for each of the 4 studies (seasonal, tidal, comparative and sulphate reduction) are described separately. The data formats and specific statistical analyses employed on the data sets resulting from each study are also presented.

2.2.1. Site Description

The criteria used for selection of a suitable study site in Bermuda (32.33°N, 64.70°W) were accessibility and the presence of a dense, resident population of *Codakia orbiculata*. This species is found in high densities in shallow-water *Thalassia testudinum* bed sediments. In addition to being representative of this most common type of habitat, the site needed to be "robust" enough, in regard to bivalve density, to withstand the long-term sampling inherent in the habitat study plus the regular collection of large numbers of specimens for physiological experiments.

The selected study site was Bailey's Bay (Fig. 6). A large *Thalassia testudinum* bed occupies the shallow regions of the Bay. This bay is on Bermuda's north shore but is offered some protection from the northerly winter storms by an island located at the head of the bay. This site is only 10 min by road from the laboratory. The depth of seawater over the grass

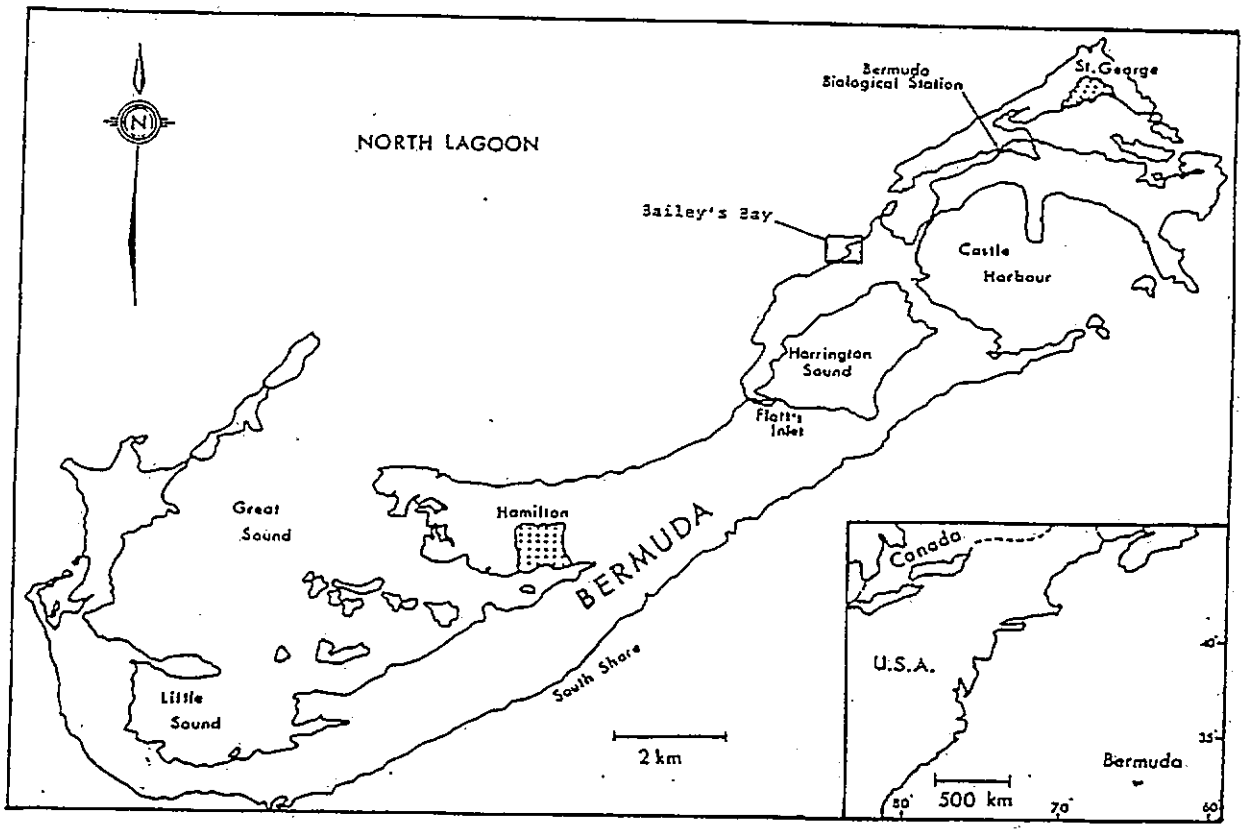

Figure 6. Map of Bermuda showing Bailey's Bay study site.

bed ranged from 1.2 to 0.1 m at high and low tides, respectively.

2.2.2. Statistical Analyses

All statistical analyses used throughout these studies follow Zar (1974); the statistical package used for data analyses throughout these studies was Systat 5.1. Statistical analyses used were linear regressions (DF=n-1), 1-way and 2-way analyses of variance (ANOVAs) (DF=n-1 and DF=n-2, respectively). ANOVAs were followed by Tukey's *post-hoc* pairwise comparisons to determine the specific location of statistically significant differences. Throughout the text, all results are reported as statistically significant where $p<0.05$ and results were considered to demonstrate no statistically significant difference where $p>0.05$. The sample size (n) for each data set is reported on figures and tables. Where linear regressions demonstrated a slope significantly different from zero, the probability (p) and the coefficient of determination (r^2) are reported.

2.2.3. General Sampling Design

Prior to initiating this study, preliminary data were collected to determine if *Codakia orbiculata* were found throughout the seagrass bed. A large aluminum core tube (200 mm diameter) was used at 10 locations throughout the bed. The core penetrated 15 cm but was not sectioned by depth. The entire core was sieved through a 1-mm mesh screen and the total number of *C. orbiculata* per core were counted. The

number of these bivalves per core ranged from 9 to 15. The *C. orbiculata* mean density for the 10 cores was 391.72/m^2 $\pm$ 75.14. Because all cores contained at least 9 *C. orbiculata* and because of the relatively low variance around the mean density ($\pm$ 1 s.d.), it is suggested that the bivalves were distributed, relatively uniformly, throughout the bed.

Dissolved sulphide in interstitial water can be difficult to measure accurately because sulphide spontaneously oxidizes in the presence of oxygen, with a maximum reaction rate at pH 8 (Chen and Morris, 1972). Prior to the initiation of this study, therefore, the optimal method for collecting interstitial water for later analysis of sulphide was investigated. A comparison of sulphide concentration in interstitial water obtained using lysimeters ("wells" or "sippers")(Short *et al.*, 1985), centrifugation and syringe extraction techniques revealed the lysimeters to be the most effective method (Appendix 1). Interstitial water for all the studies was collected from the sediments using lysimeters.

A preliminary study was carried out to determine if the seagrass bed sediments were "patchy" in regard to sediment chemistry and to determine an appropriate sampling design for collecting interstitial water, sediment and bivalve data representative of the habitat. Interstitial water samples were collected from 5, 10, 20 and 30 cm sediment depths using lysimeters placed randomly within 1 m^2 and 0.25 m^2 quadrats. The quadrats were placed randomly in the seagrass bed (using a random numbers table) and 12 lysimeters (3 for each depth) were inserted into the sediments within the quadrat.

Lysimeters were flushed, and filled, with degassed, 0.2 µm-filtered Sargasso (Sea) seawater (FSSW) prior to insertion. Lysimeters were allowed to equilibrate for 24 h prior to sample collection and, as a result, only one set of samples (3 replicates from each depth) could be collected per 24 h. A total of three sets of samples was collected from each size quadrat. All samples were collected at mid-tide, after the high tide. After sample collection, the lysimeters were removed, rinsed, filled with degassed FSSW and inserted into the next randomly-placed quadrat for 24-h equilibration prior to sample collection. Dissolved sulphide, nitrate and nitrite were measured in interstitial water samples. These variables were selected as sensitive indicators of horizontal variability within the bed. Detailed methods for collection, processing and analyses of the samples are presented in the methods for the seasonal study later in this text.

The data was analyzed using two 2-way analyses of variance, followed by Tukey's *post-hoc* pairwise comparisons. Initially, the data set was factored by quadrat size and sediment depth and results revealed no significant differences ($p>0.05$) in nitrate, nitrite or sulphide concentrations in samples collected from the 2 quadrat sizes, at each sediment depth. These results suggest that any "patchiness" in the seagrass bed sediments was either on a smaller scale than could be determined by placing lysimeters within a 0.25 m^2 quadrat (the lysimeters were placed at the minimum distance apart, for accurate sampling, in the 0.25 m^2 quadrat) or on a larger scale than 1 m^2.

Secondly, the data set was factored by quadrat and sediment depth and results revealed no significant differences ($p>0.05$) between nitrate, nitrite or sulphide concentrations in samples collected from all quadrats, for a given sediment depth. Hence, data for 3 interstitial water variables collected from 6 randomly-sampled locations within the bed were not significantly different. Because the 1 m^2 (n=3) and the 0.25 m^2 (n=3) quadrats were placed randomly in different locations throughout the seagrass bed, these results can be interpreted to suggest that the sulphide, nitrate and nitrite levels measured in 1 m^2 quadrat are representative of those found elsewhere in the seagrass bed habitat. Although each replicate for each quadrat size was collected on a different day, results of statistical analyses performed on the seasonal data set (see later in text) revealed no short-term (daily) variation in these variables. These preliminary tests did not take into account changes in patchiness due to seasonal or tidal effects but time, equipment and budget constraints did not allow collection of similar data in the seasonal and tidal studies.

All future samples were taken within 1 m^2 quadrats and results were considered representative of sediment chemistry within the seagrass bed habitat. Collecting samples within a quadrat was preferable to scattering the lysimeters throughout the entire bed because individual lysimeters proved most difficult to relocate among the seagrasses, even with mapping and triangulation. In addition, flags used as markers continuously disappeared from the sampling area and could not

be relied upon for relocation. Hence, relocating 12 lysimeters, closely positioned, proved easier than relocating widely-scattered lysimeters.

2.2.4. Seasonal Study

2.2.4.1. Sampling Design

The seasonal cycle of growth and senescence in seagrasses can influence the sediment chemistry due to changes in sediment oxygenation and organic input (Moriarty and Boon, 1989). Determination of seasonal growth rates in *Thalassia testudinum* in Bermuda had not been determined previously and, therefore, the effects of growth rate on sediment chemistry were unknown. To determine seasonal variation, environmental data were collected in March, June, August and October 1991. Seagrass growth rates were measured for each sampling episode and also for August, September and October 1990.

All samples were collected at mid-tide, after the high tide. To collect interstitial water samples, a 1 m^2 quadrat was placed randomly in the seagrass bed and 12 lysimeters were placed randomly within the quadrat. Three lysimeters collected from each of 5, 10, 20 and 30 cm depths. Because the initial processing and, in some cases, the preservation of bivalve, interstitial water and sediment samples required 24 h (see methods detailed below), only 1 set of samples could be collected per day, even after the initial 24-h equilibration period of the lysimeters.

To obtain replicate data representative of a given month, samples were collected on 3 consecutive days in each sampling month. This data set was subjected to preliminary statistical

analyses to determine if day-to-day variation was significant. The interstitial water and sediment data for the 3 days were divided into subsets by depth and then each subset of data was subjected to a 1-way analysis of variance factored by day. Tukey's *post-hoc* pairwise comparisons were used for comparisons between days for each depth and there proved to be no significant difference ($p>0.05$) between any 2 days compared in 91.7% of the pairs, for all variables. Hence, the sediment chemistry variables measured in this study appeared to demonstrate short-term (daily) stability in relation to spatial heterogeneity. Data from all lysimeters and cores at each depth were pooled for the 3 day period (n=9, except where noted).

2.2.4.2. Sample Collection

2.2.4.2.1. Interstitial and Overlying Water

Interstitial water samples were collected, using lysimeters, for analysis of dissolved sulphide, thiosulphate, nitrate, nitrite, ammonia and dissolved iron. Interstitial water pH was measured on the ammonia and iron samples prior to processing. Twelve lysimeters were used, 3 collected interstitial water from each sediment depth (5, 10, 20 or 30 cm) on each day. Sampling was carried out for 3 days. Lysimeters were flushed, and filled, with degassed, 0.2 µm-filtered FSSW prior to insertion and were allowed to equilibrate for 24 h prior to initiation of sample collection. Immediately upon arrival at the sampling site, an acid-washed 60-ml syringe was attached to the sampling tube of each

lysimeter. The cap was removed from the syringe tip and the syringe was immediately screwed onto the end of the lysimeter tube to avoid any contamination from surrounding seawater. The stopcock on the lysimeter tube was opened to allow interstitial water to be sucked into the syringe. Before collection of the first samples, 15 ml of sample was collected in the syringe and discarded. This is the volume of the lysimeter collecting chamber which was filled with degassed, FSSW prior to insertion. The syringe was re-attached and when the volume of interstitial water reached 60 ml, the lysimeter stopcock was closed and the syringe was removed, immediately capped and placed in an argon-flushed, air-tight container for transport back to the laboratory.

During each sampling episode, the oxygen concentration in the overlying water was measured using a portable YSI dissolved oxygen meter, Model 58 with a polarographic electrode equipped with stirrer. The accuracy of this method was $\pm$ 0.3% oxygen. The salinity of the overlying water was measured using a portable YSI Model 33 with a YSI 3300 series conductivity/ temperature probe. The accuracy of this method was $\pm$ 0.3% of the reading on the meter. Temperatures of the overlying water were measured $\pm$ 0.1°C, as were sediment and air temperatures.

2.2.4.2.2. Sediment Cores

Sediment samples for analysis of acid-volatile sulphides (AVS), elemental sulphur, percent water and percent organic content were collected using hand-held plexiglass cores. Sediment cores were collected in the vicinity of the 1 m^2 area

that contained the lysimeters. Three sediment cores were collected each day for 3 days. The cores measured 7.5 cm inside diameter and 30.0 cm length and the average length of sediment core obtained was 15 cm. Although the penetrating edge of the core was bevelled, the cores were very difficult to push through the rhizosphere. To overcome this problem, a device similar in design to a pile-driver was built to force the cores into the sediments. The core was pushed into the sediment a few cm and a wooden "platform" attached to a long pole fitted to the top of the core. Keeping the core and wooden pole vertical, a heavy steel pipe was slipped over the pole and lifted repeatedly and dropped onto the platform, so that the weight of the steel pipe pushed the core into the sediments. When the core had reached maximum penetration, the top of the core was fitted with a PVC cap. Caps were taped to the core tube to reduce the possibility of leaks. The core was then loosened and lifted from the sediment and the bottom of the core was capped as soon as possible. The bottom cap was also taped to the core tube. All cores were kept vertical in a bucket for transport to the laboratory.

2.2.4.2.3. Bivalves

Bivalves were collected from the sediment cores sampled for analysis of sediment variables, as described above.

2.2.4.2.4. Seagrass Growth Rate

The growth rates of *Thalassia testudinum* plants were measured as part of the seasonal sampling study following the

procedure of Powell *et al.* (1989). Three days prior to field sampling, 4 PVC rings (15 cm diameter x 5 cm high) were inserted into the seagrass bed so that approximately 2 cm protruded above the sediment surface. A ruler was placed across the ring, seagrass blades of one plant were stacked together and a needle was inserted through all the blades at the level of the ruler. This was repeated for each plant within the ring. Four days after field sampling, seagrass plants from three of the four rings were harvested. Each plant within the ring was cut at the level of the ruler placed across the ring. The blades from each plant were placed in an individual Whirlpak plastic bag and plants from each ring were kept separate.

2.2.4.3. Laboratory Processing

2.2.4.3.1. Interstitial Water

Immediately upon return to the laboratory, the argon-flushed container with the syringes was placed in an argon-filled glove bag in the laboratory. Although only the sulphide and iron samples required an oxygen-free environment for processing, it was found to be most efficient to filter and subdivide the 60-ml interstitial water samples at one time. The sample from each of the syringes was ejected through a 0.2-µm Nuclepore membrane filter (in a Gelman syringe filter holder) into acid-washed scintillation vials from which exact volumes for analyses were measured using Finnpipettes. The 60-ml sample was divided as follows: 5 replicate samples each of 1 ml were pipetted into individual pre-labelled scintillation

vials, each containing 20 µl of 2 M zinc acetate dihydrate ($(CH_3CO_2)_2Zn.2H_2O$) (ZnAc). The ZnAc precipitated the sulphide as ZnS for later analysis of sulphide using the colourimetric technique of Cline (1969). The ZnAc present in each vial was sufficient to preserve sulphide concentrations up to 40 mM sulphide, far in excess of expected sediment interstitial water maxima (Fisher and Hand, 1984). In addition, 5 replicate samples each of 100 µl for analysis of thiosulphate and sulphide were pipetted into pre-labelled Eppendorf tubes containing 100 µl degassed HEPES buffer solution (200 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazinemethanesulphonic acid) with 5 mM EDTA (ethylenediaminetetraacetic acid, 99.5%), pH 8) for later analysis using the "HPLC-bimane" technique (Newton and Fahey, 1987; Vetter *et al.*, 1989). The HEPES buffer solution ensured that the reaction occurred at optimum pH. From the remaining volume of interstitial water, 5 replicate 5 ml samples for ammonia analysis were pipetted into 10 ml disposable, polypropylene test tubes with snap-top caps. In addition, 2 replicate 5-ml samples for nitrate and nitrite analyses and a 5-ml sample for analysis of iron and manganese were pipetted into 5-ml acid-washed polyethylene vials. The nitrate/nitrite samples were placed immediately into the freezer for storage at -4°C until analysis. The remaining samples required either immediate analysis, or processing prior to storage, as outlined in the analytical methods section.

2.2.4.3.2. Sediment Cores

The cores were kept vertical in the laboratory and processed after the interstitial water samples had been stabilized. Cores were extruded into an argon glove bag fitted with a plexiglass sheet below the glove bag and another inside the glove bag. These sheets provided a stable, horizontal working surface within the bag. A hole was bored through both plexiglass sheets and a hole made in the glove bag through which a PVC ring was fitted. The PVC ring had an internal diameter equal to the external diameter of the core tubes and was kept in place by a threaded collar which screwed into threads on the outside of the ring, effectively anchoring the plexiglass sheets together with the glove bag between. The inside of the PVC ring had an O-ring seal. A PVC plunger fitted with an O-ring seal was fitted into the bottom of the core immediately after the bottom cap was removed. The core and plunger were then fitted into a car jack apparatus. As the top of the core approached the glove bag platform, the top core cap was removed quickly to avoid oxygen contamination. The core was jacked up so that the top of the core tube was pushed against the plexiglass platform and the O-ring in the PVC ring formed an effective seal against the outside barrel of the core tube. Hence, the inside of the core and the glove bag were isolated from the external environment. As the plunger was forced up the core tube, sediment was extruded into the glove bag. A sliding door, made of thin, bevelled plexiglass, was fitted into grooves on either side of the core opening inside the glove bag and was slid into place to slice

off a section of sediment core. This door also effectively sealed the glove bag when no core was in place.

The cores were quickly sectioned into 0-2 cm, 2-4 cm, 4-6 cm, 6-9 cm and 9-11 cm; longer cores were sectioned into 11-15 cm and >15 cm subsections. Using a 5-ml syringe with the needle attachment end sliced off, 3 ml of sediment were removed from each sediment section and ejected into acid-washed plastic scintillation vials for weighing, freeze drying and then analysis of acid-volatile sulphide (FeS) and elemental sulphur. From the remaining sediment in each core subsection, 2 samples of 5-10 g each were placed in pre-weighed, acid-washed glass Petri dishes for later analysis of percent water and percent organic content. Where sediment within a core subsection was composed of light and dark sections, samples of both colours were taken for later analysis of percent water, percent organics, AVS and elemental sulphur.

2.2.4.3.3. Bivalves

Bivalves found in the sediment chemistry cores were identified to species (Abbot, 1974). *Codakia orbiculata* found at each sediment depth were counted for later calculation of vertical distribution and density. *C. orbiculata* were maintained in ambient sediment until later processing for tissue analyses.

2.2.4.3.4. Seagrass Growth Rate

After returning to the laboratory, seagrass blades were refrigerated until after all interstitial water, sediment and bivalve samples had been stabilized or analyzed.

2.2.4.4. Analytical Methods

2.2.4.4.1. Interstitial Water

(i). Dissolved Sulphide Analysis Using a Colourimetric Assay.

Dissolved sulphide in interstitial water was analyzed using the colourimetric assay of Cline (1969). This method is applicable to sample concentrations of 1-1000 µM sulphide and uses a reagent containing N,N-dimethyl-p-phenylenediamine sulphate (referred to as "diamine" throughout this text). This method gives a precision of ±2% at the 95% confidence level and accuracy was estimated at ±2% at the 95% confidence level (Cline, 1969). This technique requires a range of reagent concentrations and sample dilutions prior to reading the absorbance, dependant on the concentration of sulphide in the sample (250-1000 µM, 40-250 µM, 3-40 µM and 1-3 µM). Sulphide in the 5 replicate 1-ml samples had been preserved as ZnS. 5 replicate samples were required because 1-2 samples were needed to determine which reagent concentration was appropriate. The remaining samples were then analyzed using the correct strength reagent. The technique was modified for small volumes (80 µl volume of the appropriate diamine reagent was added to the 1-ml sample). After complete colour development (20 min), the samples were diluted as required using MilliQ purified distilled water (MQwater). All sample

absorbances were measured on a Milton Roy Spec21 spectrophotometer at 670 nm.

Standards and blanks were prepared using FSSW which was first oxygenated for 20 min to remove any possible trace of sulphide and was then degassed with argon for 20 min. Standards were prepared using sodium sulphide nonahydrate ($Na_2S.9H_2O$) washed free of oxidation products and dried with a cellulose tissue prior to weighing. A minimum of 5 standard concentrations was prepared within each reagent range. Sulphide concentrations of unknown samples were determined by comparison with the appropriate standard curve. The replicate samples per lysimeter (3-4) were averaged, resulting in one datum point per lysimeter in the final data set.

(ii). Thiosulphate and Sulphide Analysis Using HPLC and Fluorometry.

The following procedure for analysis of thiosulphate and sulphide in interstitial water is from Newton and Fahey (1987), as modified by Vetter *et al.* (1989). This method is highly reproducible for reduced sulphur compounds ranging from high nannomolar to tens of millimolar in concentration (Vetter *et al.*, 1989). Prior to removing the 5 replicate samples for thiosulphate and sulphide analyses (100 µl interstitial water plus 100 µl degassed HEPES buffer solution) from the glove bag, 10 µl of 10 mM monobromobimane (referred to as "bimane" throughout the text) were added to each of the 1.5-ml Eppendorf tubes. The tubes were then removed from the glove bag and placed in the dark for 15 min for derivatization.

Once the reaction was complete, 100 µl acetonitrile was added to each tube and the samples heated to 60°C for 10 min to precipitate any proteins present. Following protein precipitation, 300 µl of 25 mM methane sulphonic acid was added to each tube to dilute the acetonitrile. Samples were then centrifuged for 1 min at 14,000 rpm to pellet any protein present and stored frozen (-4°C) until analysis. Samples remained stable for several months if stored in the freezer but significant amounts of thiols were lost from samples repeatedly thawed and refrozen (P. Barnes, pers. obs.).

Bimane reacted with the thiol group to produce covalently bonded fluorescent adducts which could be separated on an Altex C-18 reversed-phase column using an increasing hydrophobic gradient of methanol and water. The adducts were detected using a broad excitation filter of 305-395 nm and a narrow band emission filter centered at 480 nm. All analyses were performed at Scripps Institution of Oceanography (San Diego, California) using a Waters WISP automatic injector, a Gilson high-performance liquid chromatograph and a Gilson fluorometer. Reagent blanks and pyridine disulphide (PDS) controls were also run for each interstitial water sample. The latter were prepared identically to the unknown samples except that 10 µl PDS was added prior to the addition of bimane. PDS forms a stable derivative with thiols and prohibits their binding with the bimane.

The sulphide and thiosulphate concentrations in the 5 replicates were averaged, resulting in one datum point per lysimeter in the final data set.

(iii). Ammonia

The 5 replicate interstitial water samples from each lysimeter were removed from the argon glove bag for further processing. The colourimetric assay for ammonia in seawater (Strickland and Parsons, 1977) is very sensitive to the presence of sulphide. Briefly, to remove sulphide prior to analysis, the samples were acidified to reduce all sulphide present to H_2S and then degassed to remove H_2S. Samples were then buffered to optimum pH for the reaction (Dennison *et al.*, 1987). This method has a precision ranging from ± 0.10 - ± 0.15 µM, depending on the number of replicates, and can be used to detect ammonia in concentrations ranging from 0.1-10 µM (samples with ammonia concentrations >10 µM were diluted prior to reading extinction).

The pH of 1 replicate sample from each sediment depth (5, 10, 20 and 30 cm) was measured and lowered to approximately pH 2 using 1.2 N HCl. The HCl was added in 10 µl increments; 80-100 µl total volume was usually required to acidify a 5-ml sample. At pH 2, all the ammonia should be present as ammonium, which does not disappear through de-gassing. Sulphide at this pH, in contrast, should be present mostly as H_2S which can be removed from the sample by degassing with argon for 20 min. After de-gassing, 0.5 M sodium bicarbonate ($NaHCO_3$) was added to bring the pH up to neutral; approximately 350 µl was required for a 5 ml sample. Following this last buffering step, the analysis followed the procedure

in Strickland and Parsons (1977) except modified for 5-ml, rather than 50-ml, volumes.

The following steps were carried out in the fumehood due to the use of phenol. The 10-ml test tubes were uncapped immediately prior to adding each reagent and capped immediately after the addition to avoid atmospheric contamination. 200 µl of phenol reagent (20 g of crystalline analytical reagent grade phenol in 200 ml of 95% v/v ethyl alcohol) were added to each sample. After shaking, 200 µl of nitroprusside reagent (1.0 g of $Na_2Fe(CN)_5NO.2H_2O$ in 200 ml MQwater) was added to each sample. This was followed by the addition of 500 µl of oxidizing solution, consisting of 100 ml of alkaline reagent (100 g sodium citrate and 5 g sodium hydroxide (analytical reagent grade) in 500 ml MQwater) and 25 ml of sodium hypochlorite solution (commercial Chlorox bleach). Full colour development takes 1 h and the colour is stable for 24 h; after 12 h at 22°C, the absorbance of the samples was read at 640 nm on a Milton Roy Spec21 spectrophotometer.

Reagent blanks were prepared exactly as described above for samples, including the acidification, degassing and re-buffering steps, except 5 ml of FSSW was used in place of the interstitial water. The standard ammonia solution was prepared by dissolving 0.100 g of analytical reagent quality ammonium sulphate in 1000 ml of MQwater (1 ml = 1.5 µg-atN). 1 ml of chloroform was added and the solution was stored sheltered

from strong light. 1.00 ml of this solution was made up to 500 ml with FSSW (low nutrient) so that the resulting concentration was 3.0 µM. Standards were acidified, degassed and buffered following the procedure described for interstitial water samples and some standards were prepared without these steps. Addition of reagents and absorbance measurements for standards treated in both ways followed that outlined for the interstitial water samples. Analysis of the results revealed no significant difference between standards prepared with or without the modifications for the removal of sulphide (ANOVA; $p<0.05$). The F factor (F) = 3.0/(Standard absorbance - Blank absorbance). The concentration of ammonia in the unknown samples was, therefore, calculated in µM as F x Absorbance. Concentrations were then adjusted for sample dilution, where necessary.

The 5 replicate ammonia samples from each lysimeter were averaged, resulting in one datum point per lysimeter in the final data set.

(iv). Nitrate

Analysis of nitrate in interstitial water from Bailey's Bay using the colourimetric method of Strickland and Parsons (1977) proved to be problematical. Because of the small volume of interstitial water available for analysis, traditional cadmium columns designed for seawater samples were too large. In addition, although much smaller glass columns were blown and assembled, sulphide in the interstitial water samples bound to the cadmium and poisoned the column. Sulphide

could be removed from the interstitial water samples by the addition of zinc acetate for precipitation as zinc sulphide. However, this initial removal of sulphide, combined with utilization of the small cadmium columns, made nitrate analysis especially time consuming and alternate analytical methods were investigated. It was determined that the chemiluminescent method for detection of combined nitrate and nitrite in seawater (Garside, 1982) as modified by Yoshizumi *et al.* (1985) was not affected by the presence of sulphide in concentrations up to 1 mM (Barnes and Lipshultz, unpubl. data). This method utilizes a NO_x chemiluminescent analyzer to determine nitrate and nitrite in solution. Nitrate and nitrite are reduced to nitric oxide in a phosphoric acid, ferrous ammonium sulphate and ammonium molybdate solution. Interstitial water samples can be injected directly into this acidic solution, maintained at 90-100°C, and the resulting nitric oxide is detected by a NO_x chemiluminescent analyzer. Standards with and without sulphide were tested. Interstitial water samples treated with zinc acetate to remove the sulphide and those which had not been treated yielded identical results for nitrate plus nitrite. Also, this technique required only small sample volumes; an injection volume of 100-200 µl was usually adequate to measure the nitrate plus nitrite concentration (injection volume was increased if necessary). The minimum detection limit for this technique was 5 ng of nitrate plus nitrite per injection volume.

Chemiluminescent detection can be used also to determine the individual nitrate and nitrite concentrations in seawater.

After addition of sulphanilamide (prepared as described in Strickland and Parsons, 1977) to remove nitrite from solution, the sample can be run to determine the nitrate concentration; the nitrite concentration can be determined by substraction. The addition of sulphanilamide to the sulphide-rich interstitial water, however, yielded variable results and inadequate time was available to solve this problem. Instead, nitrite samples were analyzed using the colourimetric assay (Strickland and Parsons, 1977) after removal of sulphide using zinc acetate (see next section). The resulting nitrite data were substracted from the combined nitrate and nitrite concentrations obtained using the chemiluminescence technique to obtain nitrate concentrations.

All frozen water samples for nitrate plus nitrite analysis were thawed at room temperature (22°C) and then vortexed immediately prior to injection into the reaction flask. Three injections were performed for each sample to determine the precision of the technique. Injection replicates demonstrated insignificant (<2%) variation. Reagent blanks and standards were prepared using FSSW. A 100 µM working standard solution was prepared daily from a 10 mM potassium nitrate (KNO_3; reagent grade). A standard curve was generated by changing the injection volume from 100 to 2000 µl. Hence, the resulting peaks represented absolutes for nitrate plus nitrite ranging from 0.2 nmol to 4 nmol. Since injection volumes of the interstitial water samples varied in order to obtain a sufficiently large peak, calculation of absolutes and then conversion to concentration based on injection volume proved

optimal. The final data set of nitrate concentrations (µM) contained one datum point for each lysimeter.

(v). Nitrite

Sulphide interferes with colourimetric nitrite analysis (Strickland and Parsons, 1977) and was removed prior to analysis by precipitation as zinc sulphide (by the addition of zinc acetate). This method was tested by comparing nitrite standards (1 and 2 µM) with and without spikes of 100 to 1000 µM sulphide. ANOVA demonstrated no significant difference between the 2 treatments ($p<0.05$) (data not shown). The detection range for the colourimetric nitrite assay is 0.01-2.5 µM, with precision ranging from ± 0.023 to ± 0.032 depending on the number of replicates (Strickland and Parson, 1977).

The 5-ml interstitial water samples frozen for nitrite analysis were thawed at room temperature (22°C) and vortexed prior to subsampling for analysis. Three 1-ml subsamples were pipetted from each interstitial water sample and the precision of the technique was tested and found to be comparable with that of Strickland and Parsons (1977). Samples were pipetted into 1.5-ml Eppendorf tubes each containing 10 µl 2 M ZnAc. Following the addition of 10 µl 2 N sodium hydroxide (NaOH), to increase the flocculation and to help precipitation, the tubes were centrifuged for 1 min at 13,000 rpm on an Eppendorf Microcentrifuge 5415. The supernatant (800 µl) was transferred to another 1.5-ml Eppendorf tube and after this point, the method follows that of Strickland and Parsons (1977) with modifications for small volumes. The nitrite in seawater

reacted with sulphanilamide in an acid solution. The resulting diazo compound reacts with N-(1-naphthyl)-ethylenediamine and forms a highly coloured azo dye, the extinction of which is measured using a spectrophotometer. 160 µl of sulphanilamide solution (5 g sulphanilamide ($4-(H_2N)C_6H_4SO_2NH_2$) in 50 ml of concentrated hydrochloric acid (HCl) and 450 ml of MQwater) were added and, after 5 min, 160 µl of N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{10}H_7NHCH_2CH_2NH_2.2HCl$) (0.5 g in 500 ml MQwater). After allowing 1-2 h for full colour development, the absorbance of each sample was measured at 543 nm on a Milton Roy Spec21 spectrophotometer.

Nitrite concentrations were determined by comparison with a standard curve. Standards ranging from 1-5 µM nitrite were prepared from 5 mM stock solution prepared using anhydrous analytical reagent grade sodium nitrite ($NaNO_2$). Standards and reagent blanks (MQwater) were processed as described above for interstitial water samples. The final data set had one nitrite concentration (µM) datum point per lysimeter.

(vi). Iron and Manganese

In interstitial waters, the dissolved iron may be mostly Fe^{2+}, in contrast to the mostly Fe^{3+} in seawater (Boulegue *et al.*, 1982). The 5-ml interstitial water samples for dissolved iron and manganese analyses were acidified to aid preservation prior to freezing (Jickells and Knap, 1984). Acidification reduces oxidation of the sample; at neutral pH, iron oxidizes rapidly (Troup *et al.*, 1974). The pH of one sample from each sediment depth (5, 10, 20 and 30 cm) was measured and 1.2 N

HCl added until pH 2. All samples were acidified in the argon glove bag. Samples were stored at -4°C for up to 12 months prior to analysis.

After thawing at room temperature (22°C), all samples were vortexed prior to analysis. Analyses were carried out at the Plymouth Marine Laboratory by graphite furnace atomic absorption spectrophotometry (AAS) using a Varian SpectrAA 300/400. Working conditions for iron were 248.3 nm wavelength and a 0.2 nm slit width, with an optimum working range of 60-15,000 ppb (Varian, 1988). Working conditions for manganese were 279.5 nm wavelength and a 0.2 nm slit width, with an optimum working range of 52-20,000 ppb (Varian, 1988). The injection volume for each sample was 10 µl and triplicate injections were run for each sample to calculate percentage precision. A percentage precision $\leq$20% was considered acceptable.

Stock standard had been prepared by dissolving 1.0 g of iron metal (or manganese) in 20 ml 1:1 HCl and then diluted to 1 litre final volume (final concentration = 1000 ppm Fe (or Mn)). Iron and manganese concentrations in interstitial water samples were calculated from a standard curve using standards ranging from 0.5 to 10 ppm (G. Burt, pers. comm.). Standard additions were also run on random samples. FSSW blanks were run every 10-20 samples. The final data set had one Fe^{2+} concentration (ppb) datum point per lysimeter. Manganese levels proved below detection limit (samples were of inadequate volume to pre-concentrate) and are not discussed further.

2.2.4.4.2. Sediments

(i). Elemental Sulphur

The freeze-dried sediment samples for analysis of elemental sulphur were stored in a desiccator in the freezer until analysis. Each freeze-dried sample was homogenized with a mortar and pestle and 2 g of sediment was weighed into an acid-washed scintillation vial. 5 ml of HPLC grade hexane or heptane (no difference noted in extraction efficiency of elemental sulphur (S. Niven, pers. comm.)) were added to the vial. The vial cap was lined with a Teflon/silcon seal and after capping, the vial was vortexed to ensure adequate mixing and then placed on a roller for 12 h (overnight) to extract the elemental sulphur. After centrifuging at 4500 rpm for 10 min, 2 ml of supernatant was removed into a 2-ml borosilicate glass HPLC vial with polypropylene screw-top cap with silicone/PTFE seal. Since the supernatant was considered uniform, only one supernatant subsample was analyzed for each sediment sample. HPLC analysis followed the procedure described by Lauren and Watkinson (1985) and used a Hamilton PRP-1 10 µm particle (polymeric reverse phase-plastic beads) 150 x 4.1 mm column and methanol/chloroform (80-70:30) eluent at 1 ml/min flow rate. An autoinjector was used, set to a 40-µl injection volume and 12-min cycle time. Solvent was delivered with a Kontron 420 HPLC pump and elemental sulphur peaks were detected with a programmeable absorbance detector at 254 nm wavelength (flow cell 9 µl and 6 mm path length). This method could detect 2-4 ng of elemental sulphur in 1 injection.

A standard curve was generated from peak heights of standards prepared using analytical grade elemental sulphur extracted in heptane. Repeated injections of a standard solution revealed a precision of ±3% and comparison of replicate standards against the standard curve suggest the technique is accurate within ±5%. Elemental sulphur concentrations in the unknowns, expressed as µg-atoms S/l, were converted to absolutes (µg-atoms S) in the 5.0 ml extraction volume and then to µgS/mgdw (sediment). The final data set contained one elemental sulphur datum point for each sediment sample and therefore, one datum point for each core subsection (except where 2 samples were taken as representative of different sediment colours).

The above procedure was repeated on the sediments remaining after analysis for AVS (see below) to determine AVS driven to elemental sulphur due to acidification (S. O'Hara, pers. comm.).

(ii). Acid-volatile Sulphide (AVS) (or ferrous sulphide, FeS)

This procedure follows that of Morse and Cornwell (1987). AVS was driven out of the sediment through acidification, as sulphide gas, and into zinc acetate traps. The sulphide was then analyzed using the colourimetric procedure of Cline (1969), with precision and detection limits as listed above for colourimetric analysis of sulphide in interstitial water.

After extraction of elemental sulphur, the sediments for AVS analysis were allowed to dry (heptane was evaporated), and were capped and stored until analysis. Two sulphide traps were set up in series for each sample. Traps consisted of scintillation vials containing 5 ml of 5% degassed zinc acetate. All scintillation vial caps were lined with teflon/silicon septa through which holes were made for the tubing into and out of the vial. The sample vial was connected to the traps via Teflon tubing and bubbled with argon for 5 min. 5.6 N HCl was added drop-wise from a syringe attached to the the argon line until foaming stopped. The sample was then gassed with argon for 2-3 h. White precipitate (ZnS) formed in the first trap and, rarely, in the second zinc acetate trap. Both traps were analyzed, however, to ensure accurate measurement of all ZnS. After trapping, samples were stable for several days. The zinc acetate traps were analyzed colourimetrically using Cline's (1969) procedure as outlined for analysis of dissolved sulphide.

Standards, ranging from 50 to 1000 µM sulphide, were used to generate a standard curve as described for colourimetric analysis of dissolved sulphide (Cline, 1969), with the following modification. Standards were prepared in 5 ml 5% degassed zinc acetate to mimic sample conditions. 5% degassed zinc acetate was run as a blank. In addition, a series of standards was run through the AVS trap system to determine %recovery following acidification. 30 standards were run at concentrations ranging from 100 to 1000 µM, averaging 94.3% recovery.

The resulting concentration data (µM) were converted to absolutes (µmoles) and combined for the two traps. Data were then converted to µmoles/gdw (sediment). The final data set contained one AVS datum point per sediment sample. Hence, there was one AVS datum point per core subsection, except where 2 sediment samples were taken as representative of different sediment colour.

Any sediment remaining after acidification was re-extracted in heptane and re-analyzed for elemental sulphur (see procedure outlined earlier). These values were then combined with AVS levels obtained from acidification.

(iii). Percent Organic Content

The wet sediment sample for percent organics was weighed and then oven dried at 60°C to constant weight. The weight of the dry sediment was substracted from the wet weight to determine water content and data were converted to percent water (by weight). The dry sediment was then homogenized using an acid-washed mortar and pestle and a sub-sample (1-2 g) of the homogenized sediment weighed (± 1 mg) into an acid-washed crucible that had been pre-muffled at 600°C for 12 h in a Lindberg Model 51748 furnace. The samples were ashed at 600°C until constant weight (10-12 h), removed from the muffle furnace and stored in a desiccator until cool. Samples were re-weighed (± 1 mg) and the loss in weight, expressed as a percentage of the total sample weight, represents percent organic content.

2.2.4.4.3. Bivalves

Bivalves removed from sediment cores were maintained in ambient sediment until analysis. The estimated maximum time between removal from the sediment core to dissection was 60 min.

(i) Mantle Fluid Sulphide and Thiosulphate

Codakia orbiculata were placed into a large plastic weighing boat in the argon-flushed glove bag. The bivalves were quickly blotted dry with a cellulose tissue and gently prized open with a scalpel. The following procedure was modified from Newton and Fahey (1987) and Vetter *et al.* (1989). Detection limits and precision of this technique were as listed for interstitial water analyses of sulphide and thiosulphate.

Mantle fluid to be analyzed for sulphide and thiosulphate was pipetted immediately in 5-µl aliquots into a 1.5-ml Eppendorf tube containing 100 µl degassed HEPES buffer solution (pH 8). The HEPES buffer solution ensured that the reaction occurred at optimum pH. Ten µl of 10 mM monobromobimane (referred to as "bimane" throughout the text) was added and the tube placed in the dark for 15 min. Once the reaction was complete, 100 µl acetonitrile was added and the sample heated to 60°C for 10 min to precipitate the protein. Following protein precipitation, 300 µl of 25 mM methane sulphonic acid was added to dilute the acetonitrile. Samples were then centrifuged for 1 min at 14000 rpm to pellet the protein and stored frozen (-4°C) until analysis. Bimane

reacts with the thiol group to produce covalently bonded fluorescent adducts which are separated on an Altex C-18 reversed-phase column using an increasing hydrophobic gradient of methanol and water. The adducts are detected using a broad excitation filter of 305-395 nm and a narrow band emission filter centered at 480 nm. All analyses were performed at Scripps Institution of Oceanography using a Waters WISP automatic injector, a Gilson high-performance liquid chromatograph and a Gilson fluorometer. Reagent blanks and pyridine disulphide (PDS) controls, where volume was sufficient, were run also. The latter were prepared identically to the unknown samples except that 10 µl PDS was added prior to the addition of bimane. PDS forms a stable derivative with thiols and prohibits their binding with the bimane. After sampling of mantle fluid, bivalves were removed from the glove bag.

(ii). Gill Tissue Elemental Sulphur

Codakia orbiculata were removed from the argon glove bag after removal of mantle fluid. Gills were removed carefully, to avoid contamination with other tissues, under magnification of a Wild M8 dissecting microscope. For each bivalve, one gill was prepared for elemental sulphur analysis and the other for either protein content or enumeration of bacterial symbionts. The wet weight of each gill was recorded. Gills for protein or elemental sulphur analysis were freeze-dried, re-weighed and stored frozen until analysis. A comparison of preservation methods for gills prior to extraction of stored globular

elemental sulphur (SGES) using hexane or heptane revealed freeze-drying and freezing to be the most effective preservation method (Chapter 3). Gills used for bacterial counts were preserved in Lillie's buffered (pH 7.4) formalin (100 ml 40% formaldehyde, 900 ml MQwater, 4 g sodium phosphate monohydrate, 6.5 g disodium phosphate).

Freeze-dried gills for elemental sulphur analysis were placed into 2-ml borosilicate glass HPLC vials with polypropylene screw-top caps with silicone/PTFE seals containing 0.5 ml HPLC grade hexane or heptane. There was no difference in the extraction efficiency of hexane or heptane for SGES (S. Niven, pers. comm.). Gills were broken into small fragments in the solvent using a PTFE micro-probe. Vials were placed on a shaker table and extracted for 12 h (overnight). Following extraction, vials were centrifuged at 5000 rpm using an IEC Clinical centrifuge to pellet the tissue fragments and 0.5 ml of solvent was removed into a second HPLC glass sampling vial. HPLC analysis followed the procedure described by Lauren and Watkinson (1985) with solvent gradient, equipment, detection limits and precision as listed above for elemental sulphur analysis in sediments.

A standard curve was generated from peak heights of standards prepared using analytical grade elemental sulphur extracted in heptane. SGES concentrations in the unknowns, expressed as µg-atoms S/l, were converted to absolutes (µg-atoms S) in the 1.0 ml extraction volume and then to µg-atoms S/gww (gill tissue).

(iii). Gill Protein

One from each pair of freeze-dried gills was stored frozen prior to protein analysis. Protein content in freeze-dried gills was determined with the BCA protein assay (Pierce, Rockford, IL 61105, USA, 1989). Using the room temperature protocol, the applicable protein range was 20-1200 µg/ml with a typical sensitivty of 0.010 Absorbance Units per µg.

The homogenization solution used followed Vetter (1990). Each gill was homogenized in 1.0 ml 100 mM Tris-HCl, pH 7.4 with 1% Triton X-100 of MQwater using a low clearance hand homogenizer (Dounce). 100-µl replicates of the sample were pipetted into labelled test tubes, to which 2.0 ml of "working reagent" (50 parts reagent "A" (1000 ml of base reagent containing sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.2N NaOH) and 1 part reagent "B" (25 ml of 4% copper sulphate solution)) was added. After vortexing for 10 sec, the tubes were incubated at room temperature (22°C) for 2 h. The extinction was measured at 562 nm against a MQwater reagent blank. Protein concentrations were determined from a standard curve generated using standards ranging from 200 to 1200 µg/ml prepared by diluting stock 2 mg/ml BSA standard (bovine serum albumin) in MQwater.

(iv). Enumeration of Bacterial Endosymbionts

Immediately after dissection, gills for later enumeration of symbionts were preserved in Lillie's buffered formalin (pH 7.4). Gills were homogenized and centrifuged in Lillie's buffered formalin and the final bacterial pellet was

resuspended in a DAPI (4'6-diamidino-2-phenylindole) solution with a final concentration of 0.01 µg/ml. Procedures for preparation of DAPI stock solution, slides and examination of slides using epifluorescent microscopy were identical to those described by Porter and Feig (1980). These methods are applicable to both fresh and formalin-preserved material (Porter and Feig, 1980). Slides were examined using an Olympus BH-2 epifluorescent microscope but the clarity with which symbionts could be discerned and counted varied significantly. Due to time constraints, it was not possible to diagnose or solve the problem in order to include the data as part of this study. Samples have been stored for re-examination at a later date.

(v). Thiosulphate and Sulphide in Bivalve Blood

Blood can be obtained from large bivalve specimens by scoring the mantle tissue with a scalpel and collecting the blood in microcapillary tubes, after blotting the mantle cavity dry (R. Vetter, pers. comm.). *Codakia orbiculata* specimens (<10 mm shell length) were too small for this procedure. The mantle tissue was extremely fragile and small; the wet weight of mantle tissue was commonly <5 mg. Although an alternative method to obtain blood samples was attempted, results of replicate samples were disappointing and these analyses were not continued. The alternative method attempted was as follows. After opening the valves, the body of the bivalve was removed, leaving only the thin mantle tissue covering the inside of the shells. The mantle tissue was

blotted dry, removed from the shell and weighed quickly. The tissue was then placed into a Dounce glass tissue homogenizer containing 100 µl of degassed HEPES buffer solution and 10 µl of 10 mM bimane. The tissue was homogenized in this solution and then the homogenate transferred to a 1.5-ml Eppendorf tube. After centrifugation for 3 min at 10,000 g, the supernatant was removed to another 1.5-ml Eppendorf tube. Processing then followed the same procedure described above for analysis of mantle fluid sulphide and thiosulphate. Detection limits and precision were the same as described for analysis of sulphide and thiosulphate in interstitial waters.

Because the volume of blood extracted could not be determined, the resulting data were expressed as absolutes per milligram wet weight mantle tissue. Hence, these data could not be used for comparison with the literature but were, instead, intended for internal comparison within this study only. However, this technique was first tested on 5 bivalves with the mantle tissue lining one valve used as one sample and the mantle tissue from the other valve used as a replicate sample. Comparison of the blood thiol levels from the two valves revealed significant variation and this technique was not utilized further.

2.2.4.4.4. Seagrass Growth Rate

The *Thalassia testudinum* blades were examined and the distances, in mm, between the cut edge and the hole made by the needle were measured to determine growth. Growth for each blade was later converted to daily growth rate.

2.2.4.5. Data Format and Statistical Analysis

For each month, the interstitial water and sediment variable data were compared against sediment depth using linear regression analyses. The coefficient of determination (r^2) is reported where the slope was significantly different from zero ($p<0.05$). The entire seasonal data set for each variable was subjected to a 2-way analysis of variance, factored by month and sediment depth, followed by Tukey's *post-hoc* pair-wise comparisons to determine statistically significant differences between sediment depths for a given month and between months for a given sediment depth ($p<0.05$). The latter, in combination with observations on the graphs, was used to detect seasonal change for each variable. Seagrass growth rates, overlying water parameters, air and sediment temperatures were tested for significant seasonal differences using a series of 1-way ANOVAs followed by Tukey's *post-hoc* pair-wise comparisons ($p<0.05$).

Bivalve distribution proved to be clearly depth-limited (2-6 cm) and was not subjected to analysis against sediment depth. Bivalve density and tissue data were tested for significant seasonal differences using 1-way analyses of variance followed by Tukey's post-hoc *pair-wise* comparisons ($p<0.05$). Tissue data were compared with both sediment and interstitial water data by comparison with the graphed environmental data for each month.

2.2.5. Tidal cycle study

2.2.5.1. Sampling Design

To determine if environmental variables within the *T. testudinum* bed sediment habitat varied with the tidal cycle, interstitial water and sediment samples were collected and analyzed from low and high tides, over a 72-h period in March 1991. A total of 3 low tides and 3 high tides were sampled. Note that the sampling program for the preliminary sampling-design study, the seasonal study, and the comparison of seagrass bed sediments with those outside the bed were carried out at mid-tide, after the high tide.

A 1 m^2 quadrat was placed randomly within the bed and the 12 lysimeters inserted randomly within the quadrat. After an initial 24-h equilibration period, interstitial water samples were collected. Sediment variables were measured on 3 cores taken at each of 3 low and high tides. Tidal heights at the sampling times were, consecutively, 0.1 m, 1.1 m, 0.2 m, 1.2 m, 0.1 m and 1.1 m.

2.2.5.2. Sample Collection

Interstitial water samples and sediment cores were collected, for analysis of variables, as described for the seasonal study.

2.2.5.3. Sample Processing

Interstitial water, sediment and bivalve samples were processed as described for the seasonal study.

2.2.5.4. Analytical Techniques

All analytical techniques for interstitial water, sediment and bivalve samples were as described for the seasonal study. Bivalve blood samples were not taken for the tidal study, however.

2.2.5.5. Data Format and Statistical Analysis

Interstitial water and sediment data were regressed against depth for each tidal height to determine slopes significantly different from 0 ($p<0.05$). The coefficient of determination (r^2) is reported where the slope was significantly different from zero ($p<0.05$). Data for each variable were analyzed using a 2-way ANOVA factored by depth and tidal height, followed by Tukey's *post-hoc* pairwise comparisons to determine significant differences between depths for each variable at each tidal height and between tidal heights at a given depth ($p<0.05$). A series of 1-way ANOVAs were used to test for significant tidal differences in the overlying water variables and sediment temperature ($p<0.05$). Bivalve density was tested to determine significant differences between tidal heights using a 1-way ANOVA, factored by tidal height, followed by Tukey's *post-hoc* pair-wise comparisons ($p<0.05$). The tissue data set was too small for statistical analysis but bivalve distribution and tissue analyses data were compared between tidal heights and compared with graphed data for sediment and interstitial water variables. Graphed data were compared with that for the March 1991 seasonal sampling (mid-tide).

2.2.6. Comparison of sediment chemistry inside and outside the seagrass bed.

2.2.6.1. Sampling Design

The sediment chemistry inside the seagrass bed was compared with that outside the perimeter of the bed in August 1991. Sediments outside the bed were sampled for 3 days following the normal seasonal sampling within the bed. Sampling design followed that described for the seasonal study. All sediment, interstitial water and bivalve variables were measured outside the bed for comparison with the August 1991 data from inside the bed.

2.2.6.2. Sample Collection

Sample collection for inside the seagrass bed was described as part of the seasonal study. Sample collection outside the seagrass bed followed the same procedures as described for the seasonal study.

2.2.6.3. Sample Processing

All samples were processed as described for the seasonal study.

2.2.6.4. Analytical techniques

All analytical techniques were the same as those described for the seasonal study.

2.2.6.5. Data Format and Statistical Analysis

Interstitial water and sediment data were regressed against depth for each location to determine slopes significantly different from 0 ($p<0.05$). The coefficient of determination (r^2) is reported where the slope was significantly different from zero ($p<0.05$). For each variable, a 2-way ANOVA factored by depth and location, followed by Tukey's *post-hoc* pair-wise comparisons, was used to determine significant differences between inside and outside the seagrass habitat at a given depth and between depths for each location ($p<0.05$).

2.2.7. Sulphate Reduction Rates

2.2.7.1. Sampling Design

Sulphate reduction rates were measured as a function of sediment depth in the 3 sediment cores collected within the seagrass bed in October 1991.

2.2.7.2. Sample Collection

Sediment samples for measuring sulphate reduction rates were taken from the 3 sediment cores collected as part of the October 1991 seasonal study. Interstitial water samples for sulphate analysis were collected using lysimeters and syringe extraction from the sediment cores.

2.2.7.3. Sample Processing

2.2.7.3.1. Sulphate

Interstitial water samples for sulphate analysis were filtered through a 0.2-µm Nuclepore filter. Three replicate 1-ml samples were pipetted into acid-washed scintillation vials containing 20 µl of 2 N zinc acetate to precipitate sulphide. These samples were stored refrigerated for a maximum of 2 weeks prior to analysis of sulphate.

2.2.7.3.2. Sulphate Reduction

Sediment samples for the sulphate reduction experiments were removed, in the argon glove bag, from each core subsection. 4 ml of sediment from each core subsection was packed into modified 5-ml disposable syringes with the needle attachment cut off and fitted with a rubber septum. The

latter fitted snugly and prevented gas exchange. Syringes were labelled with core number and sediment depth and were kept under argon until all samples were processed. Samples were removed from the glove bag and transported to the radiation laboratory fume hood. Additional 4-ml samples were taken from each core subsection to measure interstitial water volume so that sulphate concentrations could be converted to nmoles for calculation of sulphate reduction rates.

2.2.7.4. Analytical Techniques

2.2.7.4.1. Sulphate

Sulphate levels in the interstitial water samples were measured according to Howarth (1978). Samples were filtered (0.2-µm membrane filter) prior to analysis to remove the zinc sulphide precipitate. Sulphate standards were prepared using serial dilutions of Copehagen Standard Seawater. Sulphate concentration of the standard was calculated as 2.777 g/l (24°C) assuming a sulphate-chloride ratio of 0.1400 (Morris and Riley, 1966) and a density of 1.024 kg/l (Pickard, 1963). A standard curve was prepared from the serial dilutions and the volumes of titrate added (r^2=0.9930). Standards used ranged from 28.93 mM to 0 mM (100% to 0% sulphate). Using the generated standard curve, accuracy levels averaged $\pm$0.49 mM. Precision was calculated at $\pm$0.1 mM.

To convert sulphate concentrations to absolutes in the volume of sediment used in the sulphate reduction experiments, the volume of interstitial water in a 4-ml sediment sample was calculated from the difference between the wet and dry weights

of the sample, converted to volume. The latter conversion was based on the mean weights of 10 x 1 ml samples of interstitial water.

2.2.7.4.2. Sulphate Reduction

The sulphate reduction experiment protocol most closely follows that of Hines and Lyons (1982). Stock solution of $Na_2{}^{35}SO_4$ (1 mCi/ml) was diluted with autoclaved, degassed FSSW to 3 µCi of label contained in 25 µl of solution. A 25-µl Hamilton syringe was used to inject 25 µl of label into each syringe. The label was ejected slowly as the needle was withdrawn to distribute the label evenly. The incubated syringes were maintained for 24 h under black plastic in the radiation laboratory fume hood. After 24 h, samples were placed in ziplock bags and then into a seawater ice slurry to bring the temperature down quickly and to stop the reaction. Syringes were then stored in the freezer at -4°C for 24 h.

In the radiation laboratory fume hood, frozen samples were ejected from the syringes into reaction bottles containing 25 ml of deoxygenated MQwater, forming a slurry. The reaction bottles were attached to the AVS reduction system as described in the seasonal study methods. Argon gas was run through the sediment slurry and free sulphide was trapped in two traps connected in series, each containing 5 ml of 10% zinc acetate. After 1 h, the zinc acetate traps were removed, capped and replaced. 6 N HCl (16 ml) was added drop-wise to each of the samples and argon gas was run through the system for 2 h. Traps were removed and capped. The trapped sulphide

(including $^{35}S^{2-}$) and AVS (including $Fe^{35}S$) formed a white zinc sulphide precipitate. Zinc acetate was chosen for the traps because zinc sulphide has a minimum quenching effect (Jørgensen, 1978). The remaining sediment slurry was centrifuged at 5000 rpm using an IEC Clinical centrifuge and a 5 ml aliquot of supernatant was removed for counting $^{35}SO_4^{2-}$. 5 ml of Aquasol scintillation cocktail was added to each of the zinc acetate traps and to the interstitial water (supernatant) sample. Samples were counted using a Packard Tri-Carb 4530 scintillation counter.

Dilutions of the stock $Na_2{}^{35}SO_4$ to 0.1 µCi/50 µl (0.002 µCi/µl) were prepared for quench curves. Zinc acetate quench samples were prepared with a range of ZnS precipitate. Because the $^{35}SO_4^{2-}$ remaining in the samples after conclusion of the experiment had been measured in sediment interstitial waters extracted by centrifugation (which had already been diluted with oxygen-fee MQwater (see above)), additional quench solutions included interstitial water extracted by centrifugation, MQwater and filtered, sterilized FSSW.

Sulphate reduction rate in the sediments was calculated using the following formula:

$$\text{Rate (nmoles ml}^{-1}\text{ d}^{-1}) = \frac{(\text{dpm } ^{35}S^{2-} \text{ or dpm } Fe^{35}S)\ (\text{nmoles } SO_4^{2-})\ (1.06)}{(\text{ml sample vol.})\ (\text{dpm } ^{35}SO_4^{2-})\ (\text{1 d incubation})}$$

where 1.06 is the ^{35}S fractionation factor (Hines and Lyons, 1982).

2.2.7.5. Data Format and Statistical Analysis

The final data set for the sulphate reduction experiments contained 3 reduction rates for each sediment depth range. The rates for each sediment depth were averaged and plotted (± 1 s.d.) against sediment depth for comparison with the October 1991 sediment and interstitial water data.

2.3. RESULTS

2.3.1. Seasonal Study

Data collected in this study provide a detailed description of the *Codakia orbiculata* seagrass habitat for each month, including trends across sediment depth. The data were examined to determine if *C. orbiculata* depth distribution and density were clearly related to habitat variables. In adddition, if the habitat was subject to seasonal changes, the data were examined to determine whether there were associated changes in *C. orbiculata* density or tissue parameters which were indicative of physiological condition. Because cores usually did not penetrate below 15 cm, sediment variables were representative of the rhizosphere, which had a maximum penetration of 20 cm sediment depth. The lysimeters, however, collected interstitial water from 5, 10, 20 and 30 cm sediment depth, with the 30 cm sample representative of conditions below the rhizosphere.

The data for temperature (air, sediment and seawater), salinity and dissolved oxygen in the seawater above the seagrass bed sediments are summarized in Table 5. While salinity and oxygen demonstrated no significant seasonal differences ($p>0.05$), seawater and sediment temperatures were significantly higher in August 1991 than the other months and June 1991 seawater temperature was significantly higher than March and October 1991 ($p<0.05$).

Ammonia levels in Bailey's Bay seagrass sediments ranged from 5.18 $\pm$ 2.92 µM to 30.63 $\pm$ 12.88 µM throughout the study (Fig. 7a). Ammonia concentrations demonstrated a significant

Table 5. Seasonal variation in temperature, salinity and dissolved oxygen in the overlying seawater, plus sediment and air temperatures. Data are presented as means ± 1 s.d.. n=3.

Date (1991)	Sediment Temp. (°C)	Air Temp. (°C)	Overlying Water Temp. (°C)	Overlying Water Salinity (‰)	Overlying Water Oxygen (%)
March	20.3± 0.6		21.3± 0.3	34.00± 0.00	118.87± 4.34
June	25.7± 0.8	27.3± 6.5	26.7± 1.4	33.27± 0.64	124.83± 21.16
August	29.3± 0.4	33.0± 4.5	29.9± 0.7	34.00± 0.00	102.33± 2.52
October	22.5± 3.0	22.3± 2.5	23.2± 1.3	33.93± 0.31	101.33± 2.08

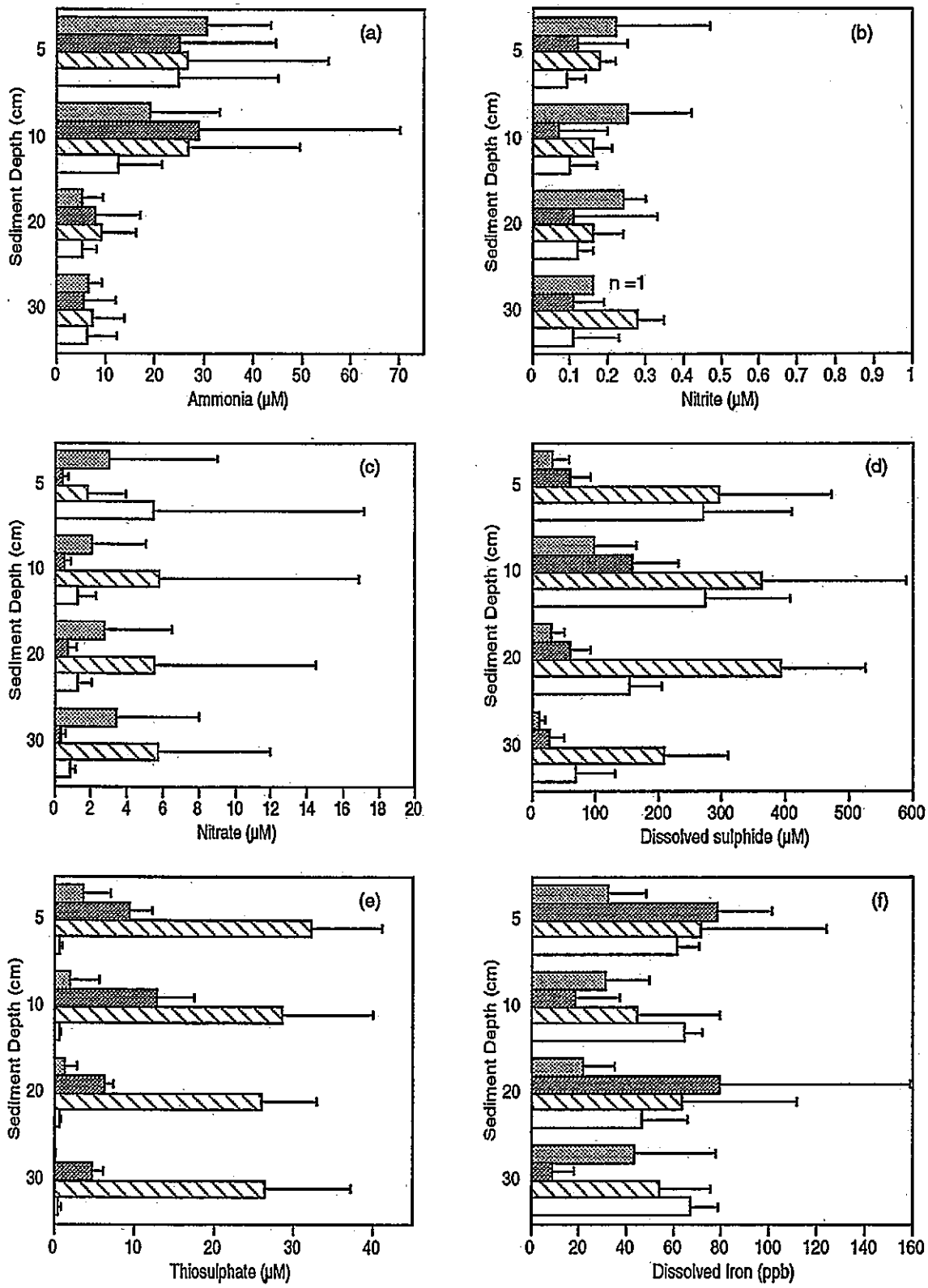

Figure 7. Interstitial water constituents in Bailey's Bay *Thalassia testudinum* sediments as a function of sediment depth for March, June, August and October 1991. (a) Ammonia (µM), (b) Nitrite (µM), (c) Nitrate (µM), (d) Dissolved sulphide (µM), (e) Thiosulphate (µM), (f) Dissolved iron (ppb). Data are plotted as means ± 1 s.d.; $6 \leq n \leq 9$, except where noted.

linear relationship of decrease with increasing sediment depth for each month ($p<0.05$; $r^2 = 0.145-0.453$). Only in March and October, however, were the ammonia levels at 5 cm sediment depth significantly higher than those at greater depths (20 and 30 cm sediment depth)($p<0.05$). The variance around the means (as evidenced by the standard deviations) were much larger in the shallower sediment depths, where concentrations were greater, suggesting greater heterogeneity. No significant seasonal differences were found, at any of the sediment depths, for ammonia ($p>0.05$).

Nitrite levels in interstitial water were low, consistently less than 1 µM (Fig. 7b). Unlike the ammonia concentrations, nitrite levels were consistent across sediment depth ($p>0.05$). Nitrate concentrations in Bailey's Bay sediment interstitial water were higher than nitrite, ranging from 0.38 ± 0.23 µM to 5.76 ± 11.17 µM. The nitrate data, similar to the ammonia concentrations, were highly variable at a given depth, as illustrated by the comparatively large standard deviations in Figure 7c. Similar to nitrite, nitrate concentrations demonstrated no significant linear relationship with sediment depth ($p>0.05$) and no significant differences between sediment depths, for any of the months ($p>0.05$). Nitrite levels demonstrated no significant seasonal trends ($p>0.05$). Although the mean nitrate levels in June were consistently lower than nitrate levels in other months, analyses revealed no statistically significant seasonal differences at any given sediment depth ($p>0.05$).

Mean concentrations of dissolved sulphide in interstitial water ranged from 11.04 ± 11.48 μM to 394.27 ± 131.22 μM. Mean sulphide levels for all months, as demonstrated in Figure 7d, were lowest at 30 cm sediment depth but analyses revealed that only in October was the 30 cm sulphide concentration significantly lower than those at shallower depths (5 and 10 cm)($p<0.05$). When mean sulphide levels at a given depth were compared between months, the August concentrations were significantly higher than the March and June levels for all sediment depths, but significantly higher than the October levels only at 20 cm sediment depth ($p<0.05$). Interstitial dissolved sulphide concentrations at 5 cm sediment depth in October were significantly higher than those in March and June ($p<0.05$).

The thiosulphate interstitial water concentrations ranged from 0.06 ± 0.19 μM to 32.27 ± 8.82 μM (Fig. 7e). Thiosulphate demonstrated a significant linear relationship with sediment depth in June 1991 ($p=0.0001$; $r^2=0.380$), decreasing with increasing sediment depth. Thiosulphate concentrations in March, June and October 1991 were minimal at 30 cm sediment depth but not statistically lower than concentrations at other sediment depths ($p>0.05$). Similar to the sulphide data, thiosulphate levels in August were statistically higher, at all sediment depths, than those from the other months ($p<0.05$). March and June thiosulphate levels at 5 cm were significantly higher than those at 5 cm in October ($p<0.05$).

Dissolved iron concentrations in Bailey's Bay interstitial water were extremely low, with means ranging from 22.01 ± 13.4 ppb to 95.22 ± 79.58 ppb (Fig. 7f). The dissolved iron concentrations in interstitial water were consistent with sediment depth for all months ($p>0.05$). Dissolved iron demonstrated no significant seasonal trends at any of the sediment depths ($p>0.05$).

The percent water content (by weight) of the seagrass bed sediments ranged from 36.0 ± 3.61% to 53.81 ± 3.03% (Fig. 8a). Water content demonstrated no significant linear relationship with sediment depth ($p>0.05$) and no significant differences between sediment depths ($p>0.05$). There proved to be no seasonal trend in water content at any of the sediment depths ($p>0.05$). The organic content in sediment core subsections ranged from 4.52 ± 0.55% to 11.09 ± 5.36% (Fig. 8b). Percent organic content of the sediments did not change significantly with sediment depth for any of the months ($p>0.05$) and demonstrated no significant seasonal change at any of the sediment depths ($p>0.05$). There was no significant difference in percent water or percent organic content between light and dark coloured sediment samples collected throughout this study ($p>0.05$).

Acid-volatile sulphide (AVS) levels in Bailey's Bay seagrass sediments ranged from 0.16 ± 0.09 µmoles/gdw to 0.96 ± 0.40 µmoles/gdw (Fig. 8c). AVS demonstrated no significant relationship with sediment depth ($p>0.05$) and analyses revealed no significant differences in AVS between sediment depths for any of the months ($p>0.05$). In addition, there was

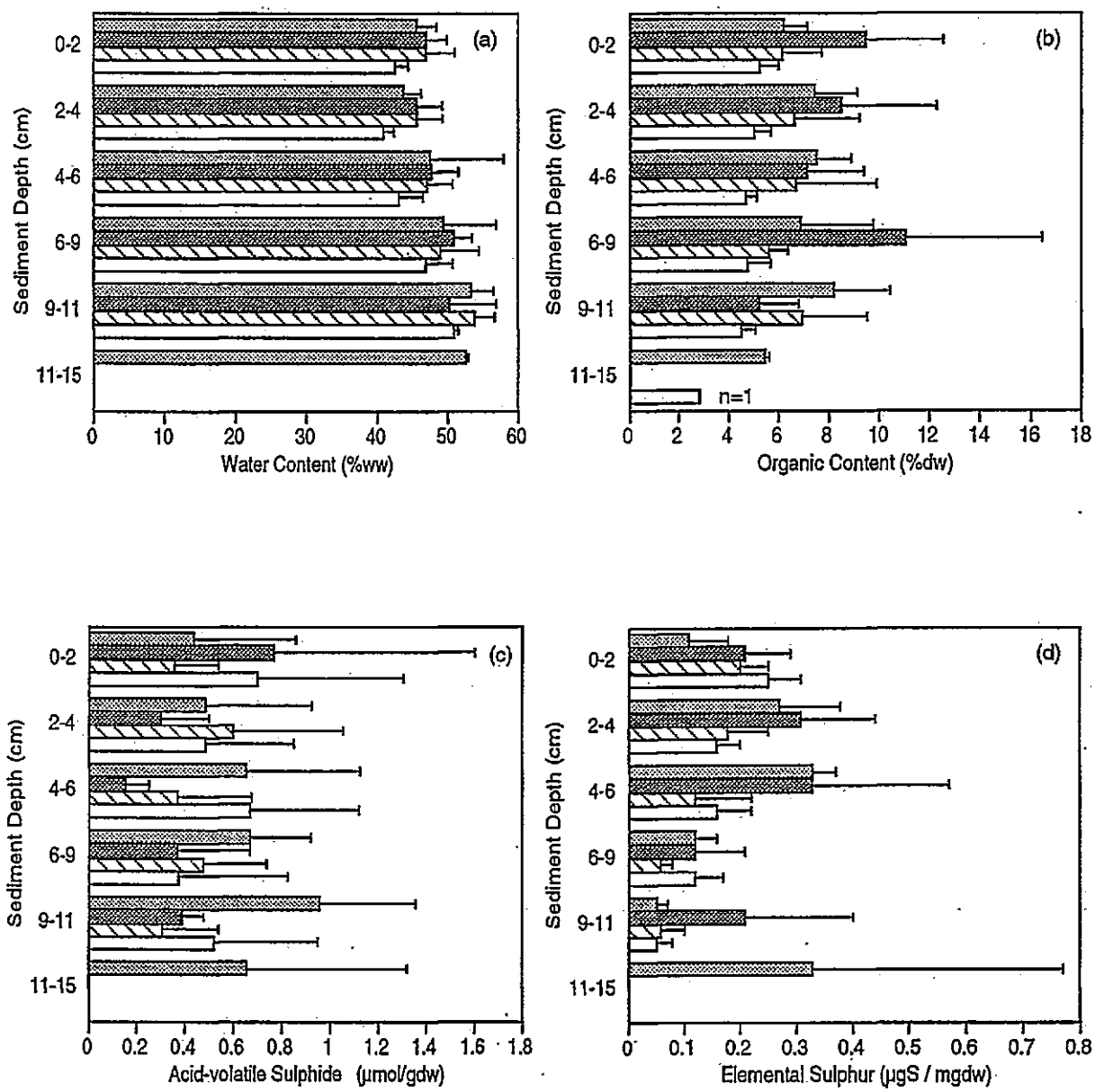

Figure 8. Sediment constituents in Bailey's Bay *Thalassia testudinum* sediments as a function of sediment depth for March, June, August and October 1991. (a) Water content (%ww), (b) Organic content (%dw), (c) Acid-volatile sulphide (µmol/gdw), (d) Elemental sulphur (µgS/mgdw). Data are plotted as means ± 1 s.d.; $3 \leq n \leq 9$ except where noted.

no significant seasonal change in AVS levels at any of the sediment depths ($p>0.05$). Mean elemental sulphur content in the seagrass bed sediments ranged from 0.05 $\pm$ 0.02 µgS/mgdw to 0.33 $\pm$ 0.45 µgS/mgdw (Fig. 8d). The August elemental sulphur data demonstrated a significant linear relationship with sediment depth, decreasing with increasing sediment depth ($p=0.001$; $r^2 = 0.329$). A similar trend was observed for other months, although was not statistically significant ($p>0.05$). Although elemental sulphur appeared to be lower in the deeper core subsections, these differences could not be proven to be statistically significant with this data set ($p>0.05$). Elemental sulphur demonstrated no significant seasonal change at any of the sediment depths ($p>0.05$). There was no significant difference in AVS or elemental sulphur content between sediments of different colour collected throughout this study ($p>0.05$).

The seagrass growth rate reached a maximum of 3.78 $\pm$ 2.00 mm/day in June 1991, while minimum growth rate was recorded in October 1991 (1.37 $\pm$ 1.03 mm/day)(Fig. 9). Seagrass growth rates in March and June 1991 were statistically higher than those in October 1990, August 1991 and October 1991 ($p>0.05$). Hence, lower growth rates were recorded at times when interstitial water sulphide levels were at a maximum (August and October 1991). Interstitial water thiosulphate concentrations, seawater temperature and sediment temperature in Bailey's Bay also reached a maximum in August.

Because *Codakia orbiculata* were only found in the 2-4 and 4-6 sediment depths, no analyses were performed on bivalve

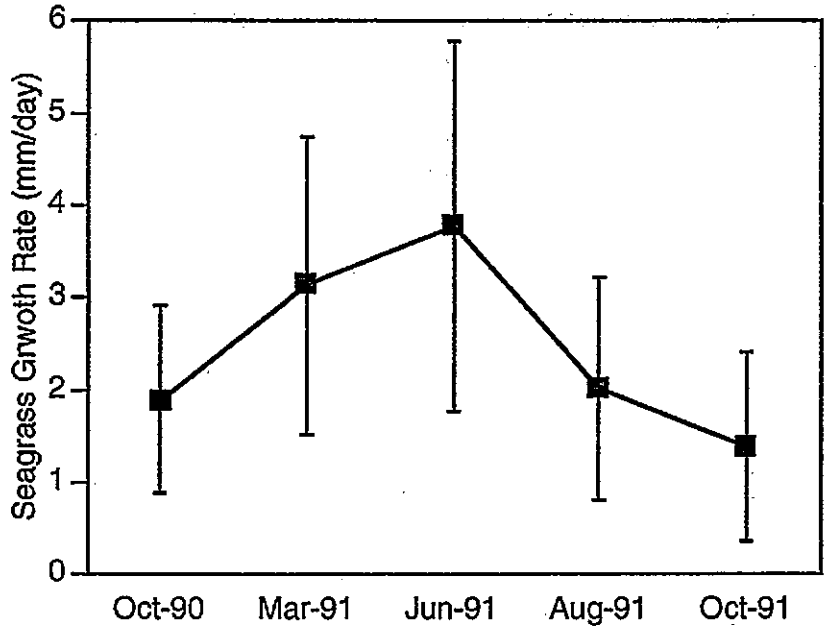

Figure 9. *Thalassia testudinum* growth rate (mm/day) for October 1990 and March, June, August and October 1991. Data are plotted as means ± 1 s.d.; n > 100.

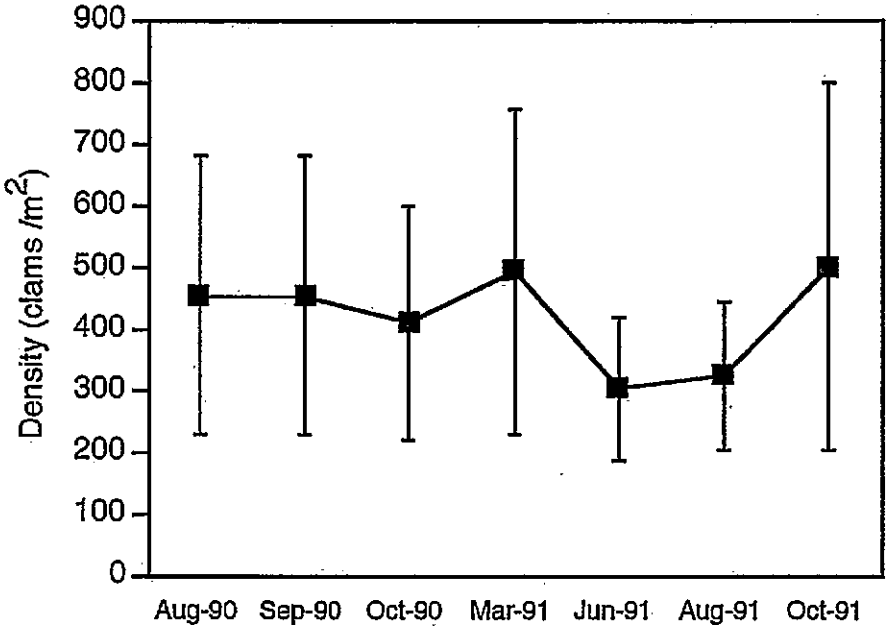

Figure 10. *Codakia orbiculata* density (clams /m^2) for August, September and October 1990, March, June, August and October 1991. Data are plotted as means ± 1 s.d.; n=3 for August 1990 through October 1990; n=9 for March 1991 through October 1991.

(note: "bivalve" is used interchangeably with "*C. orbiculata*" throughout this text since *C. orbiculata* was the only species studied) distribution, density or tissue data as a function of sediment depth. *C. orbiculata* density ranged from 303.05 ± 117.37 per m^2 in June 1991 to 500.03 ± 296.35 per m^2 in October 1991 (Fig. 10). Note that density determinations were started before the sediment chemistry study. As suggested by the graph and confirmed by statistical analysis, there were no significant differences in bivalve density with season ($p>0.05$).

The results of *Codakia orbiculata* tissue analyses are summarized in Table 6. Protein content in the gills was measured in March (0.12 ± 0.05 mg/mgww) and August (0.15 ± 0.03 mg/mgww). These protein levels were comparable with those measured in gills from bivalves used in the "starvation" experiment (Chapter 3). Seasonal trends could not be examined because of the small sample size but the Chapter 3 experiments demonstrated that protein content in *C. orbiculata* gills appeared to remain relatively stable for long periods in the absence of a reduced form of sulphur. Elemental sulphur content in bivalve gills (70.59 ± 87.71 to 185.31 ± 109.19 µg-atoms S/gww) was also comparable to that recorded in the gills of freshly collected *C. orbiculata* (Chapter 3). Elemental sulphur content in the gills of bivalves collected each month was highly variable (as evidenced by the large standard deviations in Table 6). Gill elemental sulphur content did not change significantly between sampling months ($p>0.05$).

Table 6. Seasonal variation in *Codakia orbiculata* gill tissue protein and elemental sulphur content, mantle fluid sulphide and thiosulphate concentrations. Data are presented as means ± 1 s.d.. Sample size (n) is given below data.

Date (1991)	Gill Protein (mgprotein/ mgww)	Gill Elemental Sulphur (µgatomsS/ gww)	Mantle Fluid Sulphide (µM)	Mantle Fluid Thiosulphate (µM)
March	0.12± 0.05 (13)	70.50± 87.71 (7)	88.69± 40.32 (10)	154.06± 174.96 (10)
June		91.86± 83.13 (7)	52.66± 29.35 (6)	255.82± 198.90 (6)
August	0.15± 0.03 (11)	189.14± 62.82 (4)	267.92± 105.55 (3)	375.66± 93.99 (3)
October		185.31± 109.19 (6)	268.88± 195.23 (6)	231.26± 149.07 (8)

Mantle fluid sulphide and thiosulphate data are also presented in Table 6. Both sulphide and thiosulphate levels in mantle fluid were comparable with those measured in freshly-collected bivalves used in the starvation experiments (Chapter 3). *Codakia orbiculata* collected for the latter experiments, and those collected for this study, exhibited high variation between individuals (high variance around the means). There were no significant differences in thiosulphate in bivalve mantle fluids between months ($p>0.05$). Mantle fluid sulphide levels, however, were significantly higher in August and October 1991 than the other months ($p<0.05$) and were not statistically different from each other ($p>0.05$). Comparison of mantle fluid and interstitial water thiols revealed that sulphide levels in the mantle fluid followed closely those in the interstitial water. Statistical analysis revealed no significant difference between the sulphide levels in mantle fluid and in interstitial water ($p>0.05$). In contrast, however, mantle fluid thiosulphate levels were significantly higher than interstitial water levels ($p<0.05$). The mantle fluid thiosulphate levels demonstrated no significant seasonal trend ($p>0.05$).

In summary, the above results and analyses reveal that the *Thalassia testudinum* sediment chemistry is relatively stable within the rhizosphere (maximum penetration of 20 cm sediment depth). The sediment cores did not penetrate below the rhizosphere but interstitial water levels of sulphide, thiosulphate and ammonia concentrations were generally minimal below the rhizosphere (30 cm). This could not always be

demonstrated statistically, however, possibly due to the small sample size and high variance. There appeared to be a general trend for the 0-6 cm elemental sulphur levels to be higher than those at 6-11 cm sediment depths but, again, this could not be demonstrated statistically. The spatial heterogeneity at each sediment depth with these, and other, variables may have masked (for statistical purposes) trends across sediment depth (Fourqurean *et al.*, 1992). The data for these reduced chemical species suggest, however, that the seagrass rhizosphere was not subject to the classical redox sequences with increasing sediment depth (Moriarty and Boon, 1989).

The detailed description of the sediment chemistry of the *Thalassia testudinum* bed sediments can be compared with the observed depth distribution pattern of *Codakia orbiculata* within the seagrass sediments. *C. orbiculata* was found only within the rhizosphere and the complete absence of bivalves below the rhizosphere may be due to the lower sulphide, thiosulphate and/or ammonia levels at 30 cm sediment depth. Because the sediment variables were relatively consistent across sediment depths within the rhizosphere, there appears to be no correlation between the observed bivalve distribution in the 2-4 and 4-6 cm sediment sections and the sediment variables measured in this study. Table 7 summarizes the sediment chemistry in the 2-6 cm habitat of the bivalves, including the range of interstitial water (at 5 cm sediment depth) and sediment (mean of 2-4 and 4-6 cm data) conditions for the months sampled. Other factors which may affect *C. orbiculata* depth distribution are explored in the discussion.

Table 7. Seasonal variation in interstitial water and sediment variables in *Codakia orbiculata* habitat (2-6 cm sediment depth). Data are presented as means ± 1 s.d..

(a) Interstitial water. ($6 \leq n \leq 9$).

Date	Ammonium (µM)	Nitrite (µM)	Nitrate (µM)	Sulphide (µM)	Thiosulphate (µM)	Iron (ppb)
March	30.63± 12.88	0.22± 0.13	3.04± 5.96	31.32± 27.60	3.58± 3.53	32.69± 15.62
June	25.12± 19.52	0.12± 0.04	0.47± 0.27	60.59± 31.18	9.45± 2.82	78.67± 22.61
August	26.69± 28.54	0.18± 0.05	1.84± 2.10	296.68± 176.08	32.27± 8.82	71.28± 52.62
October	24.95± 20.08	0.09± 0.03	5.45± 11.71	271.62± 139.96	0.66± 0.40	61.33± 9.24

(b) Sediments. ($6 \leq n \leq 18$).

Date	Water Content (%ww)	Organic Content (%ww)	Acid- Volatile Sulphide (µmoles/gdw)	Elemental Sulphur (µgS/mgdw)
March	45.43± 6.95	7.43± 1.97	0.58± 0.45	0.30± 0.11
June	46.89± 3.67	7.75± 2.99	0.22± 0.15	0.32± 0.18
August	46.72± 4.81	6.68± 2.78	0.59± 0.51	0.15± 0.08
October	41.98± 2.79	4.86± 0.59	0.61± 0.41	0.16± 0.05

Despite the seasonal trend for interstitial water sulphide and thiosulphate concentrations, plus overlying seawater and sediment temperature, to reach maxima in August, there appeared to be no seasonal trend in either bivalve depth distribution or density. Elemental sulphur content in the gills displayed no significant seasonal variation, suggesting an adequate and constant supply of exogenous reduced sulphur (Vetter, 1985; Chapter 3). *Codakia orbiculata* mantle fluid sulphide levels closely followed those of the interstitial water, with maxima corresponding to the interstitial water sulphide peaks and to the temperature maxima in overlying seawater and sediment. Possible interpretations of mantle fluid thiosulphate levels are examined further in the discussion.

2.3.2. Tidal Cycle Study

Seawater depths in the intertidal habitat of *Codakia orbiculata* range from 0.1 to 1.2 m. It should be noted that 2 of the low-tide and 1 of the high-tide sampling episodes were carried out in the dark. The possibility of diel effects on sediment chemistry, as a result of changes in photosynthetic oxygen shunted to the roots (Smith *et al.*, 1988), were considered *a posteriori*. The data for each variable were tested statistically for diel differences using a 2-way ANOVA factored by light/dark and sediment depth. Results revealed no significant difference, in any of the variables, between light and dark samples ($p>0.05$). It was deemed unlikely, therefore, that the diel sampling affected the determination of tidal effects using this data set. However, the possibility of interactive effects between light/dark and low/high tide could not be determined.

The sediment and water column temperatures, plus water column salinity and dissolved oxygen levels (Table 8), did not vary significantly at tidal extremes ($p>0.05$). Ammonia, nitrate and nitrite levels at both low- and high-tide (Figs. 11a-c) were comparable to those recorded at mid-tide in March 1991 (Figs. 7a-c). Low- and high-tide ammonia data, like the mid-tide data, demonstrated statistically significant linear relationships with sediment depth, decreasing with increasing depth ($p=0.001$, $r^2=0.447$ and $p=0.0056$, $r^2=0.229$ respectively). Also comparable to the March mid-tide results, ammonia levels in interstitial water at the shallower sediment depths at low (5 cm sediment depth) and high (10 cm sediment depth) tides

Table 8. Tidal variation in temperature, salinity and dissolved oxygen in the overlying water, plus sediment temperature. Data are presented as means ± s.d.. n=3.

Tide	Sediment Temp. (°C)	Overlying Water Temp. (°C)	Overlying Water Salinity (‰)	Overlying Water Oxygen (%)
Low Tide	19.1± 0.1	19.8± 1.2	34.00± 0.00	82.27± 14.77
High Tide	19.4± 0.5	19.2± 0.3	34.00± 0.00	102.00± 11.68

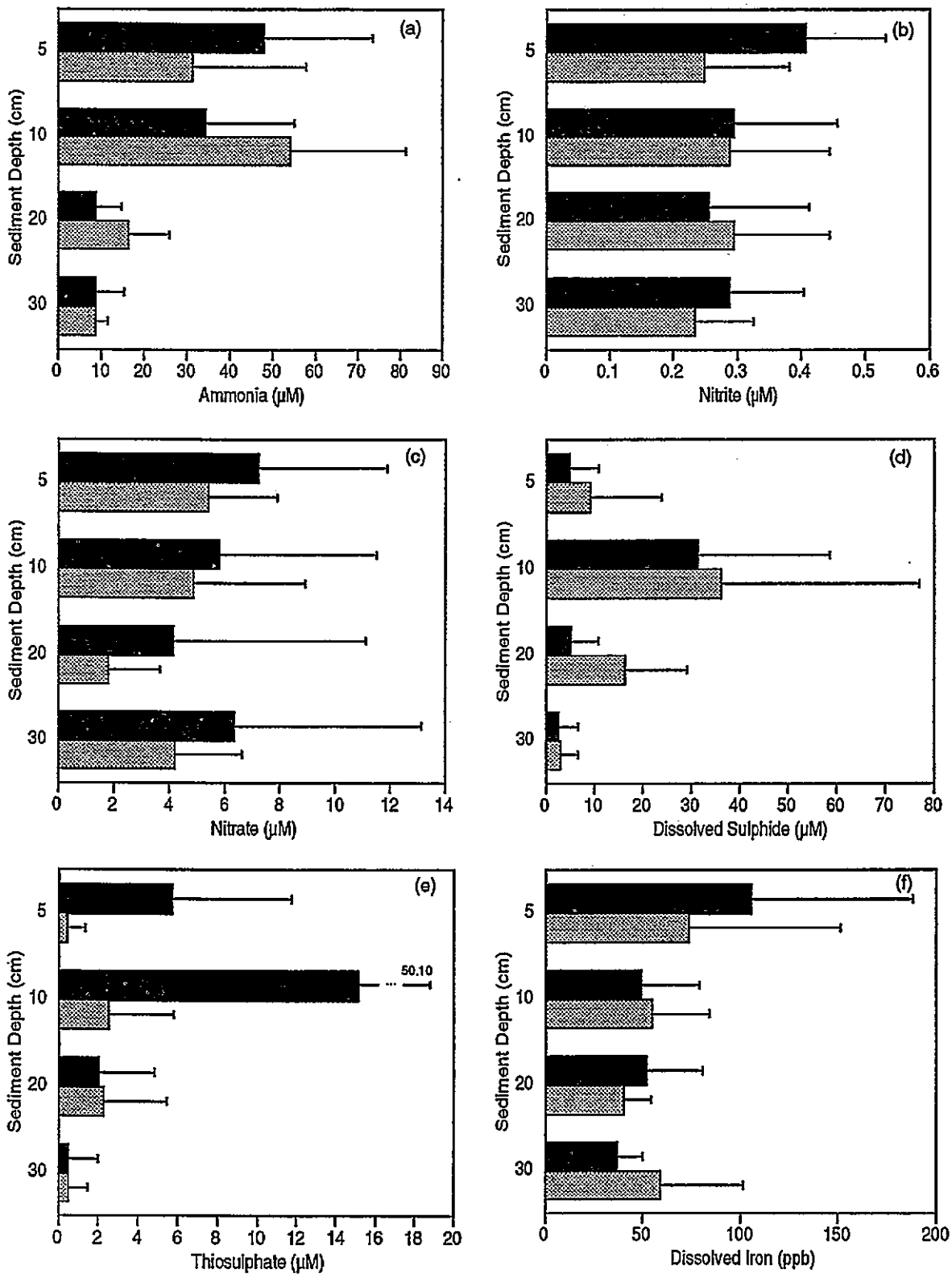

Figure 11. Interstitial water constituents in Bailey's Bay *Thalassia testudinum* sediments as a function of low and high tide in March 1991. Data are plotted as means ± 1 s.d.; $6 \leq n \leq 9$. (a) Ammonia (µM), (b) Nitrite (µM), (c) Nitrate (µM), (d) Dissolved sulphide (µM), (e) Thiosulphate (µM), (f) Dissolved iron (ppb).

■ Low Tide
▒ High Tide

were significantly different from those at 20 and 30 cm sediment depths ($p<0.05$). At all tidal heights, the variance around the mean was much greater in the shallower sediment depths, where concentrations were greater, suggesting greater heterogeneity. Nitrite and nitrate levels remained constant with increasing sediment depth at both high and low tides ($p>0.05$). Ammonia and nitrite levels were not significantly different between low and high tides at any of the sediment depths ($p>0.05$). Although mean nitrate concentrations at low tide were consistently higher than those at high tide, there proved to be no significant difference at any of the sediment depths ($p>0.05$).

Mid-tide sulphide levels (Fig. 7d) appeared higher than those measured at low and high tide (Fig. 11d) but the differences were not statistically significant ($p>0.05$). Interstitial water sulphide concentrations at 30 cm sediment depth were significantly lower than those measured at the 10 cm sediment depth at both high and low tides ($p<0.05$). A similar observation made for the mid-tide sulphide levels proved to be not statistically significant ($p>0.05$). There were no significant differences between interstitial water sulphide concentrations at high and low tide, for any sediment depth ($p>0.05$).

Thiosulphate and dissolved iron concentrations in interstitial water at low and high tide (Figs. 11e and 11f) were comparable to those measured in mid-tide interstitial water in March 1991 (Figs. 7e and 7f). Low- and high-tide thiosulphate and dissolved iron levels did not demonstrate a

linear relationship with depth ($p>0.05$). Although the thiosulphate concentrations at 30 cm sediment depth were low (similar to the mid-tide data), statistical analyses revealed no significant differences between depths at either tidal height ($p>0.05$). There also proved to be no significant differences in dissolved iron concentrations between sediment depths at either low or high tide ($p>0.05$). For both thiosulphate and dissolved iron, there were no significant differences between concentrations in interstitial water collected at high and low tide, at any sediment depth ($p>0.05$).

The water content and organic content were not measured in this study since they were deemed unlikely to demonstrate short-term, tidal variation. Similar to all the interstitial water variables, however, AVS and elemental sulphur levels in the seagrass bed sediments at low and high tides (Figs. 12a and 12b) were comparable with those measured at mid-tide (Figs. 8c and 8d). Analyses revealed that, similar to the AVS and elemental sulphur data collected at mid-tide in March, there were no significant linear relationships between these variables and depth ($p>0.05$), nor any significant changes in AVS or elemental sulphur with sediment depth for either tidal height ($p>0.05$). Neither AVS nor elemental sulphur levels were significantly different between high and low tides for any of the sediment depths ($p>0.05$).

Bivalve distribution in the sediments did not change with tidal height and *Codakia orbiculata* were found only in the 2-4 and 4-6 cm sediment depths at both low and high tides. *C.*

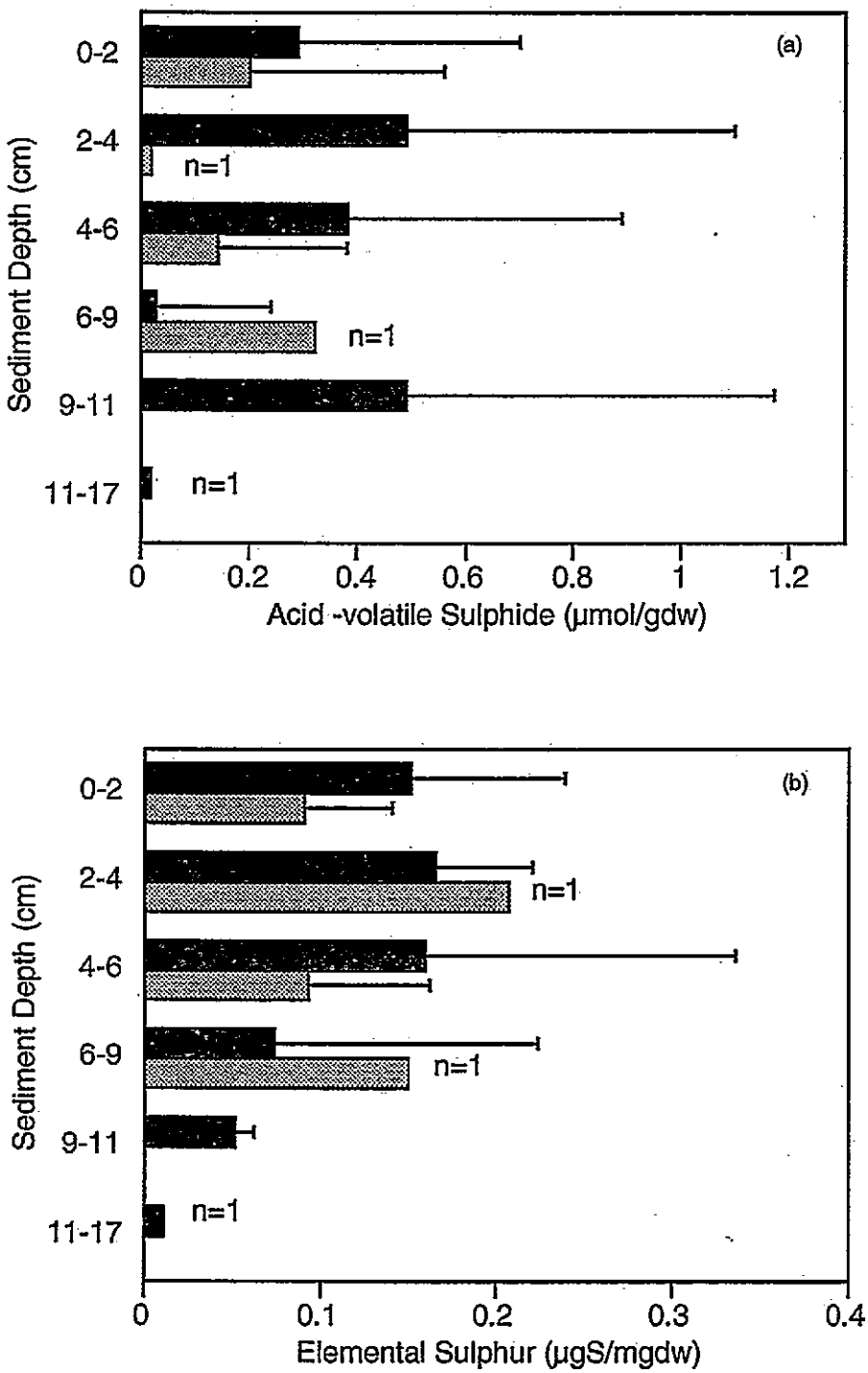

Figure12. Sediment constituents in Baley's Bay *Thalassia testudinum* sediments as a function of sediment depth for low and high tide in March 1991. Data are plotted as means ± 1 s.d.; $3 \leq n \leq 6$, except where noted. (a) Acid-volatile sulphide (µmol/gdw), (b) Elemental sulphur (µgS/gdw).

■ Low Tide

▩ High Tide

orbiculata densities, gill elemental sulphur and gill protein measured at low and high tides (Table 9) were comparable to those measured in March 1991 (mid-tide) study (Fig. 10; Table 6). Mantle fluid sulphide and thiosulphate were measured only on bivalves collected at low tide (Table 9) (high-tide mantle fluid samples were lost from the freezer at Scripps Institute) but, once again, these concentrations were comparable to those measured on bivalves collected in March 1991 at mid-tide (Table 6). Bivalve variables were not tested statistically due to the small sample size but the data suggested no obvious differences between the results of tissue analyses on bivalves collected at low- and high-tidal heights.

In summary, there appeared to be no differences in the relationships between habitat variables and sediment depth as a result of tidal height and no differences in the levels of the variables between low and high tide for any sediment depth. Hence, the *Codakia orbiculata* habitat remained relatively stable throughout the tidal cycle, as did the distribution of the bivalves within the sediment (2-6 cm).

Table 9. *Codakia orbiculata* density, gill tissue protein and elemental sulphur content, mantle fluid sulphide and thiosulphate concentrations as low and high tide. Data are presented as means ± 1 s.d.. Sample size (n) is given beneath the data.

Tide	Density (#/m2)	Gill Protein (mgprotein/ mgww)	Gill Elemental Sulphur (µgatomsS/ gww)	Mantle Fluid Sulphide (µM)	Mantle Fluid Thiosul. (µM)
Low Tide	409.1± 190.2 (9)	0.12± 0.06 (5)	87.39± 24.76 (3)	53.05± 30.82 (7)	298.72± 205.40 (7)
High Tide	304.3± 102.7 (9)	0.07± 0.02 (3)	156.60 (1)		

2.3.3. Comparison of Sediment Chemistry Inside and Outside the Seagrass Bed.

To determine which of the measured environmental variables, if any, might be pivotal to explaining why the occurrence of *Codakia orbiculata* was limited to *Thalassia testudinum* sediments, the sediment chemistry inside the seagrass bed habitat was compared with the sediment chemistry beyond the perimeter of the seagrass bed, where bivalves were not found. This study was carried out in August 1991 and sediment chemistry data inside the seagrass bed were collected as part of the seasonal study. The seagrass bed sediments have, therefore, been described in detail in the seasonal study section and are reported on only as required for comparative purposes in the following text. The data for each variable in the sediments inside and outside the bed were examined for trends across sediment depth, to provide a description of each environment, prior to determination of locational differences.

The interstitial water ammonia concentrations inside and outside the seagrass bed are presented in Figure 13a. Ammonia levels inside the bed demonstrated a significant linear relationship with sediment depth (decreasing with increasing sediment depth)(($p<0.05$; $r^2 = 0.145-0.453$), while those outside the bed did not ($p>0.05$). Despite the obviously lower ammonia levels at 20 and 30 cm sediment depth, analyses revealed no significant difference between ammonia concentrations at any of the sediment depths for either location ($p>0.05$). Variances around the mean ammonia levels

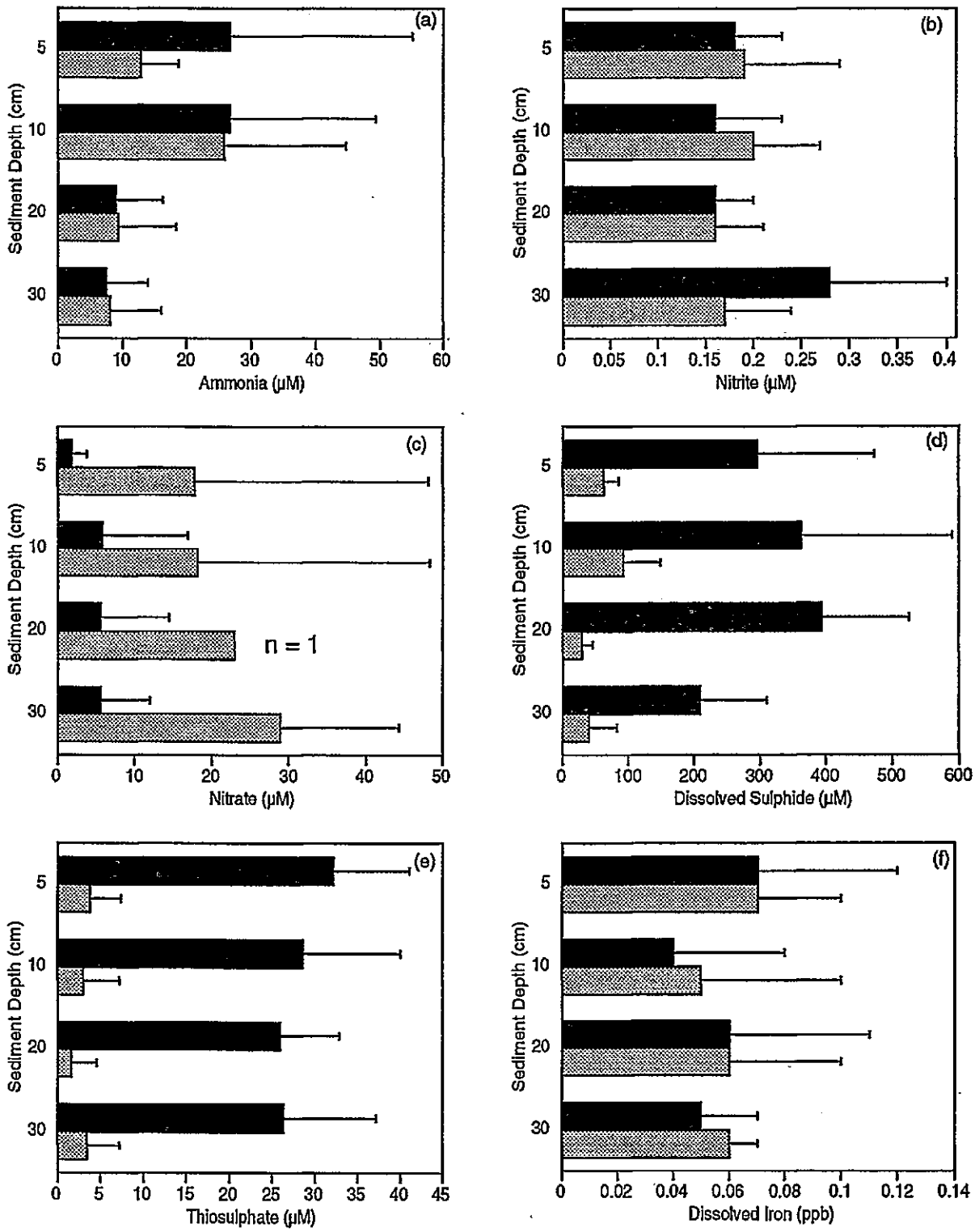

Figure 13. Interstitial water constituents in Bailey's Bay sediments inside the *Thalassia testudinum* bed and outside the perimeter of the seagrass bed as a function of sediment depth in August 1991. Data are plotted as means ± 1 s.d.; $6 \leq n \leq 9$. (a) Ammonia (µM), (b) Nitrite (µM), (c) Nitrate (µM), (d) Dissolved sulphide (µM), (e) Thiosulphate (µM), (f) Dissolved iron (ppb).

■ Inside
▨ Outside

were high, suggesting ammonia levels were highly variable spatially. At each sediment depth, concentrations of ammonia were not significantly different inside and outside the bed ($p>0.05$).

Interstitial water nitrite and nitrate levels inside and outside the seagrass bed are presented in Figures 13b and c. Nitrite and nitrate levels demonstrated no statistically significant linear relationship with sediment depth either inside or outside the seagrass bed ($p>0.05$) and did not change significantly with sediment depth at either location ($p>0.05$). Interstitial water nitrite concentrations were not significantly different between the inside and the outside of the bed at any sediment depth ($p>0.05$). Mean nitrate concentrations outside the bed were obviously much higher than those inside the seagrass bed but, due to the enormous variance around the mean in the outside samples, there was no statistically significant difference between the nitrate levels at the two locations, at any sediment depth ($p>0.05$).

Sulphide, thiosulphate and dissolved iron concentrations in interstitial water from inside and outside the *Thalassia testudinum* bed (Figs. 13d-f) did not demonstrate a linear relationship with sediment depth ($p>0.05$). There was also no significant difference in interstitial water sulphide, thiosulphate or dissolved iron levels between sediment depths at either location ($p>0.05$). Analyses revealed, however, that interstitial water sulphide concentrations inside the seagrass bed (means 208.58 - 394.27 μM) were statistically higher than those outside the bed (means 29.42 - 91.66 μM) at all sediment

depths ($p<0.05$) except 30 cm (Fig. 13d). The interstitial water thiosulphate concentrations inside the bed (means ranging from 25.97 - 32.27 µM) were an order of magnitude, and significantly, higher ($p<0.05$) than those outside the seagrass bed (1.67 µM to 3.88 µM) (Fig. 13e). Iron concentrations were not significantly different ($p>0.05$) between inside and outside the bed at any depth (Fig. 13f).

Sediment water content, organic content and AVS data are shown in Figures 14a-c. Statistical analyses of the data revealed results similar to those obtained in the seasonal study, with no significant linear relationship with sediment depth for any of the variables ($p>0.05$). In addition, there was no significant difference between the sediment water content, organic content nor AVS at any of the depths at either location ($p>0.05$). Water content and AVS were relatively constant between locations for each sediment depth and statistical analyses revealed no significant differences ($p>0.05$). Although the organic content in sediments inside the seagrass bed was higher than that outside the bed for most depths, statistical analysis revealed this difference was not statistically significant ($p>0.05$). The variances around the mean organic content were larger for sediments within the seagrass bed than for those outside the bed, most likely a result of the large rhizomal and root material in the seagrass sediments.

Elemental sulphur content in the sediments, both within and outside the seagrass bed (Fig. 14d), demonstrated a significant linear relationship with sediment depth ($p=0.0011$,

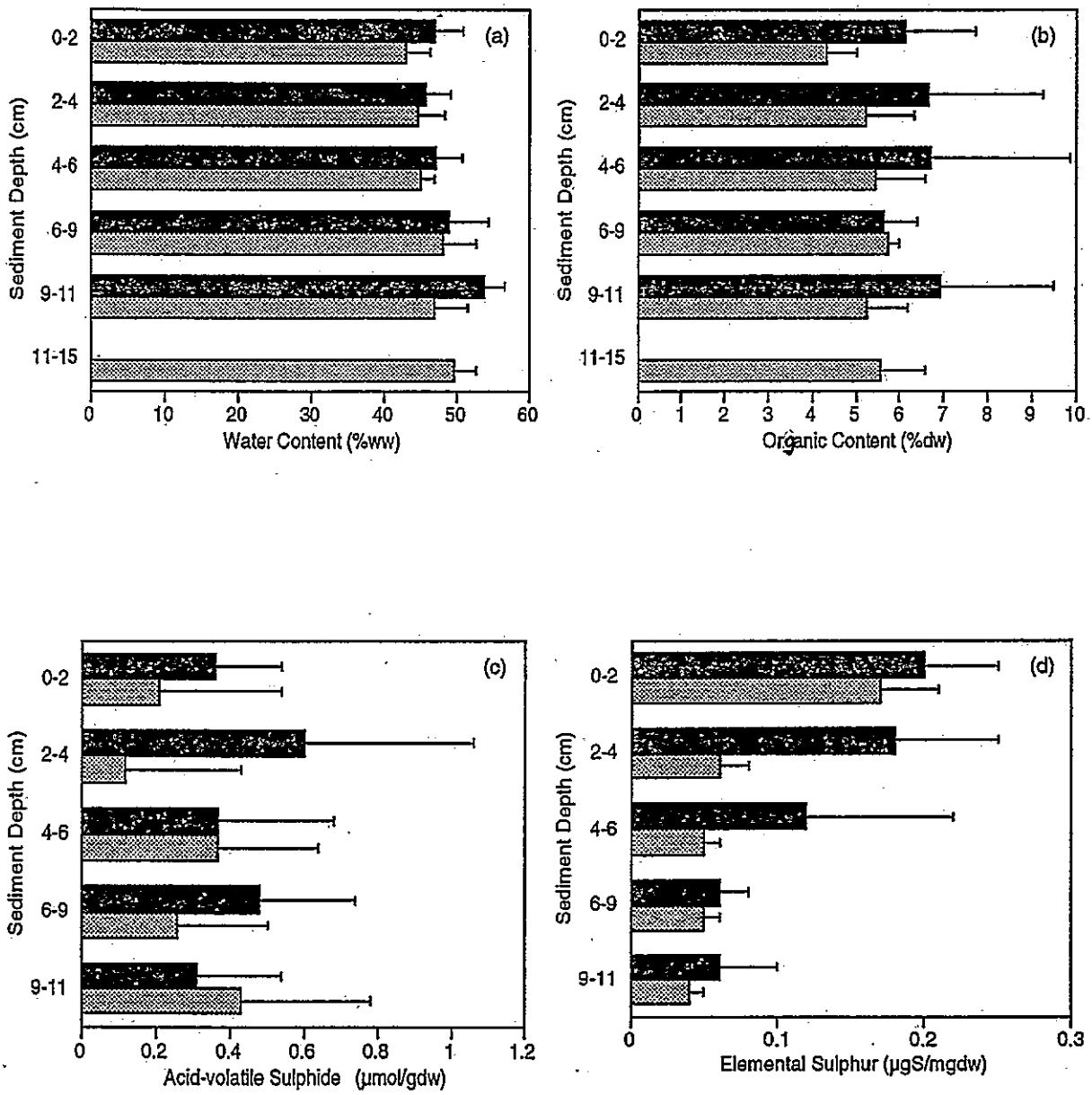

Figure 14. Sediment constituents in Bailey's Bay sediments inside the *Thalassia testudinum* bed and outside the perimeter of the seagrass bed as a function of depth in August 1991. Data are plotted as means ± 1 s.d.; $3 \leq n \leq 9$. (a) Water content (%ww), (b) Organic content (%dw), (c) Acid-volatile sulphide (µmol/gdw), (d) Elemental sulphur (µgS/mgdw).

$r^2=0.329$ and $p=0.001$, $r^2=0.459$ respectively). There was no significant difference in elemental sulphur content between depths at either location ($p>0.05$) and no significant difference between locations, at any sediment depth ($p>0.05$).

In summary, although the majority of the environmental variables measured demonstrated no statistically significant difference between the seagrass bed habitat sediments of *Codakia orbiculata* and those outside the perimeter of the habitat, there were notable exceptions. Thiosulphate was statisticaly higher in seagrass bed sediment interstitial water, at all depths, than in interstitial water collected outside the bed. This was also true for dissolved sulphide at all depths except 30 cm; the latter depth was below the maximum penetration of the rhizosphere within the seagrass bed. In contrast, mean nitrate levels were higher in interstitial water collected outside the perimeter of the bed, although enormous heterogeneity in nitrate levels at each depth outside the bed meant this could not be demonstrated statistically. These results combine to suggest that the sediments outside the seagrass bed were less reducing than those in the *Thalassia testudinum* sediments.

2.3.4. Sulphate Reduction Rates

Results of the sulphate reduction experiments suggested that only a very small quantity of the reduced $^{35}SO_4^{2-}$ was recovered prior to acidification. This was surprising in light of the low dissolved iron levels in the sediments, the relatively low concentrations of acid-volatile sulphides and

the high concentrations of dissolved sulphides (Lyons *et al.*, 1980; Thorstensen and Mackenzie, 1974; this study). Hines and Lyons (1982) also found only a small quantity of reduced $^{35}SO_4^{2-}$ was recovered prior to acidification in Bermuda carbonate sediments and suggested that flushing with nitrogen gas (or argon in this study) was insufficient to remove all of the soluble sulphides. Hence, it seems likely that the measured rate of sulphate reduction to acid-volatile sulphide incorporated some sulphate to dissolved sulphide reduction. For this reason, the sulphate reduction rates were combined and rates expressed as reduction to dissolved sulphide plus AVS.

Mean sulphate concentrations decreased from 27.2 mM in the overlying seawater to 27 mM in the top 2 cm interstitial water, down to 24.1 mM at 9-11 cm sediment depth. Sulphate reduction rates in *T. testudinum* bed sediments measured in October 1991 (Fig. 15) are expressed as the mean of rates measured on three sediment cores, with the exception of the 9-11 cm sediment depth because only one core penetrated below 9 cm. The mean sulphate reduction rates demonstrated a general trend to decrease with increasing sediment depth (998.46 $\pm$ 397.18 to 158.02 nmoles/ml/d) but there was no statistically significant trend nor any significant differences between the rates at each sediment depth. Because sulphate reduction to pyrite was not measured, the sulphate reduction rates presented here may be underestimates of true rates (Howarth,1979).

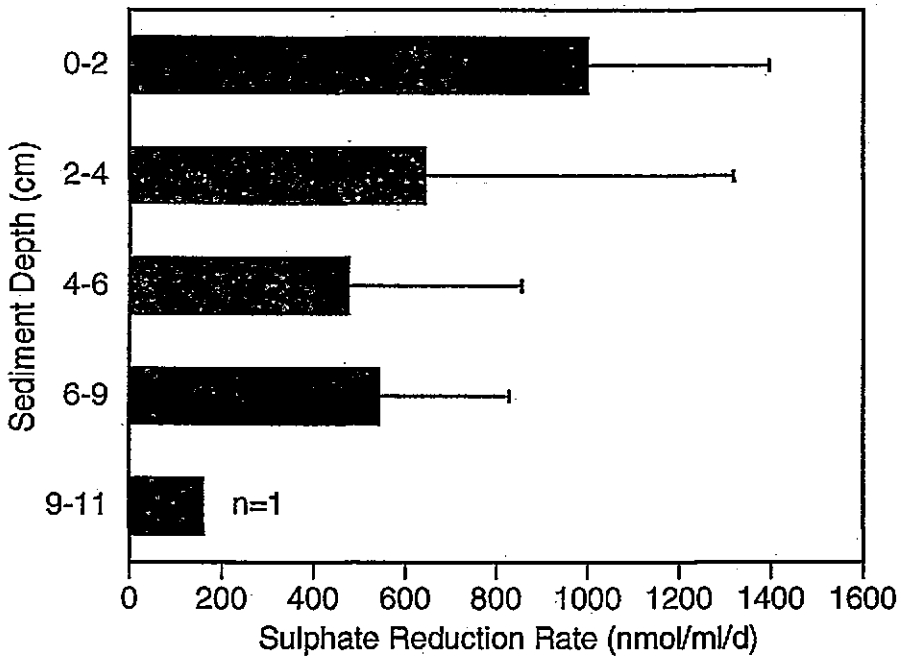

Figure 15. Sulphate reduction rate (nmol/ml/d) to dissolved sulphide and acid-volatile sulphide in *Thalassia testudinum* sediments (October 1991). Data are plotted as means ± 1 s.d.; n=3, except where noted.

2.4. DISCUSSION

2.4.1. Sediment Chemistry

Seagrasses have a marked effect on the chemical and microbiological characteristics of sediments by their production of detritus, their nutrient demand and the flux of oxygen from their roots (Moriarty and Boon, 1989). In addition to trapping external organic material within the bed due to a baffel effect (Ginsburg and Lowenstam, 1958), seagrass leaves add to the organic matter in the sediment when in the final stages of decay (Moriarty and Boon, 1989). Because the microbial community around the seagrass roots is very active metabolically (Foster *et al.*, 1983), decomposition of organic matter can be rapid and near complete, with little or no increase in organic matter in the sediment. Measurements of organic content in the sediments are often variable (Moriarty, 1980), however, due to the difficulty in completely excluding all root and rhizomal material. Organic content in the Bailey's Bay seagrass sediments did not vary with increasing sediment depth and means were generally less than 10 %dw. These sediment samples excluded all rhizomal material but roots were very fine and were difficult to exclude completely. While organic content in the shallower sediments probably represents rapid input and breakdown, organics in deeper sediments may be buried and subject to slower breakdown, depending on availability of electron acceptors (Berner, 1985). The organic content in Bailey's Bay sediments was higher than the 0.2-2.0 %dw organic carbon reported for a

mixed seagrass community in Moreton Bay, Australia (Moriarty, 1980) but samples in the latter study excluded all roots.

Sediment pH values in carbonate sediment interstitial water are usually slightly basic. In this study, mean pH values ranged from 7.24-7.36, which are comparable to values measured in other studies of seagrass carbonate sediments (Patriquin, 1972; Matson, 1985).

Because the lacunae of seagrasses are continuous from leaves to roots, oxygen can move through the lacunae and be released into the sediment from the roots (Oremland and Taylor, 1977; Sand-Jensen *et al.*, 1982; Roberts *et al.*, 1984; Smith, R.D. *et al.*, 1984). Hence, an oxic zone (usually only a few mm wide) can occur around the roots within anoxic sediments. Oxygen diffusion from the roots into the sediments may be maximum during periods of peak seagrass productivity (Howes *et al.*, 1981). Oxygenated "halos" (<1 mm) were observed around the *Thalassia testudinum* roots in sediments in Bailey's Bay (P. Barnes, pers. obs.) and in *T. testudinum* and *Ruppia maritima* beds in Florida (Fisher and Hand, 1984). Because oxygen supplied to the roots is principally derived from leaf photosynthesis (Iizumi *et al.*, 1980, Sand-Jensen *et al.*, 1982; Smith, R.D. *et al.*, 1984), diffusion from the roots at night may be inhibited and sediments in the vicinity of the roots may become anoxic (Smith, R.D. *et al.*, 1984).

Apart from the oxygenated halos observed around the roots (estimated <1 mm wide) and a fine layer of oxidized sediment observed at the sediment-surface (estimated <2 mm), the Bailey's Bay seagrass sediments were sulphide-rich and anoxic.

Redox measurements were not incorporated into this study but the Eh of nearby Bermuda *T. testudinum* sediments ranged from -105 to -305 at 0-15 cm sediment depth (Matson, 1985).

Because seagrass sediments are predominately anoxic, decomposition occurs primarily via dissimilatory sulphate reduction (Howarth and Teal, 1979; Howarth and Giblin, 1983; Moriarty *et al.*, 1985a). Dissolved sulphide produced by sulphate reduction (Jørgensen, 1982) accumulated in Bailey's Bay seagrass sediments, particularly within the rhizosphere; 30.63 µM to 394.27 µM was the maximal seasonal and depth range. These sulphide levels are unlikely to effect *Thalassia testudinum* growth rate (Pulich, 1983) since the plants are capable of maintaining sufficient oxygen concentrations within and surrounding roots to avoid hydrogen sulphide toxicity (at least on a short-term basis) (Penhale and Wetzel, 1983). Fisher and Hand (1984) reported sulphide levels in Florida *T. testudinum* and *Ruppia maritima* beds ranging from a mean of 1.67 to 2.49 mM. Concentrations in temperate seagrass bed sediments range from 0 to 3.5 mM, usually demonstrating seasonal trends with the maximum in the summer (Hines *et al.*, 1989). The interstitial water sulphide maxima in August and high levels in October in Bailey's Bay seagrass sediments suggest peak sulphate reduction rates. Sulphate reduction may be enhanced due to an increase in organic input as a result of the increase in seawater temperatures and the resulting die-off of macro-algae (McGlathery, 1992). Although the seasonal trend observed in seagrass growth rates in Bermuda was not statistically significant, the sulphide maxima do correlate

with slightly lower seagrass growth rates. Lower growth rates may be due to temperature stress (Dennison, 1987). Less oxygen is shunted to the roots during periods of low growth (Howes *et al.*, 1981; Sand-Jensen *et al.*, 1982) and the increase in interstitial water sulphide, therefore, may be partially a result of decreased oxygen release from the roots into the sediments.

The sulphate reduction rates at each sediment depth were highly variable, which may reflect heterogeneity in organic content or in the effects of the seagrass roots (due to DOM and oxygen release, discussed below). Not surprisingly, this heterogeneity in sulphate reduction is reflected in variability in dissolved sulphide concentrations at each sediment depth. In the Bailey's Bay seagrass sediments, sulphate reduction rates remained high and relatively constant down to 9 cm sediment depth. In October, when sulphate reduction rates were measured, dissolved sulphide remained relatively constant throughout the rhizosphere but decreased at 20 and 30 cm sediment depth. The high sulphate reduction rates help to explain the accumulation of dissolved sulphide in the interstitial water despite oxidation by diffusion to the oxygenated sediment-surface seawater (Troelsen and Jørgensen, 1982), by bioturbation effects (Hines *et al.*, 1982) and by release of oxygen from the seagrass roots (Howes *et al.*, 1981). A significant positive correlation between sulphide concentration and sulphate reduction rate was also noted for shallow carbonate sediments in Australia (Skyring and Chambers, 1976).

Sulphate reduction (to sulphide and AVS) rates in Bailey's Bay sediments were comparable to those measured in another *Thalassia testudinum* bed in Bermuda (Hines, 1985) but were x2-4 higher than those reported for Bermuda unvegetated carbonate sediments (Hines and Lyons, 1982). Sulphate reduction rates have been observed to be enhanced x5 (to 2.5 µmol/ml/d) by the release of DOM in the rhizosphere of seagrass beds during active growth (Hines *et al.*, 1989). A correlation between root biomass and sulphate reduction rates was found in Australian seagrass sediments, again hypothesized due to the supply of fermentable organic compounds (Moriarty *et al.*, 1985a). Hence, sulphate reduction rates in seagrass sediments are variable depending on the distribution of the root biomass; for example, in *Syringodium isoetifolium* sediments sulphate reduction was most rapid in the upper 5 cm, while the deeper-rooted *Enhalus aceroides* had rapid rates down to 30 cm (Moriarty and Boon, 1989). The *T. testudinum* rhizosphere biomass appeared relatively consistent down to 15 cm, at which point the biomass noticeably decreased until approximately 20 cm, after which no roots or rhizomes were found. Hence, the observed drop in dissolved sulphide levels between 20 and 30 cm sediment depth coincided with the maximum depth penetration of the rhizosphere and was most likely due to the lack of root biomass and reduced DOM (Foster *et al.*, 1983). It is unlikely that levels of dissolved sulphide were affected by a change in the rate of formation of iron sulphides since there was no change in dissolved iron or AVS levels with increasing sediment depth. Sulphate reduction

rates and dissolved sulphide levels would also be affected by decreased sulphate availability below the rhizosphere. Oxidation of sulphide in the micro-zone around the seagrass roots would keep sulphate supplied to the subsurface sediments in the rhizosphere (Howarth and Teal, 1979; Howes *et al.*, 1981), as would bioturbation. *Codakia orbiculata* was the dominant macro-infaunal species in Bailey's Bay ($\leq 500/m^2$) and formed inlet tubes up to the oxygenated sediment-surface seawater from a maximum depth of 6 cm. Sulphate and oxygen (the latter resulting in sulphide oxidation to sulphate) would be supplied, therefore, to anoxic sediments (Hines *et al.*, 1982; Hines and Jones, 1985). Because the sediments remained generally anoxic, any oxygen transported was probably quickly utilized. In addition, sulphide levels below the rhizosphere may also be influenced by decreased available organic material. Although the percent organic content did not decrease significantly below the rhizosphere, it must be remembered that standing pools are not indicative of the turnover rate and organic content in deeper sediments may represent burial and the absence of sufficient electron acceptors (Berner, 1985).

The above suggestions for the lower sulphide levels below the rhizosphere are also applicable to the lower dissolved sulphide concentrations in interstitial water in unvegetated sediments adjacent to the seagrass bed. Since nitrate is likely to be used prior to sulphate as an electron acceptor by bacteria in marine sediments (Berner, 1970), the higher nitrate levels in interstitial water in the unvegetated

sediments are indicative of a less-reducing environment, suggesting less organic input. Similarly, both sites had comparable levels of dissolved iron and acid-volatile sulphide, so it seems unlikely that lower sulphide levels outside the seagrass beds were a result of increased iron sulphide formation.

Sulphate reduction rates were only measured in Bailey's Bay sediments in October but peak sulphide concentrations in August suggest sulphate reduction rates may also peak at that time, as discussed earlier in this text. There appeared to be no statistically significant seasonal change in seagrass growth rate, suggesting oxygen release from the roots to the sediments remained constant.

The sulphate reduction rates reported and discussed above do not include reduction to pyrite or elemental sulphur. Sulphate reduction experiments on coastal and salt marsh sediments revealed that the short-term end-products of sulphate reduction which are present as pyrite vary from 90% to <10-15% (Howarth, 1979; Howarth and Teal, 1979; Howarth and Giblin, 1983; King *et al.*, 1985). It is difficult to predict, therefore, the extent to which the sulphate reduction rate was underestimated due to the failure to include pyrite and elemental sulphur. However, it is unlikely that pyrite levels were high in Bailey's Bay carbonate sediments. Pyrite is formed by the reaction of AVS with elemental sulphur (Berner, 1970; Goldhaber and Kaplan, 1974; Berner, 1984) or more rapidly by precipitation from solution after the reaction of ferrous iron (from dissolution of iron monosulphides) with

polysulphides that originate from elemental sulphur and dissolved sulphide (Rickard, 1975; Boulegue and Michard, 1979). Although the formation of iron sulphides in most marine sediments is limited by available organic matter for bacterial sulphate reduction, and not by sulphate or iron availability, in carbonate sediments iron is often a limiting factor (Berner, 1985). Carbonate sediments generated by calcifying organisms, like those in Bermuda, are unlikely to contain much iron (Patriquin, 1972; Entsch *et al.*, 1983) and, in addition, there is a lack of continental detrital input of iron to the carbonate sediments (Lyons *et al.*, 1980; Hines and Lyons, 1982; Hines *et al.*, 1989). Certainly, in Bailey's Bay seagrass sediments, levels of sulphate and organic matter, plus sulphate reduction rates, were high. Dissolved iron concentrations were low, however, and ranged from 22-95 ppb. Unvegetated carbonate sediments in Bermuda have previously been found to contain from 55.85-145.2 ppb (Hines and Lyons, 1982), which were comparable to those measured outside the seagrass bed in Bailey's Bay. By comparison, dissolved iron in terrigenous nearshore environments range from 56-6702 ppb in the top 20 cm sediment (Nedwell and Abram, 1978; Howarth, 1979; Hines and Jones, 1985). In sediment adjacent to a *Zostera* bed in England, Dando *et al.* (1986a) measured dissolved iron concentrations of approximately 56-335 ppb. Low levels of dissolved iron in Bailey's Bay interstitial water, in combination with the high levels of dissolved sulphide, suggest available iron may limit the formation of iron sulphides. It has been noted that in organically rich,

anaerobic sediments, sulphate reduction can result in almost complete titration of soluble iron, so that dissolved sulphide exists in excess of that precipitated as iron sulphides (Novitsky *et al.*, 1980).

Acid-volatile sulphide, formed by precipitation of dissolved iron with sulphide, was found in low levels in Bailey's Bay seagrass sediments (0.16 - 0.96 µmol/gdw) in comparison to levels of up to 25 µmole/gdw reported for coastal sediments (Jørgensen, 1977; Hines and Jones, 1985; King *et al.*, 1985). AVS may be ephemeral in sediments as a result of oxidation or pyrite formation (Luther *et al.*, 1982) but, as discussed above, pyrite formation is unlikely to be significant in the low iron carbonate sediments (Patriquin, 1972) found in Bermuda. Both AVS and pyrite formed in the *T. testudinum* beds may be oxidized if mixed with oxygen released by the seagrass rhizomes or introduced through bioturbation (Berner, 1964b). This "oxidizing power" of the seagrass roots can also encourage biotically mediated oxidation of pyrite and other reduced sulphur compounds to sulphate and thiosulphate (Howarth and Teal, 1979; Howes *et al.*, 1981; Howarth *et al.*, 1983). Hence, variation in reduced sulphur compounds, may be correlated with seagrass productivity (Luther *et al.*, 1982) and diel rhythms (Smith, R.D. *et al.*, 1984).

Thiosulphate concentrations, although low, followed generally similar trends to sulphide: no trend with sediment depth, maximum levels in August and significantly higher levels in the seagrass sediments than in adjacent bare sediments. Thiosulphate levels in seagrass interstitial water

could not be found in the literature for comparison. Bailey's Bay thiosulphate levels were comparable, however, to those reported for sediments at 500 m in Santa Barbara Basin (Cary *et al.*, 1989) but higher than those reported for an intertidal zone in the Salcombe estuary in England (Dando *et al.*, 1986a). Although thiosulphate in the interstitial water may be a chemical or bacterial oxidation product of sulphide (or a chemical oxidation product of pyrite, if present) (Jørgensen, 1982; Goldhaber, 1983), it was not inversely correlated with sulphide in *Thalassia testudinum* sediments in Bermuda. This same result was found by Howarth *et al.* (1983) in a temperate salt marsh. Although thiosulphate is chemically less reactive than dissolved sulphides, it can be rapidly reduced biologically to sulphide by sulphate-reducing bacteria (LeGall and Postgate, 1973) and it can also be bacterially oxidized to sulphate (Kuznetsov, 1970). The fact that thiosulphate concentrations follow those of sulphide in the Bailey's Bay interstitial water suggests at least a partially dynamic relationship, with thiosulphate concentration possibly controlled by an equilibrium relationship with sulphide.

Elemental sulphur data for seagrass or carbonate sediments, for comparison with Bailey's Bay seagrass sediments, was unavailable in the literature. However, Bailey's Bay elemental sulphur levels (0.05 to 0.5 µgS/mgdw or 1.48 - 14.8 µmol/cm^3 or 1,480 - 14,800 µmol/dm^3) were comparable with those reported for Danish coastal marine sediments which decreased from 14-4 µmol/cm^3 from 1-12 cm sediment depth (Howarth and Jørgensen, 1984). In contrast,

elemental sulphur in the Salcolmbe Estuary in England was much lower, ranging from 80-4000 µmol/dm^3 (Dando *et al.*, 1986a).

Elemental sulphur precipitates in sediments due to bacterial processing of sulphides, thiosulphate or both (Kuznetsov, 1970) but some of the elemental sulphur measured in the sediments may be accumulated in the cells of sulphide-oxidizing bacteria. Elemental sulphur can also be produced chemically by the rapid oxidation of sulphide (Whitcomb *et al.*, 1989) and by mixed oxidation of thiosulphate to elemental sulphur and sulphate at the surface of the grass roots (Luther *et al.*, 1982). It is not surprising, therefore, that elemental sulphur levels are higher in the shallower sediments, where sulphate reduction rates, sulphide and thiosulphate levels were also higher. The lack of negative correlation between elemental sulphur and sulphide or thiosulphate may be a result of the dynamic nature of the sediments. There is a high sulphate reduction rate, constantly producing sulphide, and thiosulphate is continually being formed by the oxidation of sulphide through oxygen available by diffusion from the sediment surface, by bioturbation or by release from seagrass roots. As a result, elemental sulphur precipitation would not affect the standing pool levels of sulphide or thiosulphate.

Although elemental sulphur is also the chemical oxidation product of ferrous sulphide in marine sediments (Nelson *et al.*, 1977), levels of dissolved iron and AVS in Bailey's Bay seagrass sediments suggest that this process did not make a significant contribution to elemental sulphur production.

Elemental sulphur produced in Bailey's Bay sediments may also be reduced back to sulphide by hetrotrophic bacteria (Pfenning and Biebl, 1976; Postgate, 1979) or oxidized by bacteria using oxygen (Suzuki, 1965a), ferric iron (Brock and Gustafson, 1976) or nitrate (Baalsrud and Baalsrud, 1954; Troelsen and Jørgensen, 1982). It is unlikely, in light of the relatively low dissolved iron and AVS levels, that the levels of elemental sulphur in the sediments are significantly affected by its combination with AVS to form pyrite (Berner, 1985).

Concentrations of ammonia in the interstitial water in Bailey's Bay sediments ranged from means of 4.26 to 30.63 μM within the rhizosphere. These levels were comparable to those reported for *Thalassia testudinum* interstitial water in the West Indies (Patriquin, 1972) and Florida (Fourqurean *et al.*, 1992) and for *T. hemprichii* interstitial water in Australia (Boon, 1986). Ammonia concentrations as high as 1.5 mM have been reported for *Zostera marina* sediments in Alaska (Iizumi *et al.*, 1982) but can be highly variable even between closely situated seagrass beds (Short *et al.*, 1985; Morse *et al.*, 1987).

Ammonia concentrations in Bailey's Bay seagrass sediments were highest in the shallow sediments, which corresponded to the zone of maximum sulphate reduction. A similar relationship between ammonia and sulphate reduction reactions in Bermuda sediments was noted by Hines and Lyons (1982). Bailey's Bay *Thalassia testudinum* sediments are largely anoxic from the sediment surface and so the accumulation of ammonia

in the shallow interstitial water is not surprising. Some diffusion into the sediment-surface seawater and oxidation by nitrifying bacteria may be occurring in the top few mm (Fourqurean *et al.*, 1992). Ammonia in the interstitial water is regenerated by the dissimilatory reduction of nitrate and as a result of rapid deamination of amino acids in the sediments (Boon *et al.*, 1986b). The latter may be dominant in seagrass sediments, particularly around the roots and rhizomes (Jørgensen *et al.*, 1981a; Smith, G.W. *et al.*, 1984). The amino acids may be derived from decomposing tissue, exudates from living seagrasses or products released by invertebrates (Boon and Moriarty, 1989), such as the abundant lucinid bivalves. The absence of these conditions below the rhizosphere would explain the observed drop in interstitial water ammonia levels in Bailey's Bay. Lower ammonia levels would be expected, therefore, in unvegated sediments but this was not the case in Bailey's Bay. It is difficult to compare ammonia levels inside and outside the bed without measuring turnover times, however. Although comparable with ammonia concentrations outside the bed, those inside the bed undoubtedly reflect both production of ammonia and uptake by the seagrasses. Whether interstitial ammonia levels are higher or lower in seagrass sediments than in adjacent unvegetated sediments depends largely on the rates of ammonia regeneration relative to those of utilization (such as nitrification and assimilation) and diffusion from the sediment (Boon and Moriarty, 1989).

Along with ammonia production, oxidation of ammonia by nitrifying bacteria may also be occurring in oxidized

microniches throughout the rhizosphere sediments as a result of oxygen transport through bioturbation (Hines *et al.*, 1989). Oxygen may also be available to bacteria, although in smaller amounts, by release from *Thalassia testudinum* roots (Howes *et al.*, 1981). This may be why interstitial water ammonia levels frequently demonstrate no uniform trend in concentration with depth within the rhizosphere. However, the lack of a uniform trend can also be partially attributed to spatial heterogeneity in ammonia levels present at each depth (Fourqurean *et al.*, 1992). In *T. testudinum* beds in the Bahamas, ammonia concentrations were noted to be more variable than those in unvegetated sediments (Morse *et al.*, 1987).

Nitrate concentrations in Bailey's Bay *Thalassia testudinum* sediment interstitial water were higher than those of nitrite and, in addition, the combined nitrate and nitrite concentrations were lower than those of ammonia. These same results have been observed for other tropical seagrass beds (Koike and Hattori, 1978a,b; Iizumi *et al.*, 1980; Horrigan and Capone, 1985). Nitrate plus nitrite concentrations reported in the latter studies and those reported by Patriquin (1972), for *T. testudinum* sediments in the Caribbean and Boon (1986), for *Thalassia hemprichii* sediments in Australia, were comparable to those found in Bailey's Bay *T. testudinum* interstitial water. Nitrate concentrations in overlying water in the summer ranged from 0.91 µM in September to 1.60 µM in June (McGlathery, 1992).

In seagrass beds, the nitrate-reducing bacteria may utilize virtually all the nitrate and nitrite produced by

nitrifying bacteria (Horrigan and Capone, 1985), resulting in relatively low, stable levels. Accumulation of nitrate, as observed in Bailey's Bay seagrass sediments, is suggestive of an increase in nitrification over nitrate reduction processes. Neither nitrate nor nitrite concentrations changed with increasing sediment depth either inside or outside the seagrass bed sediments, suggesting no change in the rates of nitrification and nitrate reduction. Nitrification has been noted to occur deeper in sediments in response to the oxygenated zones around seagrass roots (Iizumi *et al.*, 1980; Penhale and Wetzel, 1983; Thursby, 1984) and micromolar concentrations of nitrate plus nitrite are often detected deep in seagrass bed sediments (Patriquin, 1972; Iizumi *et al.*, 1980; Boon, 1986b). It is also possible that some nitrate diffused downwards from zones of active nitrification (Moriarty and Boon, 1989). Surprisingly, nitrate and nitrite levels at 30 cm sediment depth, below the rhizosphere, were comparable to those within the rhizosphere and levels in unvegetated sediments were also comparable to those within the rhizosphere. This same result was recorded in *Thalassia testudinum* sediments in the Bahamas (Patriquin, 1972). This result requires further investigation but does suggest nitrification is occurring outside the rhizosphere.

It has been suggested that most nitrate in seagrass sediments is reduced to ammonia by a dissimilatory process and that loss of nitrate through denitrification is only minor (Iizumi *et al.*, 1980; Boon, 1986c). Respiratory reduction of nitrate to nitrite may also be widespread in seagrass

sediments (Nedwell, 1975). Nitrogen cycling is more complex in seagrass sediments due to assimilatory uptake by the seagrasses, by competitition by the seagrasses with nitrifying bacteria for ammonia and by creating oxic zones aroung their roots. Nitrate uptake rate by seagrass roots is lower than that of ammonia uptake, however (Short and McRoy, 1984).

In summary, the *Thalassia testudinum* sediments in Bermuda were enriched organically and generally anoxic. Sediment chemistry within the rhizosphere was relatively uniform with regard to sediment depth, yet heterogeneous spatially. The latter may be a result of pockets of organic enrichment or oxygenation as a result of oxygen release by bioturbation or, to a lesser extent, by the seagrass roots. High sulphate-reduction rates and nutrient concentration were most likely influenced by release of oxygen and DOM from the seagrass below-ground biomass into the surrounding sediments. In contrast to the relatively low levels of acid-volatile sulphides and dissolved iron, dissolved sulphide concentrations were high in interstitial water. It is hypothesized, therefore, that iron sulphides were not major contributors to the reduced sulphur pool. Micromolar levels of nitrate, at several cm sediment depth, may be the result of nitrification occuring deeper in the sediments due to the release of oxygen from the roots. Ammonia levels were high, probably as a result of deamination. Seasonal trends in sediment chemistry were apparent only for dissolved sulphide and thiosulphate; levels for both peaked in August. It is hypothesized that sulphate reduction rates may have been

greater in August than the rates measured in October, possibly due to increased organic input as a result of macro-algae die-off due to the increased seawater and sediment temperatures (McGlathery, 1992). This may have occurred in conjunction with decreased oxygenation of sediments by *T. testudinum*, as a result of slightly lower growth rates (Howes *et al.*, 1981), possibly due to temperature stress (Dennison, 1987).

2.4.2. *Thalassia testudinum* Sediments as Habitat for Bacteria-Bivalve Symbioses

Lucinid bivalve populations reach very high densities in seagrass bed sediments. *Codakia orbiculata* density in the *Thalassia testudinum* sediments in Bermuda ranged from 300 to 500/m^2, while the density of *Lucina floridana* in seagrass bed sediments in Florida was 83 $\pm$ 11 /m^2 (Fisher and Hand, 1984). In comparison, *Myrtea spinifera* occurred in densities of $\leq$25/m^2 in fjords (Dando *et al.*, 1985). *Lucinoma borealis* was found in mean densities of 6/m^2 in shallow-water but 110/m^2 in fjord sediments (Dando *et al.*, 1986a). In contrast, *L. borealis* reaches densities of 1500/m^2 in seagrass beds on the west coast of Brittany (Monnat, 1970). It has frequently been noted, and was observed in this study, that lucinids are found in areas where there is a lack of other infaunal species (Allen, 1958; Monnat, 1970; Jackson, 1972).

The sediment chemistry of the Bailey's Bay seagrass bed sediments, described in the previous section, suggests this environment may be optimal for supporting chemoautotrophic symbioses. Sulphate reduction rates, measured in October, were high and dissolved sulphide accumulated in the interstitial water throughout the year, despite sulphide oxidation from bioturbation, from oxygen released from the seagrass roots or from utilization by free-living bacteria or by the symbiosis. Similarly, dissolved sulphide accumulated (1.67 $\pm$ 0.31 mM) in the Florida seagrass habitat of *Lucina floridana* (Fisher and Hand, 1984).

Thiosulphate concentrations in interstitial water in the *Codakia orbiculata* habitat were low, as were those reported for the habitats of *Lucinoma aequizonata* (Cary *et al.*, 1989) and *Lucinoma borealis* (Dando *et al.*, 1986a). Thiosulphate is ephemeral in the environment since it is bacterially reduced to sulphide and oxidized to sulphate (LeGall and Postgate, 1973; Kuznetsov, 1970). It has been demonstrated that neither *Solemya reidi* nor *L. aequizonata* can accumulate thiosulphate in the blood at higher levels than are found in the environment (Cary *et al.*, 1989; Wilmot and Vetter, 1992) and, hence, it appears that although thiosulphate may be an important energy source for the symbiosis as a result of host mitochondrial oxidation (Anderson *et al.*, 1987; Cary *et al.*, 1989; O'Brien and Vetter, 1990), it is unlikely to be a significant source of exogenous reduced sulphur. It should be noted that unlike mantle fluid sulphide concentrations, which closely followed interstitial water concentrations, thiosulphate concentrations in bivalve mantle fluid were an order of magnitude higher than those in interstitial water. A similar observation was made for *L. aequizonata* (Cary *et al.*, 1989) but was not interpreted. These results are difficult to explain and deserve further attention. It is suggested, however, that it is unlikely that the bivalve is releasing thiosulphate, produced by sulphide oxidation, into the mantle fluid. Thiosulphate is a charged molecule and does not diffuse easily (Holmes and Donaldson, 1969). Thiosulphate production through the oxidation of sulphide chemically, or by free-living sulphide-oxidizing bacteria (Jørgensen, 1982), in

the mantle cavity of the bivalve is a possibility but needs further research.

Unlike the *Codakia orbiculata* and *Lucina floridana* seagrass sediment habitats, many lucinids are found in sediments with barely detectable levels of dissolved sulphide (Dando *et al.*,1985, 1986a; Cary *et al.*, 1989). Elemental sulphur accumulation in the gills (Dando *et al.*, 1986a) or elevated sulphide and thiosulphate concentrations in the blood of freshly collected specimens (Cary *et al.*, 1989) suggest, however, that lucinids found in sulphide-poor habitats have access to a reduced sulphur source. These low sulphide habitats have high levels of iron sulphides in the sediments. AVS levels within the *Lucinoma borealis* habitat in shallow-water sediments near a *Zostera* bed in England (2-14 $mmol/dm^3$)(Dando *et al.*, 1986a) were an order of magnitude higher than those reported for the *C. orbiculata* habitat. This was also true for the AVS levels reported for the *Solemya borealis* habitat, where pyrite was also high, ranging from 50 to almost 200 $mmol/dm^3$ (Conway *et al.*, 1992). AVS levels reported for the *Lucinoma aequizonata* habitat in Santa Barbara Basin were comparable to those found in *C. orbiculata* habitat, <4 µmoles/gdw, while pyrite levels were substantially higher at 5-20 µmoles/gdw (Cary *et al.*, 1989). Although pyrite was not measured in the *Codakia orbiculata* habitat, the low levels of available iron suggest it is unlikely to make a significant contribution to the reduced sulphur pool. Dissolved iron concentrations in the habitats of *L. borealis* and *L. aequizonata* (Dando *et al.*, 1986a; (Cary *et al.*, 1989) were

approximately x3 and x100, respectively, those found in the *C. orbiculata* habitat in Bailey's Bay. In these low sulphide environments with high levels of iron sulphides, the pumping of oxygenated seawater through the bivalve's anterior inhalant tube (Allen, 1958) and a combination of chemical and microbial oxidation of iron sulphides could form suitable products to fuel the symbiosis (Dando *et al.*, 1986a). Thiosulphate and sulphide are produced as a result of artificially pumping oxygenated seawater through sediments high in iron sulphides (Dando and O'Hara, 1990; Southward and Southward, 1990; Barnes, unpubl. data). Access to dissolved sulphide through oxidation of sediment-bound sulphides would seem, therefore, an adequate means of fuelling these symbioses in habitats where oxygen is not limiting. Although the habitat of *L. aequizonata* had substantial pyrite deposits which could be accessed in this way, the oxygen levels in the overlying seawater in this habitat were <20 µM (Cary *et al.*, 1989) and so oxidation of the sediments seems unlikely. It has been suggested that the bivalves must be accessing short-lived pockets of sulphidic mud reached by the bivalve's burrowing foot (Cary *et al.*, 1989). This was not apparent from the sediment chemistry, however, and has yet to be demonstrated.

It is unlikely that sediment-bound sulphides, like thiosulphate, serve as a significant source of reduced sulphur for the *Codakia orbiculata* symbiosis. The availability of dissolved sulphide in the *C. orbiculata* habitat in Bermuda seagrass sediments, and the relatively low levels of AVS and

dissolved iron, suggest that sediment-bound sulphides are of comparatively little importance in supporting this symbiosis.

The minimum reduced sulphur concentration necessary to support bacteria-bivalve sulphide-oxidizing symbioses has yet to be determined. However, Dando *et al.* (1985) and Conway *et al.* (1992) have used environmental data plus literature values for conversion efficiences to speculate upon symbioses sulphide requirements *versus* availability. Both use the approach of Kelly and Kuenen (1984) to estimate the symbiont sulphide oxidation requirement to support an estimated carbon fixation rate (for each mole of reduced sulphur oxidized by the symbionts, 0.2 mole of carbon is fixed by the bacteria). Using the calculations of Dando *et al.* (1985) for *Myrtea spinifera* (adjusted for *Codakia orbiculata* wet weight and using an average density of 400 *C. orbiculata*/m^2), the sulphur utilization rate for these bivalves for growth would be 0.52 - 2.40 µmoles sulphide/l sediment/h (not including reproductive effort). The sulphate reduction rates in *C. orbiculata* habitat ranged from means of 500-1000 nmol/mlsediment/d or 20.83 - 41.67 µmoles/l sediment/h. Sulphate reduced to AVS (and possibly to pyrite), would be still available to the symbiosis after conversion to sulphide (or thiosulphate). Hence the following calculations are still appropriate. Since the probable internal pH (Wilmot and Vetter, 1992) and that of the interstitial water is 7.5, these rates must be adjusted for the proportion of dissolved sulphide which could diffuse into the cells (H_2S) at this pH (approximately 20%) (Millero, 1986). Thus, 4.16-8.33 µmoles/l sediment/h would be available

for diffusion into the cells. This is higher than the suggested sulphide flux required (0.52 - 2.40 µmoles sulphide/l sediment/h).

Conway *et al.* (1992) used the oxygen consumption rates of whole *Solemya reidi* (2.0-4.3 µmoles/gww/h)(Anderson *et al.*, 1987) as comparable to that of *Solemya borealis* in order to compare bivalve sulphide requirements with sulphide availability. By taking into account body weight and using a respiratory quotient of 1.0, they suggested 4.6-9.9 µmoles organic C/individual/h was required to support the respiration rate. Using the approach of Kelly and Kuenen (1984), the minimum symbiont sulphur oxidation rates required to support the symbiosis respiration rates would be 23-50 µmol sulphide/ individual/h. This assumes 100% carbon translocation, unlike the 20% suggested by Kelly and Kuenen (1984) and used by Dando *et al.*, (1985). Since the sulphide input into *S. borealis* sediments was measured at 220 µmol/m2/h, the sulphur demand of one individual would be 11-23% of the daily sulphur input, suggesting the bivalves must mine sediment-bound sulphides (Conway *et al.*, 1992). Oxygen consumption rates for whole *Codakia orbiculata* measured in this study were highly variable and, thus, were not incorporated into Chapter 3. The oxygen consumption rates ranged from 0.6-12 µmolesO_2/gww/h as a result of individual variation and of stimulation by thiols. The enormous range in the aerobic respiration rates suggests these calculations are of questionable utility (the maximum rate is extraordinarily high for bivalves of *Codakia orbiculata* size (R. Reid, pers. comm.) but the range is used

only for estimating purposes, as follows. Following the calculations of Conway *et al.*, (1992), 0.1-2.0 µmol sulphide/individual/h are required at 100% carbon translocation or 0.5-10.0 µmolsulphide/individual/h at 20% carbon translocation. Further calculations are based on 20% efficiency. Using a *C. orbiculata* mean density of 400/m^2, the population requires 200-4000 µmolsulphide/m^2/h. Sulphate reduction rates in the top 6 cm of sediment ranged from approximately 500-1000 nmol/mlsediment/d or 0.021-0.042 µmol/mlsediment/h. Calculating the volume (in ml) in the top 6 cm of 1 m^2, this sulphate reduction rate is equivalent to 1260-2520 µmol/m^2/h. Adjusting for pH and the proportion of sulphide present as freely diffusable H_2S results in a rate of 250-500 µmol/m^2/h, suggesting that the supply of reduced sulphur to the sediments is adequate to meet 12.5-100% of the sulphur demands of the *C. orbiculata* population. The accumulation of free sulphide in the interstitial water (Chapter 2) suggests close to 100% of the sulphur requirements of the symbiosis are met and the low 12.5% estimation is a result of the unusually high maximal respiration rate. These estimates also assume 100% dependence of chemoautotrophy and it should be noted that the lucinids, possessing a functional gut, may obtain a significant portion of their carbon from heterotrophy (Cary et al., 1989).

The environmental data clearly demonstrated heterogeneity of the sediment chemistry within the *Thalassia testudinum* sediments, implying that individual *Codakia orbiculata* may be exposed to a range of conditions. This is supported by the

variation in mantle fluid sulphide levels and the elemental sulphur content in the bivalve gills. The latter is indicative of the exogenous supply of reduced sulphur (Vetter, 1985; Chapter 3). Although the heterogeneity apparent in the environmental data suggested that individual *C. orbiculata* may be exposed to potentially toxic sulphide concentrations (Bagarinao, 1992), access to oxygenated seawater via the inlet tube would reduce the vulnerability of the animal by lowering the sulphide levels in its' immediate vicinity. Upon accessing the sulphide, however, sulphide detoxification mechanisms (host mitochondrial oxidation and non-enzymatic oxidation), as discussed at length in Chapters 1 and 3, are suggested.

In addition to the availability of adequate sources of reduced sulphur, potential sources of nitrogen for bacteria-bivalve symbioses are also of interest. It has been suggested that ammonia, which is often abundant in interstitial water, may be a primary source of N to bacteria-bivalve symbioses (Southward,1987). Lee *et al.* (1992a) suggested this to be the case for *Solemya reidi*, based on ammonia uptake rates and average interstitial ammonia concentrations of 54-64 µM in the habitat. This may also be true for *Codakia orbiculata*, whose habitat ammonia levels were also high (25-31 µM). Similar levels were reported for the Santa Barbara Basin habitat of *Lucinoma aequizonata* (Cary *et al.*, 1989) but those reported by Dando *et al.* (1986b) for the Norwegian fjord habitat of 2 species of lucinid were significantly higher. An additional potential source of nitrogen is dissolved free amino acids

(DFAA). Lee *et al.* (1992a) reported concentrations ranging from 2.60-14.84 μM in the interstitial water of *S. reidi* habitat and suggest that, given moderate uptake rates, DFAA uptake likely contributes a significant fraction of the N (and C) needs of the symbiosis. Uptake and utilization of DFAA by marine invertebrates has been well-documented (Stephens, 1988; Wright, 1988). Interstitial levels of DFAA were not measured in the *C. orbiculata* habitat but concentrations of 140 μM have been reported from seagrass bed sediments in Australia (Boon, 1986). It is important to note that unlike the gutless *S. reidi*, *C. orbiculata* and other lucinids retain a small gut and may obtain some nitrogen via heterotrophy.

Availability of oxygen is considered to be an important requirement of the habitat of bacteria-bivalve symbioses. Oxygen is essential for host respiration and may be utilized in sulphide oxidation (detoxification) by the host and sulphide oxidation by the endosymbionts (Powell and Arp, 1989; O'Brien and Vetter, 1990; Wilmot and Vetter, 1992; Chapter 3). Lucinids can extend their vermiform foot, which forms the anterior inhalant tube, up to x6 the length of the shell to maintain contact with the sediment-surface seawater and to obtain oxygen (Allen, 1953; P. Barnes, pers. obs.). Using this formula, the 6-10 mm *Codakia orbiculata* could burrow down to 3.6-6 cm sediment depth; these bivalves were found, not surprisingly, between 2-6 cm sediment depth in *Thalassia testudinum* sediments. Similar to *C. orbiculata*, *Lucina floridana* was not found below the rhizosphere (20 cm) or in areas devoid of seagrasses (Fisher and Hand, 1984). Inhalant

tubes have not been observed *in situ* for *L. floridana* and it has been suggested that the association with the roots of *T. testudinum* and *Ruppia maritima* is based on the bivalve's utilization of oxygen released from the roots (Fisher and Hand, 1984). Photosynthetic oxygen release from the seagrass roots is minimal, however, and is subject to diel change (Smith *et al.*, 1988). It seems more likely, therefore, that these bivalves obtain oxygen from the overlying seawater. An average size *L. floridana* (34 mm shell length) could burrow to a maximum depth of 20.4 cm and maintain contact with the surface (Abbot, 1974; Allen, 1953). Inhalant tubes have rarely been observed *in situ* for *C. orbiculata* in Bailey's Bay because the tubes are thin and cryptic and, as a result, are almost impossible to identify amongst the rhizomal mat. The tubes are also very fragile and are destroyed easily (pers. obs.). *C. orbiculata* can readily be observed forming inhalant tubes when burrowing in sediment in test tubes in the laboratory, however (pers. obs.). The association of *L. floridana* with the rhizomes and roots of the seagrasses (Fisher and Hand, 1984), similar to that reported for *C. orbiculata* in this study, may be due to the availability of dissolved sulphide, ammonia and nitrate, in addition to access to oxygen in sediment-surface seawater. Seagrass sediments may also be a preferred habitat since they are a physically stable environment for the bivalves (Orth, 1977) and the thick rhizomal mass may provide protection against predation (Orth, 1977; Reise, 1977).

Not all lucinids are found in habitats with unlimited oxygen supply. In an effort to explain the success of the *Lucinoma aequizonata* symbiosis in a habitat where oxygen levels in the overlying water were <20 µM and where sediments were anoxic, the use of nitrate as an alternate electron acceptor by the bacterial endosymbionts was suggested (Cary *et al.*, 1989). Hentschel *et al.* (1993) demonstrated that the intracellular symbionts of *L. aequizonata* respired nitrate but appeared incapable of respiring oxygen. It was suggested that the symbiont's ability to utilize alternate electron acceptors avoids competition with the host for oxygen, when the latter is in limited supply (Hentschel *et al.*, 1993). Certainly, symbiont nitrate respiration would seem advantageous to *L. aequizonata*, in light of the low oxygen levels and the high concentrations of nitrate (30 µM in overlying seawater) in the habitat (Cary *et al.*, 1989).

The bacterial symbionts of *Codakia orbiculata* have been demonstrated to be capable of nitrate and nitrite respiration *in vivo* with the bivalves incubated in either anoxic or oxic conditions (Chapter 3). Similar results have been reported for intact *Solemya reidi* symbioses (Wilmot and Vetter, 1992; B. Javor, pers. comm.). Unlike *Lucinoma aequizonata* symbionts, both *S. reidi* and *C. orbiculata* symbionts also have the ability to respire oxygen. The overlying seawater in the *S. reidi* sewage outfall habitat in California may have nitrate concentrations of ≥20 µM (Williams, 1986). In light of the oxygen demand at the *S. reidi* habitat, the sediment-surface seawater may periodically become anoxic and bacterial symbiont

respiration of nitrate would prove useful to the symbiosis under these conditions.

Mean nitrate concentrations in interstitial water in *C. orbiculata* habitat in Bailey's Bay ranged from 0.47-5.45 µM, approximately x5 those found in the overlying seawater (McGlathery, 1992). Nitrate concentrations as high as 36 µM were found in specific locations throughout the habitat. In contrast to the *L. aequizonata* habitat, however, oxygen seems unlikely to be limiting in the habitat of *C. orbiculata*. The bivalves had access, using their inhalant tubes, to the oxygenated sediment-surface seawater. In combination with the observation of nitrate respiration by the endosymbionts *in vivo*, when the bivalves were incubated in oxic conditions (80-100% oxygen) (Chapter 3), this suggests that the endosymbionts' ability to utilize nitrate is not necessarily dependent on oxygen concentration within the habitat.

As discussed in Chapters 1 and 3, it has been hypothesized that the lucinid symbionts are maintained in a low oxygen environment *in vivo*, as a result of the oxygen demand of the host gill cells (Fisher and Hand, 1984; Distel and Felbeck, 1987). Oxygen-binding intracellular hemoglobin in lucinid gills (Dando *et al.*, 1985; Kraus and Wittenberg, 1990) may also keep oxygen levels low in the vicinity of the bacteriocytes and this would be beneficial to RuBPC/O activity (Chollet, 1977; Dando et al., 1985). Hence, low oxygen levels in the vicinity of the bacteriocytes may not necessarily result from oxygen limitation in the habitat but, rather, may be the result of active maintainence. Presumably, therefore,

the ability of the bacterial symbionts to utilize an alternate electron acceptor other than oxygen, such as nitrate or nitrite, would be beneficial to the symbiosis. The potential role of symbiont nitrate/nitrite respiration in the *C. orbiculata* symbiosis is discussed in Chapter 3.

In summary, the *Thalassia testudinum* sediments in Bermuda have high sulphate reduction rates which appear to provide adequate reduced sulphur for the needs of the *Codakia orbiculata* symbioses at observed bivalve densities. Dissolved sulphide accumulated in the interstitial water of the rhizosphere. Sulphide concentrations remained relatively stable in regard to tidal influences. Although a seasonal peak was noted in August, levels for the rest of year were relatively consistent and were adequate to support the symbioses. Neither thiosulphate nor iron sulphides are considered a significant source of exogenous reduced sulphur for the symbioses. Ammonia levels in the interstitial water were high, suggesting this may be a source of nitrogen for the symbiosis. Unlimited oxygen was available from the sediment-surface seawater and nitrate was present in the interstitial water. Since *C. orbiculata* symbionts have the ability to use nitrate as an electron acceptor *in vivo*, even when the bivalve has access to oxygen (Chapter 3), it is suggested that nitrate availability may of importance to the symbiosis *in situ*.

CHAPTER 3

AEROBIC AND ANAEROBIC RESPIRATION, WITH EMPHASIS ON SULPHUR SUBSTRATE UTILIZATION, IN CHEMOAUTOTROPHIC BACTERIAL SYMBIONTS OF THE LUCINID BIVALVE, *CODAKIA ORBICULATA*

3.1. INTRODUCTION

Bacteria-bivalve chemoautrotrophic symbioses have been the subject of study for over 12 years yet many aspects of the physiology of some of these symbioses remain to be clarified. *In vitro* cultivation of the symbionts has yet to prove successful and symbiont-free hosts are not available, making the study of these symbioses difficult. *Solemya reidi*, the most well-studied of the bivalves containing chemoautotrophic bacterial symbionts, is found in high sulphide sediments near pulp mills and sewage outfalls (Reid, 1980; Felbeck, 1983). It has been demonstrated that *Solemya reidi* mitochondria are able to oxidize sulphide, in the presence of oxygen, to the less toxic thiosulphate for transport to the symbionts (Powell and Somero, 1985; Anderson *et al.*, 1987; O'Brien and Vetter, 1990)(refer to Fig. 5, Chapter 1). Recent investigations have demonstrated that the bacterial symbionts are also able to utilize sulphide and that they have access to sulphide *in vivo* (Wilmot and Vetter, 1992; B. Javor, pers. comm.).

Models for sulphur metabolism in the lucinid symbioses are less complete than that for the *Solemya reidi* symbiosis. In particular, evidence regarding the forms of sulphur utilized by the symbionts is lacking. In lucinid habitats studied to date, sulphide is available either in moderate concentrations in the interstitial water (Fisher and Hand, 1984; Chapter 2), through location of isolated pockets of sulphide (Cary *et al.*, 1989) or through oxidation of sediment-bound sulphides by the bivalve (Dando and O'Hara, 1990; Southward and Southward, 1990). Thiosulphate is present in

lucinid habitats in only very low levels in the interstitial waters (Dando *et al.*, 1986b; Cary *et al.*, 1989; Chapter 2).

It has been suggested that elemental sulphur, stored by the bacterial symbionts in the form of liquid-crystalline sulphur globules, can be utilized as an energy store (Vetter, 1985; Cary *et al.*, 1989) but the role of these reserves in sulphur metabolism has yet to be explored. Investigations into the sulphur metabolism of lucinids include demonstration that sulphide (<100 µM) stimulated carbon dioxide fixation in whole gills of *Myrtea spinifera* (Dando *et al.*, 1985) and *Lucinoma borealis* (Dando *et al.*, 1986a) and that growth in *Lucinoma aequizonata* was encouraged by sulphide (<10 µM) (Cary *et al.*, 1989). Gill homogenates of *L. borealis* produced ATP in response to 100 µM sulphide or sulphite but not in response to thiosulphate (up to 1 mM), suggesting thiosulphate is not utilized by the symbionts (Dando *et al.*, 1986a). In contrast, the blood of *L. aequizonata* incubated in sulphide was found to contain thiosulphate, suggesting that thiosulphate is transported to the symbionts (Cary *et al.*, 1989). Due to low levels of thiosulphate in sediment interstitial waters, it was assumed that thiosulphate was formed by sulphide oxidation, probably by host mitochondria, prior to transport to the bacterial symbionts (Cary *et al.*, 1989). Mechanisms for oxidation of sulphide to thiosulphate, similar to those described for *Solemya reidi*, may be present in the lucinid bivalves. Experiments conducted on whole gills or crude gill homogenates cannot differentiate, therefore, which thiol is utilized by the bacterial symbionts.

The considerable variation in sulphide metabolism within the family Lucinidae, suggested by these studies, may be a result of the range of experimental protocols and tissues used or of true, inter-specific physiological differences.

Recent investigations into the physiology of bacteria-bivalve symbioses have studied the potential for anaerobic respiration in the symbionts. These investigations were stimulated by the discovery of symbiotic bivalves in environments which are low in oxygen or are subject to periods of anoxia (Shepard, 1986; Cary *et al.*, 1989) but which had relatively high levels of nitrate in the sediment surface seawater and the interstitial water (Cary *et al.*, 1989), suggesting nitrate as an alternate electron acceptor. It is well-documented that many free-living bacteria found in anoxic environments utilize nitrate for respiration; nitrate is used as the terminal electron acceptor of the respiratory chain and can either be reduced to nitrite (nitrate respiration) or further reduced (nitrite respiration) to N_2 (denitrification) (Knowles, 1982).

The presence of the enzyme nitrate reductase has been noted in several bacteria-bivalve symbioses, including several species of lucinids and *Solemya reidi*, but was believed to be assimilatory (Felbeck *et al.*, 1983; Berg and Alatalo, 1984; Fisher and Hand, 1984). Recent research into the *S. reidi* symbiosis has demonstrated, however, that the bacterial symbionts, isolated and *in vivo*, are able to utilize nitrate and nitrite as alternate electron acceptors (Wilmot and Vetter, 1992; B. Javor, pers. comm.). The presence of

dissimilatory nitrate reductase and nitrate respiration (in the presence of thiosulphate) have been demonstrated in bacterial symbionts isolated from the lucinid, *Lucinoma aequizonata*, while oxygen consumption could not be demonstrated (Hentschel *et al.*, 1993). The intact *L. aequizonata* symbiosis also demonstrated nitrate respiration in the presence of both 10 µM sulphide and 10 µM thiosulphate (Hentschel *et al.*, 1993). The potential for nitrite to act as an alternate electron acceptor in lucinids in unknown.

Although *L. aequizonata* is found in a low oxygen environment, the bacterial endosymbionts of bivalves found in habitats where oxygen is not limiting may also need an electron acceptor alternate to oxygen. It has been suggested that oxygen-scavenging by the ciliated cells of the lucinid gills (Distel and Felbeck, 1987) and the presence of oxygen-binding hemoglobin in the gills (Dando *et al.*, 1985; Kraus and Wittenberg, 1990) may keep partial pressures of oxygen low in the vicinity of the bacteriocytes. Limited oxygen availability may be advantageous to the autotrophic processes of the bacteria (Dando *et al.*, 1985), reducing competition between oxygen and CO_2 for RuBPC/O active sites (Chollet, 1977).

Lucinid habitats display considerable variation in sulphide, oxygen and nitrate availability (Dando *et al.*, 1986a; Cary *et al.*, 1989; Chapter 2) and comparable variation in choice of sulphur substrates and electron acceptors may also be expected. Investigation of the aerobic and anaerobic respiratory potential of the symbionts is important,

therefore, to understanding the sulphur metabolism of the symbioses.

In the following study, a multi-directional approach was used to address the question of sulphur substrate utilization by the symbionts and their ability to use alternate electron acceptors. The small size (<10 mm) of many of the lucinids, including *Codakia orbiculata*, prevents accurate blood sampling; determination of sulphide oxidation prior to its reaching the bacteria is, therefore, difficult. Isolating the symbionts essentially uncouples symbiont metabolism from host metabolism and data on isolated symbionts must be used only to indicate the metabolic potential of the symbionts. The combination of information on the metabolic potential of the symbionts and research into the metabolism of the intact symbiosis may prove to be the most effective method for studying the symbiont metabolism *in vivo*. Carbon dioxide fixation rates, aerobic respiration rates and anaerobic respiration rates of suspensions of isolated symbionts were measured in response to sulphide and thiosulphate. Whole bivalve incubation experiments were also conducted to determine if the symbionts *in vivo* could utilize sulphur substrates and alternate electron acceptors.

This study was designed to address the following specific questions regarding sulphur metabolism and respiration in the *Codakia orbiculata* symbiosis: (1) can the isolated bacterial symbionts utilize thiosulphate, sulphide and/or stored globular elemental sulphur (SGES) as energy sources under oxic conditions? (2) can the isolated bacterial symbionts utilize

nitrate and/or nitrite as alternate electron acceptors and can thiosulphate, sulphide and/or SGES be used as energy sources? (3) do anoxic conditions and availability of sulphide, thiosulphate and/or SGES affect the metabolism of isolated bacteria as measured by $^{14}CO_2$ fixation? (4) can the symbionts *in vivo* utilize nitrate and/or nitrite as alternate electron acceptors, under oxic or anoxic conditions, when supplied with nitrate, nitrite, thiosulphate or sulphide? Because this study included the role of SGES as an energy substrate, the effect of removal of exogenous reduced sulphur on bacterial SGES was quantified. Experiments addressing questions 1-3 were conducted on bacteria with and without SGES.

3.2. MATERIALS AND METHODS

3.2.1. Gill Globular Elemental Sulphur Levels in Response to the Absence of Exogenous Reduced Sulphur

To determine the effect of the absence of an exogenous source of reduced sulphur on the stored globular elemental sulphur (SGES) in *Codakia orbiculata* gills, bivalves were maintained ("starved") in oxygenated, sulphide-free and low nutrient seawater. SGES was measured in the gills of bivalves sacrificed over a 27-day period to determine the rate at which SGES reserves were depleted. Gill protein content was measured to monitor gill condition. Mantle fluid sulphide and thiosulphate levels were measured to monitor thiol levels to which the tissues were exposed.

Bivalves were sacrificed following the schedule outlined in Table 10. To collect mantle fluid, bivalves were removed from oxygenated seawater and placed into a glove bag filled with ultra-high purity argon gas. Ultra-high purity argon gas was used throughout all experiments and is referred to throughout the remaining text as "argon". Bivalves were placed into a large plastic weighing boat, quickly blotted dry with a cellulose tissue and gently pried open with a scalpel. Mantle fluid was removed in 5 µl aliquots using a Finnpipette. The procedure for analysis of thiols in mantle fluid was modified from Newton and Fahey (1987) and Vetter *et al.* (1989) and is described in detail in the methods for Chapter 2.

After sampling of mantle fluid, bivalves were removed from the glove bag. Gills were carefully removed, to avoid contamination with other tissues, under magnification of a

Table 10. Number of samples (n) for sulphur "starvation" experiments.

Starvation Time (h or d)	Elemental Sulphur in Gill Tissue (n)	Bacteria# in Gill Tissue (n)	Protein in Gill Tissue (n)	Thiol in Mantle Fluid (n)
0 h	8	0	0	8
2 h	3	1	2	3
12 h	3	1	2	3
24 h	3	1	2	3
48 h	3	1	2	3
5 d	3	1	2	3
10 d	3	1	2	3
15 d	3	1	2	3
20 d	3	1	2	3
27 d	3	1	2	3

Wild M8 dissecting microscope. For each bivalve, one gill was used for elemental sulphur analysis and the other for either protein content or enumeration of bacterial symbionts. Wet weights of each gill were recorded. Gills used for protein or elemental sulphur analysis were freeze-dried, reweighed and stored frozen until analysis. Gills used for bacterial counts were preserved in Lillie's buffered (pH 7.4) formalin (100 ml 40% formaldehyde, 900 ml MilliQ purified distilled water (MQ water), 4 g sodium phosphate monohydrate, 6.5 g disodium phosphate).

Freeze-dried gills for elemental sulphur analysis were processed as described in the methods section in Chapter 2. Bivalves that were sacrificed at the beginning of the experiment did not have gills preserved for protein analysis or bacterial enumeration because both gills were analyzed for elemental sulphur. A different preparation method was used for each gill of the pair. Eight bivalves were sacrificed at the start of the experiment and one gill from each of the 8 bivalves was freeze-dried prior to solvent extraction. The second gill from each of 4 bivalves was solvent extracted immediately and the second gill from the remaining 4 bivalves was preserved in 1 ml of 4% (by volume) formaldehyde and 20 g/l hydrated cadmium chloride (Dando *et al.*, 1986a). After one week, the latter 4 gills were transferred to solvent and processed identically to the other samples. Results revealed that freeze-dried gills consistently had the highest SGES content. SGES content in gills preserved in $CdCl_2$/formaldehyde were 41.0% $\pm$ 27.1% of the content in the freeze-dried gills.

Fresh gills were found to have 64.9% ± 40.5% of the SGES content of freeze-dried gills. This suggests freeze-drying tissues resulted in the most efficient extraction of globular elemental sulphur. This may be because the solvent (hexane or heptane) is completely non-miscible with water and can penetrate freeze-dried tissue most effectively.

Protein content in freeze-dried gills was determined with the BCA protein assay (Pierce, Rockford, IL 61105, USA). Protocol was identical to that described in detail in the Methods section of Chapter 2.

Gills preserved for enumeration of bacterial symbionts were not processed further due to the problems encountered with this protocol in the seasonal study (Chapter 2).

Data Analysis

Means were calculated for all SGES, protein and mantle fluid thiol data and tabulated or plotted against incubation time to discern trends. The SGES data were tested for significant change over time using a 1-way ANOVA ($p<0.05$) followed by Tukey's *post-hoc* pairwise comparisons. As per Chapter 2, all statistical analyes used were based on procedures detailed in Zar (1974) and all statistical analyses were performed using the software package Systat 5.1.

3.2.2. Aerobic Respiration in Bacterial Symbiont Suspensions

Experiments were conducted to determine if sulphide or thiosulphate stimulated aerobic respiration in the isolated symbionts of *Codakia orbiculata.* Bacterial suspensions were incubated in a temperature-controlled respiration chamber and provided with either thiosulphate, sulphide (two concentrations), or no sulphur substrate. Experiments with no sulphur source were also conducted (to determine endogenous rate), as were incubations with no bacterial suspension but 0.2 µm-filtered Sargasso Sea seawater[3] (FSSW) and sulphide or thiosulphate. Experiments were conducted on bacteria isolated from freshly collected *C. orbiculata* (referred to as "fed" bacteria in text) and from bivalves maintained in oxygenated, sulphide-free and low-nutrient seawater for 10 days (referred to as "starved" bacteria in text). The gills of freshly collected bivalves were usually yellow, indicative of SGES in the bacteria. Bivalves maintained in aquaria for 10 days had dark red gills, indicative of little or no SGES. Aerobic respiration was monitored by measuring the decrease in oxygen levels in the incubation media.

Codakia orbiculata were collected immediately prior to experiments. Gills were gently removed from bivalves and placed in FSSW (0.22-µm filtered) buffered with 10 mM MOPS (3-(N-morpholino)propanesulphonic acid, pH 8) (FSSW-MOPS) and

[3] Sargasso Sea seawater nutrient levels were below detection limits using colourimetric assays in Strickland and Parsons (1977).

kept on ice. Gills were gently homogenized in FSSW-MOPS using a glass tissue homogenizer and the homogenate was filtered through 44-µm and 15-µm Nitex filters to remove large subcellular particles. The filtrate was centrifuged at 4°C for 10 min at 3000 rpm. The supernatant was discarded and the pellet washed (resuspended) in seawater MOPS. The resuspension was centrifuged for 10 mins at 3000 rpm to pellet the bacteria but not the mitochondria. The pellet was then resuspended in FSSW-MOPS. Throughout processing and until initiation of experiments, the bacterial suspension was kept on ice. The final suspension was equivalent to 3 bivalves/ml. Preliminary suspensions of isolated bacterial symbionts were examined using light microscopy and epi-fluorescent microscopy (the latter slides were stained with DAPI). Nuclei, large cellular debris and mitochondria could not be detected in the suspensions. It proved impossible, however, to exclude the pigment granules from the bacterial suspensions. The granules, which are found in the bacteriocytes *in vivo* and are believed to contain hematin (Powell and Arp, 1989), must be approximately the same density as the symbionts.

Aerobic respiration rates were determined by measuring the oxygen consumption rate of 1 ml of bacterial suspension placed in a 1.5-ml temperature-controlled (water jacketed) glass respiration chamber (Strathkelvin RC 300 respiration chamber) adjusted to 1-ml volume. Temperature was maintained at 22°C throughout the experiments and a micro-stir bar in the chamber ensured adequate mixing. Oxygen within the chamber was measured using a Clark-type oxygen electrode (Strathkelvin

Instruments 1302 microcathode electrode) which consumed negligible oxygen (10^{-11} mgO_2/sec). The electrode was modified, to reduce hydrogen sulphide interference, by etching the platinum cathode and electroplating gold onto the cathode, following the procedures of Revsbech and Ward (1983) and O'Brien and Vetter (1990). The electrode holder fitted tightly into the respiration chamber, the volume of which could be varied by changing the position of the electrode holder. The electrode holder had a groove machined down the side which allowed additions to the chamber using a Hamilton syringe. In this way, the electrode did not have to be moved, nor the bacterial suspension disturbed, when spiking the chamber. The oxygen concentration within the chamber was recorded every 10 sec for the duration of the experiments. The Strathkelvin oxygen meter was connected to an analogue to digital recorder and an IBM computer. Oxygen data were stored in ASCII files, using the data acquisition program Acquire (Labtech), for later analysis by the spreadsheet program QuattroPro.

After determining the endogenous rate of oxygen consumption, the bacterial suspension was spiked with sulphide or thiosulphate stock solutions to produce final chamber concentrations of either 100 µM, 200 µM sulphide or 500 µM thiosulphate within the chamber. Stock solutions were prepared from sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) and sodium sulphide nonahydrate ($Na_2S.9H_2O$). All stock solutions were prepared at x100 the final concentration using MQwater except for the sulphide solution, which was prepared in degassed FSSW-MOPS. The sulphide solution was neutralized to

pH 7-8 to eliminate pH effects on metabolism. All thiol stock solutions in all experiments were prepared identically. At least three replicates were performed for each set of experimental conditions except where noted. Experiments using 200 µM sulphide and 500 µM thiosulphate were also carried out on bacteria isolated from bivalves "starved" (maintained in running seawater free of reduced sulphur) for 10 days. These sulphide experiments were not replicated.

When a steady endogenous rate of oxygen consumption had been recorded, the chamber was spiked with sodium azide (NaN_3) to a final concentration 10 mM to inhibit aerobic respiration in the symbiotic bacteria. The oxygen consumption rate after this point was measured to determine non-respiratory oxygen consumption (Wilmot and Vetter, 1992). Controls using FSSW-MOPS but no bacteria, with sulphide or thiosulphate spikes followed by azide, were carried out for each concentration of thiol to determine oxygen consumed through chemical reaction.

Following experiments, the 1-ml experimental bacterial suspensions were centrifuged at 13,000 rpm in an Eppendorf Microcentrifuge 5415 to pellet the bacteria. The supernatant was removed and the wet weight of the bacterial pellet was recorded.

Data analysis

The endogenous rate, thiol-stimulated rate, and azide-sensitive rate (%oxygen/min) of oxygen consumption were calculated from the best fit slope of the data. Control rates (no bacteria) were subtracted from experimental rates to

correct for the loss of oxygen due to reaction between thiols or azide and oxygen in the chamber. The control-adjusted oxygen consumption rates were converted to absolutes (nmol oxygen) per min and then adjusted for the wet weight of 1 ml of bacterial suspension, resulting in final units of nmol oxygen/mgww/min. The azide-insensitive oxygen consumption rate (measured after addition of azide) was subtracted from the thiol-stimulated rate to determine the azide-sensitive rate, representing the true respiration rate of the bacterial symbionts. The mean oxygen consumption rates $\pm$ 1 s.d. were calculated from replicates and compared between experiments. Azide-sensitive rates resulting from thiol stimulation were also expressed as relative stimulation over endogenous rates and were tested against endogenous rates using 1-way analyses of variance (ANOVAs) for statistical difference ($p<0.05$).

3.2.3. Nitrate and Nitrite Respiration in Bacterial Symbiont Suspensions

The ability of the isolated symbiotic bacteria to utilize nitrate and/or nitrite as alternate electron acceptors under anoxic conditions was investigated using incubations of bacterial suspensions. The suspensions were provided with nitrate or nitrite and either thiosulphate, sulphide, or no sulphur substrate. Two sets of control experiments were carried out: bacterial suspensions were incubated with no exogenous electron acceptor or sulphur source and bacteria-free incubations replicating experimental conditions but using degassed FSSW-MOPS rather than bacterial suspension. Experiments were conducted on bacteria isolated from freshly collected *Codakia orbiculata* (fed bacteria) and from bivalves maintained in oxygenated, sulphide-free and low-nutrient seawater (starved bacteria) for 10 days. Nitrate and nitrite respiration rates were monitored by measuring the accumulation or disappearance of nitrite, respectively, in the incubation media.

Bacteria were isolated from host bivalves and suspensions were prepared as outlined above for oxygen consumption experiments, with the following modifications. Following the second 10 min centrifugation at 3000 rpm, the bacterial pellet was resuspended in degassed FSSW-MOPS and maintained, on ice, under an argon gas stream until used for experiments. Bacterial suspensions were used within 2 h of processing.

Experiments were carried out on aliquots of bacterial suspension transferred under an argon stream to previously

degassed 5-ml vials with screw-cap lids. Nitrate and nitrite stock solutions were prepared from either potassium nitrate (KNO_3) or sodium nitrite ($NaNO_2$). All nitrate and nitrite stock solutions for all experiments were prepared identically. Degassed stock solutions were added to the suspensions for a final concentration of 2 mM nitrate or 20 µM nitrite, respectively. These nutrient levels were in excess of levels recorded in *Codakia orbiculata* habitat (Chapter 2) in an effort to stimulate nitrite production and utilization, respectively, and to prevent complete substrate disappearance during the incubations. Following the addition of the alternate electron acceptor, the suspension was spiked to a final concentration of 200 µM sulphide, 200 µM thiosulphate or 5 mM cyanide (KCN). Sulphide incubations were re-spiked after each sample time point to ensure adequate substrate. Three replicate experiments were run for each set of conditions (except where noted). The initial volumes of the suspensions varied: 3.6 ml for 2 mM nitrate incubations to allow 1-ml subsamples at each time point for nitrite analysis, or 1.8 ml for 20 µM nitrite incubations because 500-µl subsamples required dilution to 1 ml prior to analysis. Preliminary experiments determined that suspensions required a 10-min pre-incubation period prior to the T_0 sampling to obtain linear respiration rates. Additional samples were taken at 20 and 40 min (except where noted).

Because sulphide interferes with the colourimetric nitrite analysis (Strickland and Parsons, 1977), it was removed by precipitation as zinc sulphide prior to analysis.

Samples of bacterial suspension removed for nitrite analysis (either 1 ml or 500 µl diluted to 1 ml with FSSW) were pipetted into 1.5-ml Eppendorf tube containing 10 µl 2 N zinc acetate dihydrate ($(CH_3CO_2)_2Zn.2H_2O$). Following the addition of 10 µl 2 N sodium hydroxide (NaOH), to increase the flocculation and to help precipitation, the tubes were centrifuged for 1 min at 13,000 rpm on an Eppendorf Microcentrifuge 5415. After this point, the method follows that of Strickland and Parsons (1977) with modifications for small volume. The supernatant (800 µl) was transferred to another 1.5-ml Eppendorf tube, 160 µl of sulphanilamide solution (5 g sulphanilamide ($4-(H_2N)C_6H_4SO_2NH_2$) in 50 ml of concentrated hydrochloric acid (HCl) and 450 ml of MQwater) were added and, after 5 min, 160 µl of N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{10}H_7NHCH_2CH_2NH_2.2HCl$) (0.5 g in 500 ml MQwater). After allowing 1-2 h for full colour development, the extinction of the samples was read at 543 nm on a Milton Roy Spec21 spectrophotometer. Nitrite samples with absorbances greater than 1.0 were diluted 1:10 with FSSW prior to analysis. Nitrite concentrations were determined by comparison with a standard curve.

The concentrations of sulphide and thiosulphate in the bacterial suspensions were monitored in single experimental runs. Concentrations were measured in 100 µl aliquots of suspension by HPLC after derivatization with monobromobimane (bimane). Reagent blanks and PDS controls were also analyzed.

Protocol followed the procedures of Newton and Fahey (1987) and Vetter *et al.* (1989), as described in detail in the Methods section in Chapter 2.

Data analysis

Nitrite concentrations (µM) in the incubation media were converted to absolutes (nmol) and rates of nitrite disappearance or accumulation, after adjustment for the bacteria-free controls, were calculated on a wet weight basis (nmol/gww/min). Replicate rates for each set of incubation conditions were converted to mean values ($\pm$ 1 s.d.) and tabulated. Where significant stimulation above the control rates were noted, rates were tested for statistical difference ($p<0.05$) utilizing 1-way analyses of variance.

3.2.4. Anaerobic Carbon Dioxide Fixation by Bacterial Symbiont Suspensions

To determine if *Codakia orbiculata* bacterial symbionts were able to fix CO_2 under anoxic conditions, bacterial suspensions were incubated, in the absence of oxygen, with $NaH^{14}CO_3$ and provided with either sulphide, thiosulphate, nitrate or a combination of sulphur substrate and nitrate. Experiments were also carried out with neither external electron acceptor nor sulphur source to determine endogenous anaerobic $^{14}CO_2$ fixation rates. Experiments were conducted on bacteria isolated from freshly collected *C. orbiculata* ("fed" bacteria) and from bivalves maintained in oxygenated, sulphide-free and low-nutrient seawater ("starved" bacteria) for 10 d. One incubation was conducted under oxic conditions for comparison of results with those of the experiments conducted under anoxic conditions.

Bacterial suspensions were prepared and maintained as described in the methods for anaerobic respiration experiments. Bacterial suspensions (1.2 ml) were transferred under an argon gas stream to 1.5-ml Eppendorf tubes. Sulphide (in FSSW-MOPS), thiosulphate or nitrate x100 stock solutions were added to the suspensions for final concentrations of 1 mM thiosulphate, 200 µM sulphide, 2 mM nitrate and combinations of sulphur substrates and nitrate. Bacterial suspensions were also incubated with 2 mM nitrate and 10 mM azide (fed bacteria) or 5 mM cyanide (starved bacteria). Control incubations, consisting of bacterial suspensions with no additions, and an oxic control incubation (using starved

bacteria only) were also run. At the start of each experiment, 12 µl of 400 µCi/ml $NaH^{14}CO_3$ was added to each tube, producing a final concentration of 4 µCi/ml. Samples (200 µl) were drawn from the tubes under an argon stream at 0, 15, 30, 45 and 60 min and placed in glass scintillation vials. In the fumehood, 2 ml of 2 N H_2SO_4 was added to stop fixation. The vials were left open in a 45°C sand bath over night to bring the sample to dryness. Vials were removed from the sand bath and samples redissolved in 1 ml of 0.004 N NaOH. In some cases, a PTFE scraper was used to help resuspend the precipitate. Ten ml of scintillation cocktail (ScintiVerseE, Fisher Scientific) was added to each vial. After shaking, all samples were counted on a Beckman LS6000 TA scintillation counter.

At the end of the experiments, 50-µl samples were taken from each tube to determine the total counts of each tube. These samples were not acidified but suspended in 1 ml MQwater and 10 ml scintillation cocktail. Total counts were calculated per 200 µl and used for converting counts per min, from the time point samples, to fraction of total counts. The fraction uptake of total $^{14}CO_2$ data were expressed as rates (fraction uptake/min) and rates were found to be linear over the 60-min sampling period. Rates were converted to µmolesC/gww/h and were also expressed as a ratio of the endogenous rate, to demonstrate stimulatory effects of substrates.

Data analysis

Means ($\pm$ 1 s.d.) were calculated for $^{14}CO_2$ fixation rates in experiments which were replicated. A 1-way analysis of variance was used to test for statistically significant differences ($p<0.05$) between fixation rates for fed bacteria stimulated by the addition of thiosulphate or sulphide.

3.2.5. Nitrate and Nitrite Respiration in Whole *Codakia orbiculata*

To determine the ability of the *Codakia orbiculata* bacterial symbionts to respire nitrate and nitrite *in vivo*, bivalves were incubated with alternate electron acceptors (nitrate and nitrite) and either sulphide (a range of concentrations), thiosulphate or no reduced sulphur source. Experiments were conducted under both anoxic and oxic conditions. Nitrate and nitrite respiration were measured by the increase or decrease in nitrite in the incubation media, respectively.

Codakia orbiculata were collected 24 h prior to the start of the experiments. Reduced sulphur sources in ambient sediments can be variable (Chapter 2) and this may affect thiols in the mantle fluid and blood which could be utilized by the bivalves and could, therefore, influence the results of the experiments. Bivalves were placed in oxygenated, sulphide-free seawater for 24 h in an effort to standardize the mantle fluid and blood thiol levels of the bivalves. All bivalves were of relatively uniform size (8-10 mm shell width).

Incubations were carried out in 50-ml Erlenmyer flasks sealed with rubber septa to prevent gas exchange. Flasks were covered with aluminum foil to reduce disturbance and to mimic dark, sediment conditions. Each flask contained 40 ml of incubation solution, prepared using FSSW (0.22 µm filtered), appropriate volumes of 100 mM sulphide or thiosulphate stock solutions and 4 mM nitrate or nitrite stock solutions (prepared as outlined for anaerobic respiration experiments

using isolated bacteria suspensions). Final incubation concentrations were 100 µM for nitrate and nitrite, 250 µM for thiosulphate and 50 µM, 250 µM and 1000 µM for sulphide. Anoxic and oxic incubation conditions used are listed in Tables 11a and 11b, respectively. Replicate experiments were carried out for each set of incubation conditions except where noted. A control (no bivalves) was run for each set of incubation conditions.

Trial incubations indicated a minimum of 5 bivalves in 40 ml of solution resulted in a measurable rate of thiol or nitrate/nitrite utilization at selected concentrations. Concentrations of electron acceptor and thiol were selected as compromises between habitat levels and concentrations sufficiently high to yield measurable results within a reasonable period of time. Previous experiments on suspensions of isolated symbionts had used excess nitrate (2 mM) to demonstrate the ability of the bacteria to respire nitrate. The 100 µM nitrate concentrations used in the whole animal experiments were approximately x3 higher than maximum concentrations reported from the habitat (Chapter 2) but were the minimum concentration necessary to obtain a measurable result within an experimental time period. In contrast, the 100 µM nitrite concentrations were higher than those used in the isolated bacteria experiments but, again, lower concentrations did not yield measurable results within a 6.5-h to 8-h period. Thiol levels were also selected for the same reasons. 250 µM thiosulphate yielded a measurable result but was approximately x5 the highest concentration recorded in

Table 11. Incubation conditions for whole *Codakia orbiculata* nitrate and nitrite respiration experiments. Concentrations are µM. "Thios." = Thiosulphate.

(a) Anoxic incubations.

# CLAMS	INCUBATION CONDITIONS (T_0)				# REPLICATES
	Nitrite	Nitrate	Sulphide	Thios.	
5	0	0	0	0	3
0	0	0	0	0	2
5	0	0	250	0	3
0	0	0	250	0	2
5	0	0	1000	0	3
0	0	0	1000	0	2
5	0	100	0	0	3
0	0	100	0	0	2
5	0	100	50	0	3
0	0	100	50	0	3
5	0	100	250	0	3
0	0	100	250	0	3
5	0	100	1000	0	3
0	0	100	1000	0	2
5	0	0	0	250	3
0	0	0	0	250	1
5	0	100	0	250	3
0	0	100	0	250	1
5	100	0	0	0	3
0	100	0	0	0	2
5	100	0	250	0	3
0	100	0	250	0	2
5	100	0	0	250	3

Table 11. cont'd.
(b) Oxic incubations.

# CLAMS	INCUBATION CONDITIONS (T_0)				# REPLICATES
	Nitrite	Nitrate	Sulphide	Thios.	
5	0	0	250	0	3
0	0	0	250	0	1
5	0	0	0	250	3
0	0	0	0	250	1
5	0	100	250	0	3
0	0	100	250	0	1
5	0	100	0	0	3
0	0	100	0	0	1
5	0	100	0	250	3
0	0	100	0	250	1
5	100	0	250	0	3
0	100	0	250	0	1
5	0	0	0	0	3
0	0	0	0	0	1
5	0	0	50	0	3
0	0	0	50	0	1
5	0	0	1000	0	3
0	0	0	1000	0	1
5	0	0	0	1000	1
0	0	0	0	1000	1
5	100	0	0	0	3
0	100	0	0	0	1

habitat interstitial water (Chapter 2). The sulphide concentrations used (0-1000 µM) bracketed the range of sulphide levels found in the bivalves' habitat, where mean dissolved sulphide levels ranged from 31.32 - 296.68 µM (Chapter 2). Bivalves were sacrificed after the experiments to determine total wet tissue weights for later calculation of nitrate and nitrite respiration rates for the whole organism.

Anoxic Incubations

The total incubation volume in all experiments was 40 ml (FSSW + nitrate or nitrite stock + thiol stock). FSSW was degassed with argon gas in a 50-ml Erlenmyer flask for 20 min prior to the addition of 1 ml of 4 mM nitrate or nitrite stock solution for a final concentration of 100 µM. Incubation solutions were then degassed for an additional 1-2 min while bivalves were prepared. The absence of oxygen at initiation of the experiments, immediately prior to adding the bivalves, was determined using a Strathkelvin Clark-type oxygen micro-electrode modified to avoid sulphide poisoning as described earlier. Oxygen levels were also measured at the conclusion of the experiments to determine if oxygen had diffused across the septum.

Bivalves were measured and their shells washed with clean seawater to remove adhering detritus. Immediately prior to adding the bivalves to the degassed incubation flask, they were blotted dry with a cellulose tissue. The argon bubbler was removed from the flask, the bivalves added and the flasks were immediately sealed. The appropriate volume of thiol stock

solution was injected through the rubber septum using a 1-ml or a 100-µl Hamilton syringe. The flask was swirled to mix solutions and the T_0 reading was taken less than 10 sec after the bivalves were added to the flask. The foil-covered anoxic incubation flasks were then placed in an argon glove bag, flushed continuously with argon, gently swirled once every 0.5 h to ensure a uniform incubation medium and removed only for sampling. All samples of the incubation media were removed using a Hamilton syringe. Room temperature was maintained at 22°C for the duration of the experiments.

Oxic Incubations

The experimental procedure was identical to that used for the anoxic incubations, except the solutions were not degassed with argon and the flasks were not kept in the glove bag.

Sampling and Analysis

Samples of the incubation solutions were removed at 0, 3, 6.5 (or 8) and 24 h. Trial experiments had revealed that 3 h was the minimum incubation time necessary to yield a repeatedly measurable rate of thiol and/or nitrate or nitrite utilization. Flasks were gently swirled prior to sampling to ensure uniform concentration. For nitrate analysis, 1 ml of solution was removed through the septum, using a Hamilton syringe, and immediately frozen in a 1.5 ml-Eppendorf tube for later analysis using chemiluminescence (Barnes and Lipshultz, unpubl. data). Although this method of analysis was successfully used to analyse nitrate concentrations in

standards and interstitial water samples (Chapter 2), preliminary analyses of the incubation media samples were unsuccessful. Possible sources of interference with the technique have yet to be identified and, therefore, these data could not be utilized. One ml of incubation solution was removed as above for nitrite analysis. The method used for nitrite analysis was identical to that described for anaerobic respiration experiments in suspensions of isolated bacteria, as discussed earlier.

Thiosulphate and sulphide were measured in 100-µl samples of the incubation media, removed using a 100-µl Hamilton syringe and processed following the HPLC bimane method (Fahey and Newton, 1987; Vetter *et al.*, 1989). In addition to the above method, sulphide was also measured in some of the incubation media using Cline's colourimetric assay (Cline, 1969). One ml of incubation solution was removed and added to a degassed scintillation vial containing 40 µl of 2 N zinc acetate (to preserve the sulphide as zinc sulphide). These samples were stored in the dark for 2-5 days prior to analysis with no loss of sulphide (Barnes, unpubl. data). A detailed procedure for this analysis can be found in the methods section in Chapter 2.

Data Analysis

The nitrite concentration data were converted to absolutes (nmol) and the mean rate measurements, expressed as nmol/gww/min, were calculated for the first 6.5 (or 8) h and

the mean rates compared between treatments. Where nitrite concentrations changed significantly, the response was linear over the 6.5 (or 8) h period. One-way ANOVAs were used, where appropriate, to test for statistically significant differences ($p<0.05$) between rates.

3.3. RESULTS

3.3.1. Gill Globular Elemental Sulphur Levels in Response to the Absence of Exogenous Reduced Sulphur

Only specific sulphur-oxidizing bacteria have been observed to produce and accumulate globular elemental sulphur, which is believed to be an index of bacterial condition (Vetter, 1985). Availability of reduced sulphur compounds may be reflected in stored globular elemental sulphur (SGES) in the gills of *Codakia orbiculata*, representing a physiological response by the bacterial endosymbionts. SGES was measured in the gills of *C. orbiculata* maintained in oxygenated, sulphide-free seawater over a 27-d period. The data are presented in Table 12. Because bivalves were collected immediately prior to the start of the experiment, SGES levels measured in the gills of bivalves at T_0 were considered representative of the levels found in bivalves *in situ*. Foot and mantle tissues from 5 freshly collected bivalves were also analysed but no SGES was detected (data not shown). The mean SGES content in gills at the start of the experiment was 107.28 $\pm$ 149.70 µg-atoms S/gww. SGES in the gills fluctuated over the first 48 h but dropped dramatically between 48 h (2 d) and 120 h (5 d); the 5-d gill samples contained only 4.70 $\pm$ 6.89 µg-atoms S/gww. After 5 d, the SGES remained relatively constant for the duration of the experiment. Gill colour was observed for all bivalves and was noted to change from pale yellow-cream at the start of the experiment to a dark brown-red colour at the end of the experiment.

Table 12. *Codakia orbiculata* gill globular elemental sulphur (µgatoms S/gww) in response to incubation in the absence of exogenous reduced sulphur ("starvation"). Data are presented as means ± 1 s.d..

Incubation Time (h or d)	Gill Sulphur (µgatoms S/gww)	Gill Sulphur (%d.w.)	n	Gill Colour
0 h	107.28 ± 149.70	1.52 ± 2.27	8	Creamy Yellow
2 h	55.46 ± 43.89	0.85 ± 0.67	3	Yellow
12 h	101.70 ± 23.57	1.15 ± 0.40	2	Yellow
24 h	51.92 ± 38.08	0.49 ± 0.51	3	Yellow
48 h	50.38 ± 79.37	1.10 ± 1.81	3	Yellow
5 d	4.70 ± 6.89	0.04 ± 0.06	3	Reddish Brown
10 d	4.96 ± 6.92	0.05 ± 0.08	2	Reddish Brown
15 d	1.72 ± 2.41	0.01 ± 0.02	3	Reddish Brown
20 d	4.51 ± 3.56	0.03 ± 0.02	3	Reddish Brown
27 d	5.59 ± 3.12	0.03 ± 0.01	3	Reddish Brown

Surprisingly, no statistically significant differences were found between SGES measured at each sampling time (1-way ANOVA; $p>0.05$). This is attributed to the predominately small sample size ($n=3$) and the large variation around the mean SGES content in freshly collected bivalves. The data follow a trend over time, however, with gill SGES decreasing initially and then levelling off. As the experiment progressed, the variation around the mean SGES at each sampling time decreased, reflecting the constant conditions in which the bivalves were maintained and a basal concentration found in this species.

Mean protein content ranged from 0.07 ± 0.03 to 0.17 ± 0.004 mg protein/mgww throughout the experiment with no apparent correlation to days of sulphide deprivation (Fig. 16).

Over the course of the experiment, thiosulphate and sulphide were measured in the mantle fluid of bivalves sacrificed for gill SGES analysis (Figs. 17a and b). Thiosulphate levels were high in the 0 h samples (87.54 ± 66.45) but dropped dramatically in the 24 h samples and, again, in the 48 h samples. After 48 h, the mantle fluid thiosulphate levels remained consistently low (ranging from 0.00 to 6.59 ± 4.73 µM), suggesting a low basal concentration in this species. Variance around the mean was greater in the samples taken in the first 24 h than in subsequent samples. Levels of dissolved sulphide in the mantle fluid of the bivalves followed a similar pattern to that of thiosulphate, although the decrease over the first 24 h was not as great. No

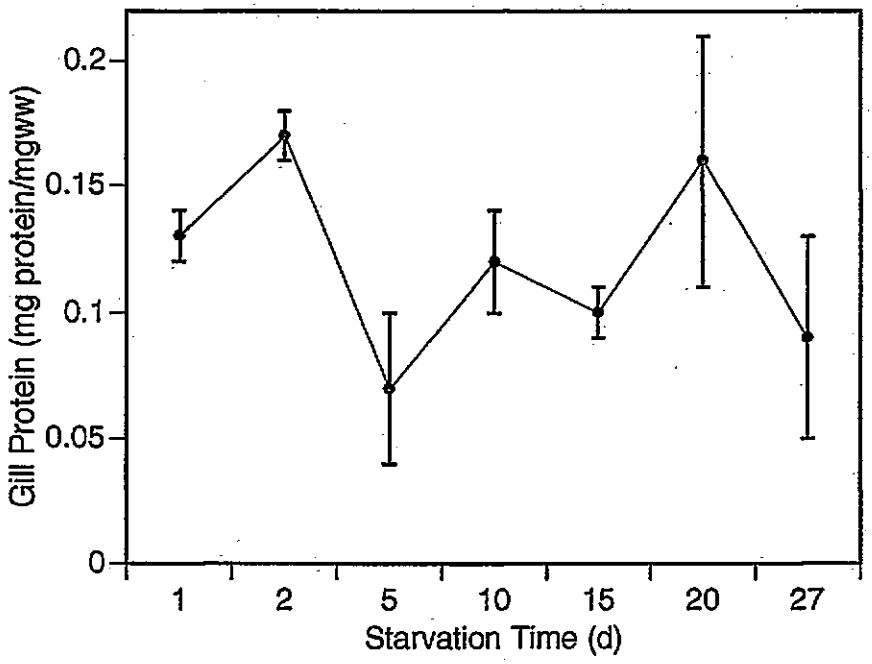

Figure 16. Protein content of *Codakia orbiculata* gills (mg/protein/mgww) in response to the absence of exogenous reduced sulphur ("starvation"). Data are plotted as means ± 1 s.d.; n=3.

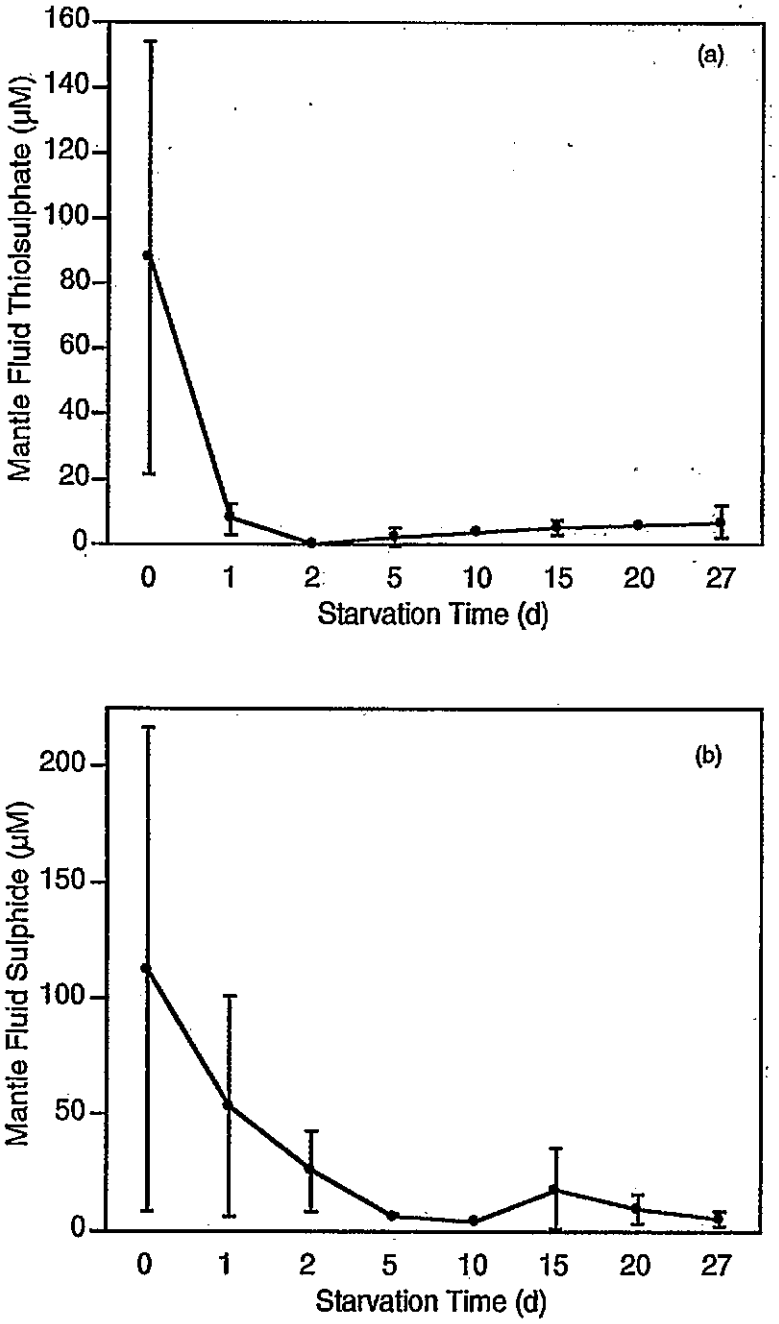

Figure 17. Mantle fluid thiol concentrations (µM) in response to the absence of exogenous reduced sulphur ("starvation"). Data are plotted as means ± 1 s.d.; n=3, except at 0 days, n=8. (a) Thiolsulphate (b) Sulphide.

samples were taken between 2 and 5 days, however, and using the 12 h to 48 h rate of sulphide disappearance to extrapolate between 48 h and 120 h, it seems likely that the sulphide would have virtually disappeared before 72 h (Fig. 17b). Mean sulphide levels in mantle fluid dropped dramatically from 112.33 ± 104.51 µM at 0 h, to 52.98 ± 47.51 µM after 24 h, to 25.34 ± 17.23 µM after 48 h (2 d) and to 6.29 ± 0.55 µM after 5 d. Between 5 d and 27 d, the mantle fluid sulphide levels appeared to remain relatively constant. Sulphide in mantle fluid in this species, similar to thiosulphate, seems to maintain a low basal concentration.

3.3.2. Aerobic Respiration in Bacterial Symbiont Suspensions

To determine if *Codakia orbiculata* bacterial symbionts were capable of respiring sulphide and thiosulphate aerobically, respiratory (azide-sensitive) oxygen consumption was measured in the presence of these thiols and compared to endogenous consumption rates. Non-respiratory (azide-insensitive) oxygen consumption was also measured; this is believed to be a result of non-enzymatic sulphide oxidation by hematin-containing pigment granules (Powell and Arp, 1989; Wilmot and Vetter, 1992). The pigment granules could not be separated from the bacteria. Oxygen consumption rates of suspensions of bacteria isolated from freshly collected *C. orbiculata* are presented in Table 13a. All rates have been corrected for oxygen consumption in the chamber with the same conditions but no bacteria. Results indicated that the bacterial symbionts were able to respire aerobically, with mean endogenous rates (no exogenous reduced sulpur) ranging from 0.42 to 0.65 nmol/mgww/min. Sulphide (100 µM) and thiosulphate (500 µM) stimulated azide-sensitive oxygen consumption but rates were not statistically different from the endogenous rate (1-way ANOVA; $p>0.05$). However, azide-sensitive oxygen consumption rates in the presence of 200 µM sulphide were statistically higher than the endogenous rate (1-way ANOVA; $p<0.05$). The azide-insensitive oxygen consumption rates for 100 µM and 200 µM sulphide remained relatively constant (means of 0.12 and 0.10 nmol/mgww/min respectively). The same rate for 500 µM thiosulphate was slightly lower, 0.09 $\pm$ 0.03 nmol/mgww/min.

Table 13. Oxygen consumption rates (nmol/mgww/min) of *Codakia orbiculata* bacterial symbiont suspensions. Data are presented as means ± 1 s.d..

(a) Bacteria isolated from freshly collected bivalves ("fed").

Thiol	Conc. (µM)	n	Endogenous Rate	Rate With Thiol	Azide-Sens. Rate	Azide-Insens. Rate
Sulphide	100	3	0.65± 0.22	0.86± 0.32	0.74± 0.37	0.12± 0.11
Sulphide	200	4	0.42± 0.09	0.97± 0.20	0.86± 0.19	0.10± 0.02
Thiosulphate	500	3	0.49± 0.12	0.74± 0.03	0.65± 0.05	0.09± 0.03

(b) Bacteria isolated from bivalves maintained in sulphide-free oxygenated seawater for 10 days ("starved").

Thiol	Conc. (µM)	n	Endogenous Rate	Rate With Thiol	Azide-Sens. Rate	Azide-Insens. Rate
Sulphide	200	1	0.21	0.79	0.66	0.13
Thiosulphate	500	3	0.33± 0.00	0.46± 0.03	0.39± 0.03	0.07± 0.00

Oxygen consumption rates of suspensions of bacteria isolated from *Codakia orbiculata* maintained in oxygenated, sulphide-free seawater for 10 d are listed in Table 13b. The mean endogenous rate was much lower than those of the fed bacteria. The presence of 200 µM sulphide increased the azide-sensitive oxygen consumption rate to x3.18 the endogenous rate, comparable to the mean rate measured for fed bacteria under the same conditions. The experiments with sulphide were not replicated, however. The presence of 500 µM thiosulphate resulted in a statistically insignificant increase (1.18X) in the azide-sensitive rate over the endogenous rate (1-way ANOVA; $p>0.05$). This level of stimulation was comparable to that measured for fed bacteria, although the respiration rate was much lower. Azide-insensitive rates, 0.13 and 0.07 for 200 µM sulphide and 500 µM thiosulphate respectively, remained constant in relation to those measured in the experiments with the suspensions of fed bacteria.

3.3.3. Nitrate and Nitrite Respiration in Bacterial Symbiont Suspensions

3.3.3.1. Nitrate Respiration

Nitrite did not accumulate in incubations in the absence of bacteria (data not shown). The results of anoxic incubations with bacteria isolated from freshly collected bivalves (yellow gills indicative of SGES) revealed that net nitrite accumulation was typically linear with respect to incubation time, so nitrate respiration rates were calculated for the 40-min incubation period (Table 14a). Nitrate respiration rates in bacterial suspensions exposed to 5 mM cyanide were not statistically different from zero (1-way ANOVA; $p<0.05$)(data not shown). The results demonstrated that the bacteria were able to respire nitrate. The mean rate of net nitrate respiration in the nitrate-free incubations was very low and variable, 6.22 $\pm$ 5.86 nmol/gww/min. Nitrate respiration in this control incubation, even at low rates, suggests that the bacteria may be capable of storing small amounts of nitrate. In the presence of 2 mM nitrate but no exogenous sulphur source, the nitrate respiration rate of the bacteria was x15.5 the control rate. This stimulation in nitrate respiration rate in the absence of exogenous reduced sulphur suggests the use of SGES as substrate.

Both 200 µM sulphide and 200 µM thiosulphate stimulated nitrate respiration in bacterial isolates in comparison to the control rates but these rates were not significantly different from the nitrate respiration rate of the bacterial suspensions in the presence of nitrate and no external sulphur (1-way

Table 14. Net nitrate respiration rates (nmol/gww/min) of *Codakia orbiculata* bacterial symbiont suspensions. Data are presented as means ± 1 s.d..

(a) Bacteria isolated from freshly collected bivalves ("fed").

Incubation Conditions (T_0)					
Nitrate (mM)	Thiosulphate (µM)	Sulphide (µM)	Time (min)	n	Respiration Rate
0	0	0	40	3	6.22 ± 5.86
2	0	0	40	3	96.27 ± 21.44
2	200	0	40	3	122.62 ± 9.40
2	0	200	40	3	108.15 ± 24.28

(b) Bacteria isolated from bivalves maintained in sulphide-free, oxygenated seawater for 10 d ("starved").

Incubation Conditions (T_0)					
Nitrate (mM)	Thiosulphate (µM)	Sulphide (µM)	Time (min)	n	Respiration Rate
0	0	0	30	1	0.14
2	0	0	40	1	0.05
2	200	0	40	3	13.93 ± 2.06
2	0	200	40	3	0.00 ± 0.00

ANOVA; $p>0.05$). This statistical result may be influenced by the small sample size ($n=3$). Analysis of sulphide and thiosulphate in the incubation medium, over the course of a single experiment, revealed that levels of both thiols decreased rapidly in the suspensions (data not shown). This finding suggests that the bacteria utilized these thiols, when available, in preference to SGES. Additional factors, such as the binding of sulphide to SGES and oxidation by hematin granules (which could not be separated from the bacteria) may have influenced the concentration of sulphide in the suspensions, however.

The results of anoxic incubations of bacteria isolated from bivalves which had been maintained in sulphide-free, oxic conditions for 10 d (starved) are presented in Table 14b. The control rate of nitrite accumulation (0.14 nmol/gww/min) was much lower than that of the bacteria isolated from freshly collected bivalves. The addition of 2 mM nitrate but no sulphur substrate, did not stimulate nitrite accumulation rate; the measured rate (0.05 nmol/gww/min) was much lower than recorded for suspensions of bacteria isolated from freshly collected bivalves. These results suggest that the low respiration rate was not due to lack of electron acceptor. The incubations with neither exogenous nitrate nor sulphur source and the incubations with exogenous nitrate only were not replicated. The nitrite accumulation rate for suspensions of starved bacteria incubated with 5 mM cyanide was not significantly different from zero ($p>0.05$) (data not shown).

When suspensions of starved bacterial symbionts were incubated anoxically in the presence of nitrate and thiosulphate, nitrite accumulation rates were much higher (13.93 $\pm$ 2.06 nmol/gww/min) than those measured in the absence of thiosulphate. This finding suggests the latter rates were low, at least in part, due to lack of sulphur substrate. Even in the presence of thiosulphate, however, nitrite accumulation rates were substantially lower (x0.11) than those recorded for suspensions of bacteria isolated from freshly collected bivalves. This finding suggests that the starvation period may have resulted in repression of nitrate respiration.

The addition of sulphide to suspensions of bacteria from starved bivalves resulted in zero detectable nitrite accumulation. It seems unlikely that 200 µM sulphide completely inhibited respiration in the starved symbionts; sulphide concentrations up to 250 µM did not inhibit anaerobic $^{14}CO_2$ fixation (next section). Sulphide concentration in the incubation medium decreased at rates only slightly lower than those recorded in the suspensions of fed bacterial symbionts (single experiment only; data not shown). This finding, combined with the fact that zero nitrite accumulated in the presence of sulphide, suggests that although repression of nitrate respiration may be a contributing factor, either the bacteria did not accumulate the intermediate nitrite and denitrification was occurring or sulphide did not stimulate nitrate respiration. The results of the nitrite respiration experiments, presented in the following section, support the former suggestion.

3.3.3.2. Nitrite Respiration

Bacterial symbionts isolated from the gills of freshly collected *Codakia orbiculata* were incubated under anoxic conditions and nitrite respiration rates were measured by the decrease in nitrite concentration in the incubation medium. Nitrite respiration rates were linear over 40 min. All incubations were repeated in the absence of bacteria and no changes in nitrite concentration were detected (data not shown). In the control incubations (bacterial suspensions incubated with neither nitrite nor external reduced sulphur), a small amount of nitrite accumulated in the medium (as discussed above), suggesting that the bacteria stored a small amount of nitrate (Table 15a) and that nitrite probably was not reduced further. Because nitrite respiration was measured by the decrease in nitrite in the incubation medium and not by the accumulation of an end-product (eg: N_2), these experiments did not determine if the bacteria were able to store nitrite.

In the presence of 20 μM nitrite and no external sulphur source, nitrite concentrations increased rather than decreased, in the incubation medium (Table 15a). The net nitrite accumulation rate in the presence of nitrite was not significantly different from the rate in the complete absence of exogenous nitrite (1-way ANOVA; $p>0.05$). It is assumed that the bacteria must have used SGES as substrate in the absence of exogenous reduced sulphur. Nitrite concentrations also increased, rather than decreased, in incubations with 20 μM nitrite and 1 mM thiosulphate. These results again suggest that a small amount of nitrate, possibly stored, must be

Table 15. Net nitrite respiration rates (nmol/gww/min) of *Codakia orbiculata* bacterial symbiont suspensions. Nitrite accumulation rates are expressed as negative values. Data are presented as means ± 1 s.d..

(a) Bacteria isolated from freshly collected bivalves ("fed").

Incubation Conditions (T_0)					
Nitrite (µM)	Thiosulphate (mM)	Sulphide (µM)	Time (min)	n	Respiration Rate
0	0	0	40	3	-6.22 ± 5.86
20	0	0	40	3	-1.52 ± 2.08
20	1	0	60	1	-6.16
20	0	200	40	3	4.84 ± 1.97

(b) Bacteria isolated from bivalves maintained in sulphide-free, oxygenated seawater for 10 d ("starved").

Incubation Conditions (T_0)					
Nitrite (µM)	Thiosulphate (mM)	Sulphide (µM)	Time (min)	n	Respiration Rate
0	0	0	30	1	-0.14
20	0	0	30	1	0.35
20	1	0	60	1	-0.88
20	0	200	30	3	26.19 ± 5.00

available to the bacteria. The data suggest that fed bacteria, in the absence of exogenous sulphur or in the presence of thiosulphate, used stored nitrate preferentially to nitrite and that nitrite was not reduced at a measurable rate.

Sulphide (200 μM) stimulated nitrite respiration: nitrite disappeared from the incubation medium at the rate of 4.84 ± 1.97 nmol/gww/min. Cyanide (5 mM) reduced nitrite disappearance to rates not significantly different from zero (1-way ANOVA; $p>0.05$) (data not shown). These results suggest that the bacterial symbionts were capable of net nitrite respiration only in the presence of sulphide.

The results of anoxic nitrite respiration experiments using suspensions of bacteria isolated from bivalves previously maintained in sulphide-free, oxic conditions for 10 d are presented in Table 15b. In the suspension incubated with neither exogenous nitrite nor reduced sulphur, the nitrite concentration increased at a barely measurable rate. This suggests SGES and stored nitrate reserves were low. In the presence of 20 μM nitrite and no sulphur substrate or 20 μM nitrite and 1 mM thiosulphate (these incubations were not replicated), nitrite concentrations remained relatively constant.

Nitrite disappearance rates in suspensions of starved bacteria were stimulated by 200 μM sulphide (26.19 ± 5.00 nmol/gww/min). These rates, measured over 30 min, were significantly higher (1-way ANOVA; $p<0.05$) than those recorded for suspensions of bacteria from freshly collected bivalves

during 60-min incubations (4.84 $\pm$ 1.97 nmol/gww/min). Assuming that the bacteria are capable of storing nitrate, the latter rates may have been mediated by the production of nitrite from nitrate stores. The nitrite disappearance rates for starved bacteria incubated with 5 mM cyanide (data not shown) were not significantly different from zero (1-way ANOVA; $p>0.05$), indicating that nitrite reduction was due to its respiration. The high rates of nitrite respiration in the presence of sulphide obviously do not suggest repression of nitrite respiration as a result of the starvation period, as indicated earlier for nitrate respiration.

3.3.4. Anaerobic Carbon Dioxide Fixation by Bacterial Symbiont Suspensions

Anaerobic carbon dioxide fixation rates for suspensions of bacterial symbionts isolated from *Codakia orbiculata* are summarized in Tables 16a and 16b. These experiments were conducted primarily to determine if the isolated bacterial symbionts were able to fix carbon dioxide anaerobically and, secondly, to provide preliminary data on the effects of nitrate (as an alternate electron acceptor) and the effects of thiosulphate and sulphide on anaerobic fixation rates. In addition, preliminary investigations were conducted on the effect of starvation (maintaining bivalves in oxygenated sulphide-free seawater for 10 d) on anaerobic carbon dioxide fixation.

The endogenous, anaerobic carbon dioxide fixation rate of suspensions of bacteria isolated from freshly collected bivalves was 23.9 $\pm$ 0.76 µmolC/gww/h (Table 16a). Neither the addition of 1 mM thiosulphate nor 200 µM sulphide significantly stimulated CO_2 fixation above endogenous levels (1-way ANOVA; $p>0.05$). Incubations with 2 mM nitrate and with combinations of nitrate and sulphide or thiosulphate were not replicated but results suggest no stimulation of the carbon dioxide fixation rate in comparison to the endogenous rate. The addition of 10 mM azide to the bacterial isolates reduced CO_2 fixation by approximately 65%, suggesting that $^{14}CO_2$ uptake was linked to bacterial respiration. These latter incubation experiments need to be repeated in order for the results to be analyzed statistically.

Table 16. Carbon dioxide ($^{14}CO_2$) fixation rates (µmolC/gww/h) of *Codakia orbiculata* bacterial symbiont suspensions. Data are presented as means ± 1 s.d.. "Endog." = Endogenous.

(a) Bacteria isolated from freshly collected bivalves ("fed").

Incubation Conditions (T_0) Nitrate (mM)	Thiosul. (mM)	Sulphide (µM)	Azide (mM)	n	CO_2 Fixation Rate	CO_2 Fixation Rate:Endog. Rate
0	0	0	0	3	23.90 ± 0.76	1.00
0	1	0	0	3	27.24 ± 0.40	1.14
0	0	200	0	3	26.08 ± 2.55	1.09
2	0	0	0	1	22.76	0.98
2	1	0	0	1	21.96	0.94
2	0	200	0	1	22.76	0.98
2	0	0	10	1	8.28	0.35

(b) Bacteria isolated from bivalves maintained in sulphide-free, oxygenated seawater for 10 days ("starved").

Incubation Conditions (T_0) Nitrate (mM)	Thiosul. (mM)	Sulphide (µM)	Cyanide (mM)	n	CO_2 Fixation Rate	CO_2 Fixation Rate:Endog. Rate
0	0	0	0	1	7.36	1.00
0	1	0	0	1	29.32	4.14
0	0	200	0	1	23.53	3.34
5	0	0	0	1	8.77	1.20
5	1	0	0	1	23.69	3.34
5	0	200	0	1	19.51	3.33
5	0	0	5	1	0.57	0.08
0*	0	0	0	1	3.81	0.50

* Aerobic Control

The same experiments were carried out on bacteria isolated from *Codakia orbiculata* maintained (starved) in oxygenated, sulphide-free seawater for 10 d prior to experimentation (Table 16b). The endogenous CO_2 fixation rate of suspensions of starved bacteria (7.36 µmolC/gww/h) was approximately x0.3 the endogenous rate of suspensions of fed bacteria. Results revealed that 1 mM thiosulphate and 200 µM sulphide increased the CO_2 fixation rate by x4.14 and x3.34 the endogenous rates, respectively. The addition of 5 mM nitrate did not appear to stimulate CO_2 fixation, while the nitrate/sulphide and nitrate/thiosulphate combinations resulted in rates x3.34 and x2.65 the endogenous rate, respectively. Cyanide (5 mM) reduced the carbon dioxide fixation rate to nearly zero, again suggesting a link to bacterial respiration. Endogenous CO_2 fixation rates were also measured under oxic conditions and rates were x0.50 those measured under anoxic conditions. These experiments on suspensions of starved bacteria should be repeated in order for results to be analyzed statistically but stimulation of carbon dioxide fixation, as a result of thiol addition as an energy source, is suggested.

3.3.5. Nitrate and Nitrite Respiration in Whole *Codakia orbiculata*

The ability of the *Codakia orbiculata* bacterial symbionts *in vivo* to respire nitrate and nitrite was examined using whole bivalve incubation experiments. Bivalves were incubated for up to 24 h in either oxic or anoxic sterilized, FSSW containing combinations of nitrate or nitrite and sulphide, thiosulphate or no sulphur substrate. Nitrate and nitrite respiration rates were determined by monitoring the change in nitrite concentration in the incubation medium over time. Respiration rates were linear over the first 6.5 (or 8) h and are presented in Tables 17 and 18. Incubations with no bivalves but with the above conditions and incubations with bivalves but no external electron acceptor or sulphur substrate resulted in no change in nitrite concentration in the incubation medium (data not shown).

The nitrate respiration data in Table 17a demonstrate that the symbionts were capable of nitrate respiration *in vivo* under anoxic conditions. Whole *C. orbiculata* incubated without an external form of reduced sulphur but with 100 µM nitrate produced nitrite at a low rate, suggesting that the bivalves may have been using SGES. The mean nitrate respiration rate recorded in the presence of nitrate and 250 µM sulphide was not statistically different from the rate measured in the absence of an external sulphur source (1-way ANOVA; $p>0.05$). The variation around the mean (expressed as $\pm$ 1 s.d.) was high and, in some incubations, no nitrate respiration could be detected. In contrast, bivalves incubated anoxically with 100

Table 17. Nitrate respiration rates (nmol/gww/min) of whole *Codakia orbiculata* incubated in anoxic and oxic conditions, plus sulphide disappearance rates (nmol/gww/min) from the incubation medium. Data are presented as means ± 1 s.d.. n=3.

(a) Nitrate respiration rates of whole *Codakia orbiculata* incubated under anoxic conditions.

Incubation Conditions (T_0) Nitrate (µM)	Thiosul. (µM)	Sulphide (µM)	Time (h)	Respiration Rate	Sulphide Disappearance Rate
100	0	0	6.5	0.39 ± 0.04	
100	0	250	6.5	0.20 ± 0.24	4.54 ± 3.49
100	250	0	8.0	2.75 ± 0.36	

(b) Nitrate respiration rates of whole *Codakia orbiculata* incubated under anoxic conditions with sulphide.

Incubation Conditions (T_0) Nitrate (µM)	Sulphide (µM)	Time (h)	Respiration Rate
100	0	6.5	0.39 ± 0.04
100	50	8.0	1.25 ± 1.56
100	250	6.5	0.20 ± 0.24
100	1000	8.0	0.04 ± 0.02

(c) Nitrate respiration rates of whole *Codakia orbiculata* incubated under oxic conditions with sulphide, thiosulphate or no thiol.

Incubation Conditions (T_0) Nitrate (µM)	Thiosul. (µM)	Sulphide (µM)	Time (h)	Respiration Rate	Sulphide Disappearance Rate
100	0	0	8.0	0.15 ± 0.02	
100	0	250	8.0	3.67 ± 0.28	105.13 ± 0.27
100	250	0	8.0	0.43 ± 0.03	

μM nitrate and 250 μM thiosulphate had the highest nitrate respiration rate in anoxic conditions, with comparatively low variation around the mean. This rate was statistically higher (1-way ANOVA; $p<0.05$) than the nitrate respiration rates measured in the absence of an external sulphur source or in the presence of 250 μM sulphide.

Table 17b summarizes the results of anaerobic respiration experiments in the presence of nitrate and a range of sulphide concentrations. The nitrate respiration rate in the presence of 1000 μM sulphide is statistically lower than the rates in the presence of the lower sulphide concentrations (1-way ANOVA; $p<0.05$). The nitrate respiration rates in the presence of 0, 50 or 250 μM sulphide are not statistically different from each other (1-way ANOVA; $p>0.05$). However, the maximum mean nitrate respiration rate occurred in the 50 μM sulphide incubations.

The whole bivalve oxic-incubation data for nitrate respiration are presented in Table 17c. The nitrate respiration rates for symbionts *in vivo*, incubated with 100 μM nitrate but no external form of reduced sulphur and for incubations with nitrate and thiosulphate, were significantly lower (1-way ANOVA; $p<0.05$) than the respiration rates measured under comparable anoxic conditions. This suggests that the oxic conditions resulted in some inhibition of nitrate respiration. It is possible that the bacteria may be using aerobic, in addition to anaerobic, respiration. In contrast, nitrate respiration rates for intact symbioses in the presence of oxygen, 100 μM nitrate and 250 μM sulphide

were significantly higher (1-way ANOVA; $p<0.05$) than those recorded under the comparable anoxic conditions.

The rate of sulphide disappearance in the incubation media was measured for both oxic and anoxic 250 µM sulphide experiments. Sulphide disappeared at the rate of 105.13 ± 0.27 nmol/gww/min in the oxic incubations and 4.54 ± 3.49 nmol/gww/min in the anoxic incubations (all rates corrected for bivalve-free controls). In addition to stimulating nitrate respiration rates, sulphide may have been utilized by the symbionts aerobically; aerobic respiration rates were comparatively much higher than anaerobic respiration rates for isolated symbionts (Tables 13, 14 and 15).

Results of whole animal nitrite respiration experiments under oxic and anoxic conditions and in the presence of nitrite and sulphide are summarized in Table 18. Bacterial symbionts *in vivo* respired nitrite only in the presence of 250 µM sulphide; comparable experiments in the presence of nitrite only or nitrite and thiosulphate yielded nitrite respiration rates which were not statistically significant from zero (1-way ANOVA; $p>0.05$; data not shown). In combination with the results of the nitrite respiration experiments on suspensions of isolated bacteria, these results suggest that the symbionts *in vivo* must have had access to sulphide. Nitrite was respired in the anoxic sulphide and nitrite incubations at the rate of 3.61 ± 2.17 nmol/gww/min and in the oxic sulphide and nitrite incubations at the rate of 2.29 ± 0.31 nmol/gww/min. These respiration rates were not statistically different (1-way ANOVA; $p>0.05$). The slight decrease in nitrite respiration

under oxic conditions may be related to the increased availability of oxygen and the additional utilization of aerobic respiration by the symbionts.

Table 18. Nitrite respiration rates (nmol/gww/min) of whole *Codakia orbiculata* incubated with sulphide under oxic or anoxic conditions, plus sulphide disappearance rates (nmol/gww/min). Data are presented as means ± 1 s.d.. n=3.

Incubation Conditions (T_0)				
Oxic/ Anoxic	Nitrite (µM)	Sulphide (µM)	Time (h)	Respiration Rate
oxic	100	250	6.5	2.29 ± 0.31
anoxic	100	250	8.0	3.61 ± 2.17

3.4. DISCUSSION

3.4.1. Gill Globular Elemental Sulphur Levels in Response to the Absence of Exogenous Reduced Sulphur

Stored globular elemental sulphur (SGES) was found in the bacteria-containing gills of *Codakia orbiculata*. SGES has been shown to be enclosed in small vesicles, formed by invagination of the cytoplasmic membrane, in the bacterial symbionts of several species of lucinid bivalve (Fisher and Hand, 1984; Dando *et al.*, 1985; Vetter, 1985; Reid and Brand, 1986; Southward, 1986; Distel and Felbeck, 1987). Foot and mantle tissues of *C. orbiculata*, like those of *Lucinoma aequizonata* (Vetter, 1985) and the thyasirid bivalve *Thyasira flexuosa* (Dando and Southward, 1986), did not contain SGES.

SGES in symbiotic bacteria, similar to that in free-living bacteria, is assumed to be a stable intermediate product of sulphur oxidation which accumulates when the bacteria receive adequate supplies of reduced sulphur compounds (Schedel and Trüper, 1980, Lawrey *et al.*, 1981; Strohl *et al.*, 1981; Vetter, 1985). It has also been suggested that SGES accumulation in the free-living *Beggiatoa* and the symbiotic bacteria of *Solemya reidi* and *Lucinoma aequizonata* may be in response to potentially toxic environmental sulphide levels (Nelson *et al.*, 1986; Anderson *et al.*, 1987; Cary *et al.*, 1989). The variation observed in SGES in the gills of freshly collected *Codakia orbiculata* is presumed, therefore, to be largely a result of variation in sulphide content in the ambient sediment interstitial water as discussed in Chapter 2. Similar variation in gill SGES has been noted in freshly

collected specimens of the thyasirids *Thyasira flexuosa* and *Thyasira sarsi* and the lucinids *Myrtea spinifera* (Dando *et al.*, 1985), *Lucinoma borealis* (Dando *et al.*, 1986a) and *Pseudomiltha sp.* (Brooks *et al.*, 1987). Freshly collected specimens of the solemyid bivalve, *S. reidi*, also demonstrated variation in gill SGES (Anderson *et al.*, 1987).

The gills of freshly collected *Codakia orbiculata* contained 17.35 to 455.57 µg-atoms S/gww. In contrast, the SGES content of the gills of an aposymbiotic bivalve, *Dosinia lupinus*, contained only 1.1 ± 0.7 µg-atoms S/gww (Dando *et al.*, 1986a). The SGES content in gills of freshly collected *C. orbiculata* was greater than those reported for the lucinid bivalves, *Myrtea spinifera* and *Lucinoma borealis* (Dando *et al.*, 1985; Dando *et al.*, 1986a). Although there may be inter-specific differences in the ability to accumulate globular elemental sulphur, it seems likely that the high levels of SGES found in *C. orbiculata* gills are a result of access to environmental concentrations of reduced sulphur forms higher than those available to the other lucinid species. Both *M. spinifera* and *L. borealis* are found in sediments with barely detectable levels of dissolved sulphide in the interstitial water (Dando *et al.*, 1985, 1986a). In contrast, mean sulphide levels in the *Thalassia testudinum* bed sediment habitat of *C. orbiculata* ranged from 31.32 to 296.68 µM. The vent vesicomyid bivalves, *Calyptogena magnifica* and *Calyptogena ponderosa*, had the highest reported globular elemental sulphur reserves with maximum levels of 1021.9 µg-atoms S/gww (Fisher *et al.*, 1988) and 2593.8 µg-atoms S/gww (Brooks *et al.*, 1987), respectively.

Although vent water at the base of the bivalves contained only 20-40 µM (Johnson *et al.*, 1988), it has been hypothesized that the bivalves insert their foot into seawater with substantially higher sulphide concentrations (Fisher *et al.*, 1988).

In the absence of exogenous thiols, the SGES of *Codakia orbiculata* gills was observed to decrease. Gill SGES levels reached a consistently low background level after 5 d; this background level was comparable to SGES content reported for European species of lucinids, discussed earlier, found in low sulphide habitats (Dando *et al.*, 1985, 1986a). The observed decrease in SGES levels in *C. orbiculata* gills is assumed to be a result of utilization of SGES as an energy source by the bacterial symbionts (Anderson *et al.*,1987; Cary *et al.*, 1989). The ability of free-living sulphur bacteria to utilize SGES as an energy source has been well documented (Schedel and Trüper, 1980; Kelly, 1982; Javor *et al.*, 1990).

Codakia orbiculata gills changed from yellow to red, as the yellow SGES was utilized, due to the increasingly visible pigment granules and intracellular hemoglobin. Hence, as suggested by Vetter (1985), gill colour appears to be a useful general indicator of sulphur content. Although *Myrtea spinifera* gills have never been reported as yellow, those reported to be deep pink had higher SGES than those which were dark red and the gills were observed as uniformly dark red after 4 weeks in laboratory conditions (Dando *et al.*, 1985). Dando *et al.* (1986a) reported no correlation between gill colour and globular elemental sulphur content in *Lucinoma*

borealis (data on gill colour not shown) but it has been noted that the gills lose the whitish "bloom" (presumed by this author to be SGES) obvious in freshly collected specimens and show a darkening of the gills when maintained in sulphide-free conditions in the laboratory (Southward, 1986). Change in gill colour in response to availability of a reduced sulphur source has also been observed for the solemyid bivalve, *Solemya reidi*. Maintaining bivalves in oxygenated seawater may also result in a reduction in the number of intracellular bacteria (Dando *et al.*, 1986a) and, hence, a reduction in SGES.

There appears to be considerable variation in the rate at which SGES is utilized by bivalve bacterial symbionts. In *Codakia orbiculata*, SGES was utilized relatively rapidly: dropping from approximately 1.5% dry weight to 0.5% dry weight after 2 days. In contrast, *L. aequizonata* gill elemental sulphur dropped from 1% dry weight to 0.5% dry weight in 28 days (Cary *et al.*, 1989). This may be indicative of a higher rate of metabolism in *C. orbiculata* symbionts, perhaps associated with a higher metabolic rate in the host bivalve. Gill elemental sulphur content in the thyasirid bivalve, *Thyasira sarsi*, decreased over 2 days (Dando and Southward, 1986) at a rate comparable to that observed in *C. orbiculata*. After 2 days starvation, *C. orbiculata* and *T. sarsi* gills had 33% and 50% of the elemental sulphur reserves remaining, respectively. *Thyasira flexuosa* gills had only 5% of the sulphur reserves remaining after 2 days starvation (Dando and Southward, 1986), suggesting *T. flexuosa* symbionts utilize

elemental sulphur reserves more rapidly than either *C. orbiculata* or *T. sarsi*.

The rate of utilization of SGES may be influenced by thiols present in the mantle fluid at initiation of the experiment, which may be used as energy substrates. Mantle fluid thiosulphate and sulphide levels decreased rapidly in *Codakia orbiculata* incubated in sulphide-free conditions, approaching zero after 48 and 72 h respectively. The most significant drop in gill SGES occurred between 48 and 120 h. After 120 h reserves were virtually exhausted and mean levels remained consistently low. It seems probable, therefore, that the mantle fluid thiols were used in preference to SGES. Bivalves placed in seawater lacking exogenous thiols were observed to close their valves so that mantle fluid was not exchanged with the surrounding medium. The subsequent drop in sulphide and thiosulphate levels in the mantle fluid suggests rapid utilization of these thiols in the absence of an exogenous source of reduced sulphur.

The contribution of globular elemental sulphur reserves to the overall sulphur flux of *Codakia orbiculata* has yet to be determined but can be hypothesized. Anderson *et al.* (1987) suggested that for the solemyid bivalve, *Solemya reidi*, elemental sulphur reserves do not represent a significant amount of the total sulphur flux that supports net CO_2 uptake. The mean quantity of elemental sulphur found in the gills was calculated to represent only 0.19 to 13.7% of the sulphide oxidized in 12 h by an average-sized *S. reidi*. However, using these sulphide oxidation rates to calculate how long the

elemental sulphur reserves would last, *S. reidi* bacterial sulphur reserves would last for 1.4 to 98.7 min. It is feasible that the mean SGES in gills of freshly collected *C. orbiculata*, which are x5 greater (per unit dry wgt) than those of *S. reidi*, would last significantly longer. Hence, as suggested for *L. aequizonata* (Vetter, 1985), the SGES of *C. orbiculata* symbionts could be used as a short-term energy source, permitting the symbiotic bacteria to function during the absence of external sulphide. Results of aerobic respiration, $^{14}CO_2$ fixation and nitrate/nitrite respiration studies on suspensions of isolated bacterial symbionts of *C. orbiculata* (Sections 3.4.2.-3.4.4) and nitrate/nitrite respiration studies on whole *C. orbiculata* suggest that globular elemental sulphur is used as a sulphur substrate in aerobic and anaerobic respiration and that the energy released can stimulate carbon dioxide fixation in the symbionts.

3.4.2. Aerobic Respiration in Bacterial Symbiont Suspensions

Bacterial symbiont suspensions of *Codakia orbiculata* respired oxygen, suggesting that the symbionts may also utilize aerobic respiration *in vivo*. *C. orbiculata* has access to oxygen *in situ*; the seawater overlying the bivalves' habitat, which the animals can access with an inhalant tube (Allen, 1958), is saturated in oxygen (Chapter 2). However, as discussed in Chapter 1, the position of the bacteriocytes within the lucinid gill is such that incoming seawater must first pass across oxygen-scavenging cells in the ciliated zone (Fisher and Hand, 1984; Distel and Felbeck, 1987). The symbionts would have access, therefore, to lower oxygen concentrations *in vivo* than are available in the habitat. It has been suggested that maintaining the bacterial symbionts under very low oxygen, or even anoxic conditions, *in vivo* would be beneficial to CO_2 fixation (Fisher and Hand, 1984; Dando *et al.*, 1985). Since RuBPC/O can exhibit both oxygenase and carboxylase activities, low oxygen conditions would reduce competition for the same active site (Chollet, 1977). Certainly, free-living sulphur-oxidizing bacteria are frequently microaerophilic (Jørgensen, 1982). The oxygen-binding intracellular hemoglobin found in lucinid gills may also help to protect the symbionts from excess oxygen which would interfere with their autotrophic processes (Dando *et al.*, 1985; Kraus and Wittenberg, 1990)). It is probable, therefore, that the aerobic respiration rates of symbionts *in vivo* are lower than those measured for suspensions of isolated

bacterial symbionts in this study, which were maintained at >80% oxygen.

In apparent contrast to the bacterial symbiont suspensions of *Codakia orbiculata*, those of the lucinid *Lucinoma aequizonata* did not demonstrate measurable aerobic respiration (Cary and O'Brien, unpubl. data.). The inability to respire aerobically may be related to the *L. aequizonata* symbionts' lack of access to oxygen *in vivo*. In contrast to the *C. orbiculata* habitat, *L. aequizonata* habitat is oxygen-depleted with an oxygen concentration, 1 m above the sediment-water interface, of approximately 18 - 20 µM (Cary *et al.*, 1989). If the bivalves' habitat is oxygen-depleted, the bacteria may be exposed frequently to anoxic conditions. Hentschel *et al.* (1993) reported that incubating *L. aequizonata* in oxygenated conditions did not change the inability of the symbionts to respire oxygen, suggesting that these bacteria are dependent on anaerobic respiration.

The suspensions of *Codakia orbiculata* bacterial symbionts were able to respire oxygen in the absence of exogenous reduced sulphur, suggesting utilization of SGES. Both sulphide and thiosulphate stimulated respiratory oxygen consumption, demonstrating that the bacterial suspensions were capable of aerobically utilizing both thiols as energy sources. Sulphide (200 µM) did not inhibit respiration in the bacterial suspensions and, in fact, had the greatest stimulatory effect on respiratory oxygen consumption, suggesting that the bacteria may have access to sulphide *in vivo*. The extent to which lucinid bacterial symbionts encounter sulphide *in vivo*

has yet to be established, however. As discussed in Chapter 1, host cell mitochondrial oxidation of sulphide to thiosulphate has been demonstrated for *Solemya reidi* when the bivalves are maintained in oxic conditions (Powell and Somero, 1985; Anderson *et al.*, 1987; O'Brien and Vetter, 1990; Wilmot and Vetter, 1992). Preliminary evidence, based on analysis of blood thiols, suggests that the lucinid host cell mitochondria may also oxidize sulphide to thiosulphate in the presence of oxygen (Cary *et al.*, 1989). Non-enzymatic sulphide oxidation, involving the hematin-containing granules, may also produce thiosulphate which could be used as an energy source by the bacterial symbionts (R. Vetter, pers. comm.). Thiosulphate stimulated aerobic respiration in the bacterial suspensions of *C. orbiculata*, but since thiosulphate is only present in very low concentrations in *C. orbiculata* habitat (means range from 0.66-32.27 µM), it is presumed that thiosulphate available to the symbionts *in vivo* is largely the product of host oxidation of sulphide (Powell and Somero, 1985; Anderson *et al.*, 1987; Cary *et al.*, 1989; O'Brien and Vetter, 1990).

Host mechanisms for oxidation of sulphide may not be 100% efficient, however, particularly when the bivalves are exposed to high sulphide concentrations. The bacteria may have access to some sulphide, therefore. This suggestion is supported by the findings that nitrite respiration, stimulated only by sulphide in isolated symbionts, occurred in whole *Solemya reidi* (Wilmot and Vetter, 1992) and *Codakia orbiculata* (this study) in oxic conditions. In addition, the presence of sulphide-binding intracellular hemoglobin in *Lucina pectinata*

gills (Kraus and Wittenberg, 1990) and the location of the bacteria towards the exterior part of the bacteriocyte (near the bacteriocyte channels, the inhalant water flow and, therefore, the sulphide source) (Distel and Felbeck, 1987) support the *in vivo* utilization of sulphide by the bacterial symbionts. However, host detoxification mechanisms, in addition to cell permeability to dissolved sulphide as a function of pH, would ensure that the bacterial symbionts would be exposed to lower sulphide concentrations *in vivo* than in the environment (Chen and Morris, 1972; Cary *et al.*, 1989; Bagarinao, 1992; Wilmot and Vetter, 1992).

Unfortunately, there are no comparable data in the literature on thiol stimulation of aerobic respiration in the bacterial symbionts of other species of lucinids. However, aerobic $^{14}CO_2$ fixation was stimulated in isolated bacteriocytes of *Lucina floridana* in the presence of 1 mM thiosulphate (Hand, 1987). This finding supports the results of this study that the symbionts are able to utilize thiosulphate aerobically. All other experiments showing stimulation of aerobic carbon dioxide fixation in lucinids were conducted on isolated gills (Dando *et al.*, 1985, 1986a). In these experiments the amount of oxygen available to the symbionts *in vivo* is unknown, which makes comparison with this study difficult. In addition, only sulphide was tested as a substrate. In light of potential oxidation of sulphide to thiosulphate prior to reaching the symbionts, the observed stimulation may have been due to thiosulphate and not sulphide.

The non-enzymatic (azide-insensitive) oxygen consumption rates for *Codakia orbiculata* symbiont suspensions remained relatively constant over a range of sulphide concentrations. Non-enzymatic sulphide oxidation is the result of oxidation by the hematin-containing (ferric iron) pigment granules (Powell and Arp, 1989; Vismann, 1991; Wilmot and Vetter, 1992). The results of this study suggest that the sulphide-oxidizing capacity of pigment granules in *C. orbiculata* symbiont suspensions was maximal at 100 µM sulphide. In contrast, azide-insensitive oxygen consumption rates of *Solemya reidi* symbiont suspensions increased linearly with sulphide concentration up to 1 mM sulphide, reaching a maximal rate at >1 mM (Wilmot and Vetter, 1992). The oxygen consumption rates of fed *S. reidi* symbionts at 100 µM and 200 µM sulphide were approximately x100 and x200 greater, respectively, than the rates recorded for *C. orbiculata* symbiont suspensions. Hence, *S. reidi* bacterial suspensions appear to have the greater capacity for non-enzymatic oxidation. This may be due to a higher density of, or larger size of, pigment granules in *S. reidi*. The size of pigment granules has been observed to increase in the gills of starved *S. reidi*, accompanied by an increase in azide-insensitive sulphide oxidation rates in bacterial suspensions (Wilmot and Vetter, 1992). Similar observations have been made on the size of pigment granules in the gills of starved *Lucinoma borealis* (Southward, 1986). Although similar observations have not been made for *C. orbiculata*, an increase in azide-insensitive sulphide

oxidation rates was observed in starved *C. orbiculata* bacterial suspensions.

The role of the pigment granules in sulphide oxidation is undetermined for intact bacteria-bivalve symbioses. Granular non-enzymatic oxidation of sulphide appeared to prevent poisoning of aerobic respiration in *Solemya reidi* bacterial suspensions, enabling respiration to continue at a maximal rate at sulphide levels >150 µM (Wilmot and Vetter, 1992). As discussed earlier, however, the bacteria are probably not exposed to levels of sulphide *in vivo* comparable to those in the environment. Respiratory oxygen consumption at sulphide levels <150 µM was greater than azide-insensitive oxygen consumption in *S. reidi* bacterial enrichments (Wilmot and Vetter, 1992). This suggests that, at the lower levels of sulphide likely to be found in the cells, the role of pigment granules in sulphide oxidation is less significant. Furthermore, the location of the pigment granules in the bacteriocytes eliminates any possible "peripheral defense" strategy for protecting the host cells or the bacteria from high sulphide levels (R. Vetter, pers. comm.). The large granular shape of the pigment granules, in contrast to a diffused distribution, would also reduce their effectiveness in oxidizing sulphide (R. Vetter, pers. comm.). The pigment granules have other hypothesized "identities" including lysosomes (Giere, 1985; Southward, 1986; Distel and Felbeck, 1987; Le Pennec *et al.*, 1988) and remants of host mitochondria and lysosomes occluded by iron (D. Wilmot and J. O'Brien, pers. comm.).

Although the results of this study demonstrate the ability of suspensions of isolated *Codakia orbiculata* bacterial symbionts to aerobically respire both sulphide and thiosulphate, their ability to utilize these thiols aerobically *in vivo* remains to be determined. If oxygen levels are low in the vicinity of the bacteriocytes, as hypothesized and discussed earlier, the bacteria also may utilize anaerobic respiration. The *Codakia orbiculata* bacterial symbionts have been demonstrated to respire nitrate and nitrite *in vivo* when the host bivalve is exposed to oxic conditions. The potential for *in vivo* aerobic respiration and sulphur substrate utilization in the symbionts is discussed, in light of evidence for nitrate and nitrite respiration by the symbionts *in vivo*, in section 3.4.5 and is summarized and related to habitat conditions in Chapter 4.

3.4.3. Nitrate and Nitrite Respiration in Bacterial Symbiont Suspensions

Many free-living, facultatively anaerobic bacteria utilize nitrate as a terminal electron acceptor for respiration (Adams *et al.*, 1971; Knowles, 1982). Nitrate (NO_3^-) is reduced to nitrite (NO_2^-) or may be further reduced, via nitric oxide (NO) and nitrous oxide (N_2O), to nitrogen gas (N_2). The conversion of nitrate to gaseous nitrogen compounds is termed denitrification (Knowles, 1982; Brock and Madigan, 1988). The symbiotic bacteria of *Codakia orbiculata* appear to resemble these free-living bacteria in their ability to utilize nitrate and nitrite as electron acceptors.

Fed bacteria isolated from *Codakia orbiculata* were able to respire nitrate in the absence of an external source of reduced sulphur but starved bacteria were not, suggesting that SGES can be utilized as substrate for nitrate respiration. Similar observations have been made for *Solemya reidi* bacterial suspensions (B. Javor, pers. comm.). Although no comparable data for symbiotic bacteria could be located in the literature, some free-living bacteria are able to oxidize globular elemental sulphur to sulphate using nitrate (Troelsen and Jørgensen, 1982).

The addition of sulphide or thiosulphate to suspensions of fed *Codakia orbiculata* bacteria did not significantly increase the nitrate respiration rate above the respiration rate measured in the absence of exogenous reduced sulphur. Similarly, nitrate respiration in purified symbionts from fed *Lucinoma aequizonata* was not stimulated by thiosulphate or

sulphide (Hentschel *et al.*, 1993). In contrast, sulphide and thiosulphate stimulated nitrate respiration in isolated symbionts from fed *S. reidi* by x6 and x4 the control rate, respectively (B. Javor, pers.comm.). Stimulation of respiration in suspensions of fed bacteria due to exogenous thiols must be interpreted with caution, however. SGES in freshly collected lucinids and *Solemya reidi* exhibit significant variation (Dando *et al.*, 1985, 1986a; Anderson *et al.*, 1987; Brooks *et al.*, 1987; Section 3.4.1.). If SGES is utilized as a sulphur substrate, the size of the reserves would affect the nitrate respiration rate in the absence of exogenous sulphur sources. The level of stimulation resulting from the addition of exogenous sulphur sources would also be affected, therefore. Hence, lack of stimulation of nitrate respiration in fed *C. orbiculata* symbionts by exogenous thiols could be interpreted to mean either these thiols are not utilized in nitrate respiration or exogenous thiols fuel nitrate respiration at a rate comparable to SGES.

The thiosulphate concentration decreased in suspensions of fed *Codakia orbiculata* symbionts suggesting that thiosulphate added to the suspension was used in preference to SGES to fuel nitrate respiration. It would appear, therefore, that SGES and exogenous thiosulphate fuel nitrate respiration at comparable rates. As discussed in section 3.4.2 on aerobic respiration, because thiosulphate concentrations in the bivalves' habitat are minimal, thiosulphate utilized by the symbionts *in vivo* probably results from host oxidation of sulphide, possibly by host cell mitochondria (Powell and

Somero, 1985; Anderson *et al.*, 1987; Cary *et al.*, 1989). Since thiosulphate is not gaseous, it presumably does not penetrate the bacterial cells easily (Holmes and Donaldson, 1969) and utilization of thiosulphate by the bacteria implies the presence of a transport mechanism (R. Vetter, pers. comm.).

Thiosulphate clearly stimulated nitrate respiration rates in starved *Codakia orbiculata* symbionts. Thiosulphate concentrations decreased in these experiments, although more slowly than in the fed bacterial suspensions. The nitrate respiration rates were significantly lower in starved bacteria than those measured in the fed symbionts, suggesting that nitrate respiration may have been repressed by the 10-day incubation in oxygenated, low nitrate (<1 μM) conditions. Nitrate respiration was repressed in whole *Solemya reidi* and the isolated symbionts, but not completely, after prolonged incubation in oxygenated, low-nutrient seawater (Wilmot and Vetter, 1992; B. Javor, pers.comm.).

Repression of nitrate respiration observed in *Codakia orbiculata* symbionts may be the result of either the oxygenated, or the nitrate-free, conditions of the 10 day incubation. In free-living heterotrophic denitrifying bacteria, evidence suggests that nitrate is required for the production of nitrate reductase and also, that nitrate reductases are repressed by oxygen but can be derepressed by decreases in oxygen (Chalamet, 1985). Repression of nitrate reductase in *Lucinoma aequizonata* symbionts has been shown, however, to be caused by the absence of nitrate but was not affected by the presence of oxygen (Hentschel *et al.*, 1993).

However, unlike the symbionts of *C. orbiculata* and *S. reidi*, those of *L. aequizonata* did not appear to respire aerobically. *C. orbiculata* symbionts respired nitrate *in vivo* when bivalves were maintained in oxic conditions, although the respiration rate was also lower than that measured in anoxic conditions (Section 3.4.5). This suggests that neither the low levels of oxygen present in the vicinity of the bacteriocytes nor the 24 h pre-incubation in oxygenated, low-nitrate seawater completely inhibited nitrate respiration. To determine the immediate effect of oxygen on nitrate respiration in symbionts, nitrate respiration rates should be measured in oxic suspensions of bacterial symbionts.

The addition of sulphide to fed *Codakia orbiculata* bacterial suspensions did not increase the nitrate respiration rate. Unlike for thiosulphate, there is no direct evidence for the role of sulphide in nitrate respiration; the addition of sulphide to starved *C. orbiculata* bacterial suspensions incubated with nitrate resulted in a nitrite accumulation rate consistently zero.

Sulphide concentrations decreased, however, in both fed and, at a slower rate, in starved *Codakia orbiculata* bacterial suspensions. The measured decrease in sulphide concentrations has a number of possible explanations. Sulphide may bind to elemental sulphur present in the suspensions, forming polysulphides (B. Javor, pers. comm.). The disappearance of sulphide from the starved bacteria suspensions due to the formation of polysulphides would be expected to be less, since starved bacteria have less SGES than fed bacteria. Analysis of

polysulphide formation would aid in interpretation of the sulphide disappearance data obtained in this study.

The decrease in sulphide levels in the suspension, in combination with undetectable nitrite accumulation, can best be explained by the occurrence of denitrification, with no accumulation of the nitrite intermediate. Nitrite produced from nitrate reduction (using sulphide) may be used immediately in nitrite respiration, producing N_2 (Knowles, 1982). Nitrite respiration in *Codakia orbiculata* bacterial suspensions was measured only in the presence of sulphide. Hence, denitrification seems probable. Further experiments, in which the end product of denitrification is measured, are required for confirmation. If denitrification is occurring in the sulphide incubations, then the reduction in the rate of sulphide disappearance observed in suspensions of starved bacterial symbionts may be partially a result of repression of nitrate respiration, as suggested earlier.

Inhibition of nitric oxide (NO) reduction in free-living bacteria begins at nitrate concentrations in the mM range (Packard *et al.*, 1983), so the nitrate concentration in the incubations (2 mM) may have resulted in reduced production of N_2 (Payne and Riley, 1969). Concentrations of nitrate found in seawater (1-40 µM), however, have been found to have little or no inhibitory effect on nitric oxide reduction (Packard *et al.*, 1983). Denitrification may be particularly important, therefore, for *in situ* bivalves, since nitrate levels in the interstitial water of *Codakia orbiculata* habitat sediments reached 36 µM but were commonly <10 µM (Chapter 2). Further

investigations of denitrification potential using other techniques (Payne, 1981) are suggested.

It must be noted that, since denitrification is hypothesized to be occurring in the presence of sulphide, caution must be used in comparing "nitrate respiration" rates for incubations with sulphide to rates measured in other incubations. Measuring nitrite accumulation as a method to assess nitrate respiration may be applicable to experiments in which thiosulphate is the reduced sulphur source but appears to be inappropriate when applied to experiments with sulphide. The possibility of denitrification is considered, throughout, for experiments in which sulphide was the reduced sulphur substrate.

Low nitrate respiration rates were measured for fed bacterial suspensions in the absence of added nitrate or sulphur substrate. This finding suggests that the fed symbionts may store a small amount of nitrate which can be used as an electron acceptor in the oxidation of globular elemental sulphur reserves. Interestingly, nitrite concentrations increased in suspensions of fed bacteria in the presence of nitrite, or nitrite plus thiosulphate, suggesting respiration of stored nitrate in preference to, or at a faster rate than, nitrite respiration. Hence, although further research is required, it is possible that the low nitrate respiration control rates measured in starved bacteria may be a result of both depleted SGES and nitrate stores, in combination with possible repression of nitrate reductase.

The ability of free-living bacteria to store nitrate is unreported in the literature. Purified symbionts from *Lucinoma aequizonata* did not demonstrate nitrate respiration in the absence of an external nitrate source but whole bivalves incubated in nitrate-free oxic or anoxic seawater for 8 days had 6.4 and 8.3 µM nitrite in their mantle fluid, respectively (Hentschel *et al.*, 1993). This suggests a small source of nitrate. The symbionts may utilize nitrate present *in vivo* in the mantle fluid, the blood or, as suggested by this study on *Codakia orbiculata* isolated symbionts, within the bacterial cells.

In contrast to *Codakia orbiculata* bacterial suspensions, purified *Lucinoma aequizonata* symbionts were reported to lack the ability to respire nitrite (Hentschel *et al.*, 1993). The experimental conditions were not reported, however. Assuming that the bacteria were isolated from fed bivalves and that the exogenous thiol, similar to those reported by the authors for nitrate respiration experiments, was 100 µM thiosulphate, then *L. aequizonata* lacks the ability to respire utilizing thiosulphate as substrate. *L. aequizonata* symbionts, like those of *C. orbiculata*, may respire nitrite only in the presence of sulphide and the possiblity of nitrite respiration should not be eliminated. Bacterial suspensions of *Solemya reidi* were also found to respire nitrite only in the presence of sulphide (B. Javor, pers comm). The bacterial symbionts may need a low oxidation-reduction potential, provided by sulphide, for denitrification (B. Javor, pers. comm.).

Nitrite respiration, in contrast to nitrate respiration, did not appear to be repressed afer a 10-day incubation in oxygenated, low nutrient seawater. In fact, starved bacterial suspensions respired nitrite at approximately x5 the rate measured for suspensions of bacteria from fed *Codakia orbiculata*. However, if the fed bacteria had access to a small amount of stored nitrate, then lower nitrite respiration rates (measured by the disappearance of nitrite from the incubation medium) in fed bacteria suspensions may have been influenced by respiration of stored nitrate to nitrite; ie. nitrite reduced to N_2 may be replaced (partially) by nitrite produced from nitrate reduction. In the absence of nitrate stores (starved bacterial suspensions), the nitrite respiration rate would appear to be greater, as was observed.

Nitrite is present in low amounts in seawater (<10 nM) (McGlathery, 1992) and in *C. orbiculata* habitat, nitrite in sediment interstitial water was consistenly below 0.3 µM (Chapter 2), suggesting bacterial symbiont nitrite respiration *in vivo* is most likely fuelled by nitrite produced by symbiont nitrate respiration. It should be noted that nitrite concentrations used in these experiments, like those of nitrate, were 2 orders of magnitude greater than those encountered by the bivalves *in situ* (Chapter 2). Hence, this study demonstrated the potential for nitrate and nitrite respiration in the bacterial symbionts but respiration rates measured in this study are likely to be greater than those of the intact symbiosis *in situ*.

The potential roles of nitrate, nitrite and aerobic respiration in the physiology of *Codakia orbiculata* symbionts *in vivo* are discussed in Section 3.4.5. of this chapter (the intact symbiosis) and summarized, together with habitat characteristics, in Chapter 4.

3.4.4. Anaerobic Carbon Dioxide Fixation by Bacterial Symbiont Suspensions

Suspensions of bacterial symbionts from fed *Codakia orbiculata* were able to fix $^{14}CO_2$ under anaerobic conditions. Surprisingly, the symbiont suspensions fixed carbon dioxide anaerobically in the absence of an exogenous electron acceptor. Addition of nitrate as an electron acceptor did not stimulate the carbon dioxide fixation rate. This was also observed for purified fed symbionts of *Lucinoma aequizonata* but not interpreted (Hentschel *et al.*, 1993). The low $^{14}CO_2$ fixation rates observed in suspensions of bacteria from starved *C. orbiculata* were stimulated by the addition of exogenous reduced sulphur but not by the addition of nitrate. The addition of both thiol and nitrate did not result in higher fixation rates than those observed with the thiols alone. Similar results have been observed in carbon dioxide fixation experiments on bacteria isolated from *Solemya reidi* (B. Javor, pers. comm.) and suggest the presence of an unidentified electron acceptor. In contrast, $^{14}CO_2$ fixation activity in gill homogenates of the vent mussel, *Bathymodiolus thermophillus*, was completely inhibited in deoxygenated samples (Belkin *et al.*, 1986) suggesting no endogenous electron acceptor.

In the absence of direct evidence, the identity of the electron acceptor can only be hypothesized by systematically examining possibilities, as follows. The experimental protocol for this study on *Codakia orbiculata* was carefully designed to avoid oxygen contamination, so the consistent

presence of free oxygen in the incubations was not considered as a possibility. The *C. orbiculata* symbionts, as suggested by nitrate respiration data discussed in the previous section, are able to utilize nitrate as an electron acceptor and may be able to store small amounts of nitrate. It is possible, therefore, that stored nitrate is the unidentified electron acceptor for the bacteria. However, the low nitrate respiration rate suggests that these stores are not large. Hence, it is unlikely that sufficient nitrate would remain, after 10 days starvation, to act as electron acceptor for the starved bacteria. Carbon dioxide fixation in starved *C. orbiculata* symbionts was stimulated by exogenous thiols, without an added electron acceptor, suggesting the presence of an unidentified electron acceptor in the suspensions of starved bacteria. Since the nitrate respiration experiments demonstrated nitrate is utilized as an electron acceptor, it can be assumed that the symbionts use nitrate as an electron acceptor when it is available or, possibly, that nitrate is used in addition to the unidentified electron acceptor.

SGES, interacting with sulphide to produce polysulphides, can act as an "internal" electron acceptor and this has been suggested for *Solemya reidi* bacterial symbionts (R. Vetter, pers. comm.). Experimental results demonstrate this is unlikely for *Codakia orbiculata* bacterial symbionts, however. *C. orbiculata* symbionts are able to fix $^{14}CO_2$ anaerobically in incubations with no sulphide added. In addition, globular elemental sulphur reserves are known to decline rapidly

(Section 3.4.1.), suggesting the formation of polysulphides would be unlikely in the starved bacteria.

It has been suggested that the bacterial symbionts of *Lucinoma aequizonata* may use a sulphur-oxidation strategy similar to *Thiobacillus denitrificans*, a free-living bacteria (Cary *et al.*, 1989). *T. denitrificans* utilizes a reaction catalyzed by a siroheme-containing enzyme, with the required oxygen derived from water molecules, to oxidize elemental sulphur (Schedel and Trüper, 1980). This possibility has not been examined experimentally for the lucinid bacterial symbionts and may be feasible for the fed bacteria but, again, would not be feasible for the starved bacteria due to their lack of SGES. It also does not address the question of oxidation of exogenous sulphide and thiosulphate.

Intracellular Fe^{3+} could be a respiratory-linked electron acceptor for sulphide or elemental sulphur oxidation in the symbiotic bacteria of *Codakia orbiculata*. Both sulphide and elemental sulphur can be further oxidized by free-living bacteria using Fe^{3+} (Berner, 1970; Brock and Gustafson, 1976). Obviously, further experimentation is required to investigate this possibility.

Finally, the possibility that some energy is coming from a fermentative process cannot be completely excluded; addition of azide to the fed bacteria incubations did not completely reduce CO_2 fixation rates. However, this experiment was not replicated and, in addition, azide proved to be ineffective in eliminating respiration in whole bivalves (P. Barnes, pers.

obs.). Further research into the role of fermentative pathways in these symbionts seems justified.

Bacteria isolated from freshly collected bivalves, with yellow globular elemental sulphur deposits in the gills, had a high anaerobic $^{14}CO_2$ fixation rate in the absence of exogenous reduced sulphur. Bacteria isolated from the gills of starved *Codakia orbiculata* had endogenous $^{14}CO_2$ fixation rates x0.31 the endogenous rates for fed bacteria. This reduction in carbon dioxide fixation rate was presumed to be a result of the reduction in SGES observed in bivalves maintained in oxygenated, sulphide-free seawater for >2 days (Section 3.4.1.). A similar result and explanation were reported for isolated gills of *Lucinoma borealis* (Dando *et al.*, 1986a). Bouvy *et al.* (1989) hypothesized that $^{14}CO_2$ fixation rate in the gills of *Spisula subtruncata* (experiments conducted in the absence of external reduced sulphur) may have been related to the levels of reduced compounds in the habitat. Since an abundance of reduced sulphur in the environment leads to the accumulation of elemental sulphur in lucinid gills (Cary *et al.*, 1989; P. Barnes, pers. obs.), the results of Bouvy *et al.* (1989) support the use of elemental sulphur as an energy source for carbon dioxide fixation.

Further evidence of the link between oxidation of reduced sulphur and CO_2 fixation is provided by the stimulation of the $^{14}CO_2$ fixation rate observed in starved bacteria after the addition of sulphide or thiosulphate, to x4.14 and x3.34 the endogenous rate for starved bacteria, respectively. The resulting CO_2 fixation rates were comparable to those measured

for fed bacteria. Starved gills of *Lucinoma borealis* responded similarly to 100 μM sulphide (thiosulphate was not tested) (Dando *et al.*, 1986a). Since the addition of sulphide or thiosulphate to fed bacteria did not stimulate the $^{14}CO_2$ fixation rate, presumably SGES fuels carbon dioxide fixation at a rate comparable to that obtained using exogenous reduced sulphur. These data, in agreement with the results of the aerobic and anaerobic respiration experiments on isolated symbionts and the anaerobic respiration experiments on whole *Codakia orbiculata*, support the utilization of SGES as an energy source, for short periods, in the absence of all external sulphur sources. As discussed in Section 3.4.1., elemental sulphur utilization has been suggested for the bacterial symbionts of several species of lucinid bivalve (Vetter, 1985; Dando *et al.*, 1986a; Cary *et al.*, 1989) and has been recorded for free-living bacteria (Schedel and Trüper, 1980; Kelly, 1982; Javor *et al.*, 1990).

The endogenous aerobic CO_2 fixation rate of suspensions of bacterial symbionts isolated from starved bivalves was found to be approximately x0.5 the endogenous rate in the anoxic incubations. A similar result was obtained for bacterial symbionts isolated from *Solemya reidi* (B. Javor, pers. comm.). Because RuBPC/O exhibits both oxygenase and carboxylase activities, due to competition between oxygen and carbon dioxide for the same active site, carbon dioxide fixation will proceed most efficiently when the intracellular ratio of CO_2 and O_2 is high (Chollet, 1977). The data presented here support the suggestions of Fisher and Hand

(1984) and Dando *et al.* (1985) that a low oxygen partial pressure maintained in the vicinity of the bacteriocytes would be beneficial to chemoautotrophy by preventing the oxygenase activity of RuBPC/O from competing with carboxylase activity.

This is the first study to determine the stimulation of anaerobic carbon dioxide fixation in bacterial symbiont suspensions of a lucinid bivalve in response to either sulphide or thiosulphate. The bacterial symbiont suspensions of *Codakia orbiculata* were able to utilize both sulphide and thiosulphate as electron donors, with no obvious preference. Stimulation of $^{14}CO_2$ fixation rate has been observed for isolated bacteriocytes from *Lucina floridana* in response to thiosulphate (1 mM) (Hand, 1987) and for isolated gills of *Lucinoma borealis* and *Myrtea spinifera* (Dando *et al.*, 1985, 1986a) in response to sulphide (100 µM and 23-31 µM, respectively). These studies tested only one thiol, however. Among other families of bivalves possessing bacterial symbionts, both sulphide and thiosulphate were found to stimulate CO_2 fixation rates in whole gills of *Solemya velum* (Cavanaugh, 1983) and in the intact symbiosis and isolated symbionts of *Solemya reidi* (Anderson *et al.*, 1987; Wilmot and Vetter, 1992; B. Javor, pers. comm.). In contrast, *Bathymodiolus thermophilus* gill homogenates were found to use thiosulphate, but not sulphide, as the electron donor. Results of studies using isolated gills or the intact symbiosis must be interpreted with caution, however, due to the possible oxidation of sulphide to thiosulphate by host mitochondria (and, potentially, other oxidation mechanisms), prior to

reaching the symbionts (Powell and Somero, 1985; Anderson *et al.*, 1987; Cary *et al.*, 1989; Powell and Arp, 1989). Hence, thiosulphate, rather than sulphide, may be responsible for observed stimulation.

Sulphide inhibition of *Codakia orbiculata* bacterial suspensions did not occur at 200 µM sulphide, which is in agreement with such free-living sulphur-oxidizing bacteria as *Thiomicrospira acna*. *T. acna* fix $^{14}CO_2$ optimally with 100-300 µM sulphide and fixation rate is still high at 800 µM sulphide (Ruby and Jannasch, 1982; Jannasch *et al.*, 1985). The symbionts of *C. orbiculata* are unlikely to be exposed to sulphide concentrations *in vivo* comparable to 200 µM, however, due to host detoxification mechanisms (Cary *et al.*, 1989; Bagarinao, 1992) and cell permeability to dissolved sulphide as a function of pH (Wilmot and Vetter, 1992). *C. orbiculata* bacterial symbionts may have a higher tolerance for sulphide than the intact symbiosis. For example, sulphide concentrations >100 µM have been shown to have an inhibitory effect on aerobic metabolism in *Solemya reidi* and the bivalve switches to anaerobic pathways (Anderson *et al.*, 1990). In isolated bacterial symbionts of *S. reidi*, however, stimulation of aerobic and anaerobic CO_2 fixation and relatively high rates of aerobic and anaerobic respiration have been demonstrated using up to 500 µM sulphide (Wilmot and Vetter, 1992; B. Javor, pers. comm.).

The carbon dioxide fixation rates measured in suspensions of bacteria isolated from the gills of *Codakia orbiculata* cannot be compared directly with those reported in the

literature for other species of lucinids since the latter report the carbon dioxide fixation rates of the bacteria *in vivo* in the isolated gills or in the intact symbiosis. In addition, the condition of the animals and bacteria, or the experimental protocol (eg. oxygen concentration of incubation medium), can affect the $^{14}CO_2$ fixation rates. Comparison of rates measured for aposymbiotic and symbiotic bivalve gills, reported in the literature, suggests that technique may be an important factor and that comparisons of rates obtained in different studies must be treated with caution. For example, Felbeck (1983) measured $^{14}CO_2$ fixation rates in the isolated gills of the aposymbiotic *Mytilus edulis* and *Hinnites multirugosus* of 0.16 $\pm$ 0.05 and 0.28 $\pm$ 0.03 µmolC/gww/h, respectively. These fixation rates are comparable to those recorded for isolated gills of the symbiotic lucinids *Lucinoma borealis* and *Myrtea spinifera* (Dando *et al.*, 1985; Dando *et al.*, 1986a). In contrast, $^{14}CO_2$ fixation rates in the gills of the aposymbiotic *Corbula gibba* were an order of magnitude lower (Dando *et al.*, 1985).

In summary, the results of this study suggest that bacteria isolated from freshly collected *Codakia orbiculata* are capable of anaerobic CO_2 fixation utilizing energy released from oxidation of (loss of electrons from) SGES and utilizing an electron acceptor which has yet to be identified. The isolated bacterial symbionts are able to use oxidation of both sulphide and thiosulphate to fuel anaerobic $^{14}CO_2$ fixation.

3.4.5. Nitrate and Nitrite Respiration in Whole *Codakia orbiculata*

This study demonstrated that the bacterial symbionts of *Codakia orbiculata* were capable of nitrate and nitrite respiration *in vivo*, when the bivalves were maintained in oxic or anoxic conditions. Nitrite respiration occurred only in bivalves incubated, in oxic or anoxic conditions, with sulphide. Experiments using anoxic suspensions of isolated *C. orbiculata* bacterial symbionts, discussed earlier, also demonstrated that the symbionts were capable of nitrite respiration only in the presence of sulphide. This has also been reported for the intact *Solemya reidi* symbiosis in the presence of sulphide (Wilmot and Vetter, 1992). The results of the whole bivalve nitrite respiration experiments demonstrate, therefore, that the symbionts must have access to sulphide *in vivo* and that they oxidize sulphide. Gaseous H_2S may diffuse across membranes and may be transported across cells through binding to specific hemoglobin (Kraus and Wittenberg, 1990; D. Kraus, pers. comm.).

There proved to be no statistically significant difference between the nitrite respiration rates in the oxic and anoxic incubations in the presence of sulphide. These results suggest that the *in vivo* symbionts' exposure to low levels of oxygen did not result in immediate or significant repression of nitrite reductase. This has also been observed for a few free-living bacteria (Robertson and Kuenen, 1984; Robertson *et al.*, 1988). A decrease in nitrite respiration rates might be expected in oxic conditions since thiosulphate

may be produced as a result of host oxidation of sulphide (Cary et al., 1989; Wilmot and Vetter, 1992) and thiosulphate does not stimulate nitrite respiration. The similarity of nitrite respiration rates measured in the whole animal under anoxic and oxic conditions in this study suggests further research into the role of thiosulphate and sulphide in the symbionts physiology *in vivo* is required. However, the demonstration that the symbionts have access to sulphide when the host is in oxic conditions reveals that host oxidation of sulphide (to thiosulphate) is not 100% efficient. This was hypothesized earlier on the basis of sulphide-binding hemoglobin in lucinid gills (Kraus and Wittenberg, 1990).

In *Codakia orbiculata* habitat, the low nitrite levels in sediment interstitial water (<0.3 µM) and overlying seawater (<0.01 µM) (McGlathery, 1992) suggest the symbionts obtain nitrite from bacterial nitrate respiration *in vivo*, rather than directly from the environment. In nitrite incubations using whole bivalves, nitrite must enter the bacteriocyte either from the blood or directly from seawater.

The bacterial symbionts of *Codakia orbiculata* were found to respire nitrate *in vivo*, in the absence of an external sulphur source. This finding suggests that the bacteria *in vivo*, similar to suspensions of isolated bacteria, are able to use SGES as a substrate for nitrate respiration. In the intact symbiosis, nitrate must enter the bacteriocyte either from the blood or directly from seawater.

Thiosulphate stimulated nitrate respiration in the intact symbiosis in anoxic conditions. Since thiosulphate does not

have a gas phase and, therefore, does not diffuse across cells as freely as sulphide (H_2S) (Holmes and Donaldson, 1969; Wilmot and Vetter, 1992), these results suggest the presence of an active transport mechanism (Vetter, pers. comm.). It should also be noted that *Codakia orbiculata* generally has access to thiosulphate in concentrations <10 µM *in situ* (Chapter 2). Hence, thiosulphate utilized by bacterial symbionts *in vivo* is believed to arise predominately from host oxidation of sulphide (Powell and Somero, 1985; Anderson *et al.*, 1987; Cary *et al.*, 1989; O'Brien and Vetter, 1990; Bagarinao, 1992).

Whole *Codakia orbiculata* were incubated anoxically with nitrate and a range of sulphide concentrations. Because host sulphide oxidation (host mitochondria or hematin-granule oxidation) requires oxygen, these mechanisms could not function under anoxic conditions (Cary *et al.*, 1989; Powell and Arp, 1989; O'Brien and Vetter, 1990; Wilmot and Vetter, 1992). Hence, sulphide was not oxidized to thiosulphate and was the only exogenous sulphur source available. Because there was no statistically significant difference between the nitrate respiration (net nitrite accumulation) rates measured in the 0 µM, 50 µM and 250 µM sulphide anoxic incubations, there was no direct proof that sulphide was used as a substrate in nitrate respiration in the intact symbiosis. Sulphide levels in the surrounding medium decreased slightly, suggesting either some sulphide utilization or binding of sulphide to SGES to form polysulphides.

The low rate of nitrite accumulation in the incubation medium in the presence of sulphide, as suggested for suspensions of isolated bacterial symbionts, may be due to denitrification without accumulation of the nitrite intermediate. Both suspensions of isolated bacteria and the symbionts *in vivo*, demonstrated nitrite respiration in the presence of sulphide. As discussed earlier, concentrations of nitrate in seawater (1-40 µM) have been found to have little or no inhibitory effect on nitric oxide reduction (Packard *et al.*, 1983) and it is, therefore, unlikely that the 100 µM concentration of nitrate used in these experiments inhibited nitrite reduction. Further experiments which measure the products of nitrite reduction, as suggested for the isolated bacteria experiments, are required to confirm the hypothesis that denitrification occurs in whole *Codakia orbiculata* in the presence of sulphide.

The lack of statistical difference in net nitrite accumulation rates between the 0 µM, 50 µM and 250 µM sulphide incubations suggests sulphide concentrations up to 250 µM do not inhibit nitrate or nitrite respiration. Sulphide inhibition of host metabolism seems unlikely at these concentrations (<250 µM), which are comparable to those found in the bivalves' habitat (Chapter 2). The rate of net nitrite accumulation in the 1000 µM sulphide incubation was near zero, however, and statistically lower than those obtained using incubations with other sulphide concentrations. This suggests sulphide inhibition of the host bivalve metabolism. In their habitat, the bivalves are exposed to sulphide concentrations

lower than 1000 µM (Chapter 2). Although some sulphide may be bound to intracellular hemoglobin in the gills (Kraus and Wittenberg, 1990), host defences may be overloaded in the absence of additional detoxification mechanisms resulting from the lack of exogenous oxygen. Cilial activity in isolated gills of the lucinid *Lucinoma borealis* demonstrated decreased activity when exposed to 300 µM sulphide and complete inhibition at 1000 µM sulphide (Dando *et al.*, 1986a). *Solemya reidi*, found in high-sulphide sediments (often >1 mM) (Childress and Lowell, 1982), has been shown to switch to sulphide-stimulated anaerobiosis at sulphide concentrations >100 µM (Anderson *et al.*, 1990).

When whole *Codakia orbiculata* were maintained under anoxic conditions, the bacteria would have been exposed to anoxic conditions *in vivo*. When whole *C. orbiculata* were maintained under oxic conditions, the symbionts would probably have had access to only low levels of oxygen *in vivo*, as discussed earlier, as a result of host oxygen demand, the lucinid gill structure and the presence of oxygen-binding hemoglobin (Fisher and Hand, 1984; Distel and Felbeck, 1987; Kraus and Wittenberg, 1990). Some oxygen would be available to the bacteria, however, for aerobic respiration. The results of this study reveal that the bacterial symbionts were capable of nitrate respiration *in vivo* when the whole bivalves were maintained under oxic conditions. The presence of oxygen, as discussed in Section 3.4.3., does not completely inhibit nitrate respiration in all bacteria. Isolated bacterial symbionts of *Lucinoma aequizonata*, and whole *L. aequizonata*,

demonstrated only slightly lower nitrate respiration rates to nitrite when maintained under oxic conditions (Hentschel *et al.*, 1993).

The nitrate respiration rates for whole *Codakia orbiculata* maintained under oxic conditions in the absence of exogenous thiol, or in the presence of thiosulphate, were statistically lower than rates measured in comparable, but anoxic, conditions. Nitrate respiration may be partially inhibited by the presence of oxygen, therefore. Isolated symbionts from *C. orbiculata* maintained for 10 days in oxygenated and nitrate-free seawater also demonstrated reduced nitrate respiration rates. Access to both oxygen and nitrate suggests that *C. orbiculata* bacterial symbionts may respire both oxygen and nitrate when maintained under these conditions. Co-respiration of oxygen and nitrate has been recorded for free-living bacteria (Bonin and Gilewicz, 1991) and for *Solemya reidi* isolated symbionts (B. Javor, pers. comm.). Aerobic and anaerobic respiration may be utilized by the symbionts alternately, dependent on availability of electron acceptors.

In contrast to the above results, the rates of nitrate respiration to nitrite under oxic conditions with sulphide were statistically higher than those measured under anoxic conditions with sulphide. This is surprising, initially, in light of the potential for co-occurring aerobic and anaerobic respiration under oxic conditions and the possibility of slight inhibition of nitrate reductase by oxygen, which, as suggested for the other oxic incubations, may result in a

reduced nitrate respiration rate. In addition, nitrite respiration occurred in intact symbioses in both anoxic and oxic incubations with sulphide and it would be expected, therefore, that the nitrate respiration rate (as measured by the net nitrite accumulation rate) would be lowered by nitrite reduction.

A possible explanation for the observed high respiration rates, however, is that the presence of oxygen in the incubation medium allowed host oxidation of sulphide to thiosulphate (Wilmot and Vetter, 1992) and the resulting supply of thiosulphate, transported to the symbionts, stimulated nitrate respiration. As discussed in Chapter 1, host cell mitochondrial oxidation is believed operative in the lucinids (Cary *et al.*, 1989). In addition, thiosulphate supplied to the symbionts would not stimulate nitrite respiration. However, the nitrite respiration rates in whole *C. orbiculata* in oxic conditions with sulphide revealed that some sulphide reached the symbionts, suggesting that some denitrification, and further reduction of nitrite produced by thiosulphate-stimulated nitrate respiration, may occur. The net accumulation of nitrite suggests that these processes are less significant than nitrate respiration stimulated by thiosulphate. Access to exogenous sulphide, rather than thiosulphate, may be preferable for the intact symbiosis when oxygen is available. Gaseous H_2S, unlike the charged thiosulphate molecule (Holmes and Donaldson, 1969), freely diffuses across host cells and results in an increased internal supply of reduced sulphur. If oxygen is available,

presumably the toxic effects of sulphide would be mediated by host oxidation, in addition to hemoglobin-binding and bacterial utilization.

Sulphide levels in the incubation media, after adjustment for chemical oxidation, decreased rapidly in the oxic incubations. This may be attributed to a combination of direct utilization of sulphide, host oxidation of sulphide to thiosulphate and possibly, non-enzymatic oxidation (Powell and Arp, 1989) and some binding to SGES (Vetter, pers. comm.). The relative importance of direct uptake of sulphide across the gill and thiosulphate formation by host oxidation, to the sulphur metabolism of the intact symbiosis has yet to be determined.

The results of these investigations into symbiont respiration and sulphur utilization and the hypotheses put forward are summarized in Chapter 4.

CHAPTER 4

AEROBIC AND ANAEROBIC RESPIRATION, AND SULPHUR SUBSTRATE UTILIZATION, IN BACTERIAL SYMBIONTS OF THE LUCINID BIVALVE, *CODAKIA ORBICULATA*: IMPLICATIONS FOR THE INTACT SYMBIOSIS

4.1 SUMMARY

The isolated bacterial symbionts of *Codakia orbiculata* have the ability to respire nitrate and nitrite, in addition to oxygen. Nitrate respiration in *Lucinoma aequizonata* and *Solemya reidi* symbionts has been suggested to be advantageous to the symbioses as a result of reduced competition, between symbiont and host, for oxygen (Wilmot and Vetter, 1992; Hentschel *et al.*, 1993). Certainly, *L. aequizonata* is found in low oxygen environments where oxygen may become limiting (Cary *et al.*, 1989). The overlying waters of the habitat of *C. orbiculata*, which the clam can access with its' inhalant tube, are saturated with oxygen and it seems unlikely that oxygen availability would be limited in this environment. Nitrate and nitrite respiration were found to occur in whole *C. orbiculata* maintained in oxic (>80% oxygen), in addition to anoxic, conditions. This suggests that the advantages of nitrate respiration to the symbiosis may not be strictly limited to a reduction in competition for oxygen with the host clam but, instead, may occur even when oxygen is readily available in the habitat.

It seems probable that, regardless of external oxygen concentrations, the endosymbionts are maintained in a low oxygen environment. The structure of the lucinid gill (Fisher and Hand, 1984; Distel and Felbeck, 1987) suggests that the oxygen level in the water which reaches the bacteriocytes will have been greatly reduced. The presence of oxygen-binding intracellular hemoglobin in the gill may keep the partial pressure of oxygen low in the vicinity of the bacteriocytes

and this has been suggested to be advantageous for bacterial chemoautotrophic processes (Dando *et al.*, 1985; Kraus and Wittenberg, 1990). Reduced competition between oxygen and carbon dioxide for the same active site on RuBPC/O, which exhibits both oxygenase and carboxylase activities (Chollet, 1977) would benefit bacterial carbon dioxide fixation. Hence, oxygen available for the symbionts may be limited and the ability to utilize an alternate electron acceptor may be required. The standard free energy gained from oxygen as the terminal electron acceptor is greater than from nitrate (Stouthamer, 1988), suggesting the potential advantages to the chemoautotrophic symbiosis, of maintaining the bacteria in a low oxygen environment *in vivo*, must be substantial.

In the isolated symbiont respiration experiments, aerobic respiration rates were higher than nitrate respiration rates. In both aerobic and anaerobic respiration experiments, bacterial suspensions were supplied with an excess of electron acceptor. Nitrate levels in interstitial water in the habitat of *Codakia orbiculata* were as high as 36 μM but mean concentrations ranged from 0.46-5.45 μM, while oxygen available in the overlying water was >100%. This suggests that nitrate was less available to the symbioses *in situ* than oxygen. It is difficult to hypothesize regarding the significance of symbiont aerobic respiration *in vivo*, however, in light of the potentially low oxygen environment of the symbionts *in vivo*. To determine the relative importance of aerobic and anaerobic respiration to the symbionts *in vivo*, it

is critical to define the availability of specific electron acceptors *in vivo*.

It has been demonstrated that nitrate respiration in the isolated bacterial symbionts of *Solemya reidi* and *Lucinoma aequizonata* is only slightly inhibited by the presence of oxygen (Wilmot and Vetter, 1992; Hentschel *et al.*, 1993; B. Javor, pers. comm.). The ability to respire nitrate under oxic conditions is uncommon and, in addition to these bivalve symbionts, has been reported in only a few free-living bacteria (Robertson and Kuenen, 1984, Robertson *et al.*, 1988). Although *Codakia orbiculata* isolated symbionts were not tested for nitrate respiration under oxic conditions, bacteria isolated from *C. orbiculata* maintained for 10 days in oxygenated, nitrate-free seawater were able to respire nitrate in the presence of thiosulphate. Nitrate respiration measured in the intact symbiosis in oxic conditions suggests that nitrate respiration in the symbionts is not completely repressed in the presence of low levels of oxygen.

It is possible that the *Codakia orbiculata* symbionts utilize a combination of aerobic and anaerobic respiration to maximize energy production. Co-respiration of nitrate and oxygen has been demonstrated for *Solemya reidi* symbionts and some free-living bacteria (Bonin and Bilewicz, 1991; Wilmot and Vetter, 1992; B. Javor, pers.comm.). Alternation of aerobic and anaerobic respiration is also a possibility, dependent on internal availability of electron acceptors. Although an ability to maintain both respiratory chains is suggested for the *C. orbiculata* symbionts, their ability to

co-respire, either isolated or *in vivo*, requires further experimentation.

Investigations into sulphur substrate utilization in the isolated bacterial symbionts of *Codakia orbiculata* revealed that stored globular elemental sulphur can be used as an energy source for carbon dioxide fixation and as a sulphur substrate for aerobic and nitrate respiration. Similarly, nitrate respiration was measured in freshly collected whole bivalves in the absence of exogenous sulphur. Stored globular elemental sulphur disappeared from the gills within 72 hours in the absence of exogenous reduced sulphur. Despite the high sulphate reduction rates and accumulation of dissolved sulphide in the *Thalassia testudinum* sediment habitat of *C. orbiculata*, habitat data suggests spatial heterogeneity within the environment. The variation in SGES levels in the gills of freshly-collected *C. orbiculata* also suggested that some bivalves may lack access, temporarily, to exogenous reduced sulphur.

Thiosulphate stimulated anaerobic carbon dioxide fixation, aerobic respiration and nitrate respiration in isolated symbionts, and nitrate respiration in the intact symbiosis. Thiosulphate in the interstitial water of *Codakia orbiculata* habitat was present in low levels only (0.66-32.27 μM) and it seems unlikely, therefore, that thiosulphate utilized by the symbiosis is obtained externally. As discussed at length in Chapter 1, host mitochondrial oxidation of sulphide to thiosulphate is well-documented for *Solemya reidi* (Powell and Somero, 1986; Anderson et al., 1987; O'Brien

and Vetter, 1990; Wilmot and Vetter, 1992) and has been suggested for lucinid bivalves (Cary et al., 1989). In light of thiosulphate stimulation of *C. orbiculata* symbiont respiration and anaerobic carbon dioxide fixation, and the low levels of this thiol in the habitat, host oxidation of sulphide would seem to be a suitable hypothesis for the supply of thiosulphate for the *C. orbiculata* symbiosis.

Dissolved sulphide, present in abundance in the *Codakia orbiculata* habitat, also stimulated anaerobic carbon dioxide fixation and aerobic respiration in isolated bacterial symbionts but there was no direct proof that sulphide was used as a sulphur substrate in nitrate respiration in any of the experiments. However, only sulphide-stimulated nitrite respiration. Because nitrate respiration rates were determined by measuring nitrite accumulation in the incubation medium, it is suggested that sulphide stimulated denitrification, a 5-electron transfer from nitrate to N_2 which would yield more energy for the bacteria, was occurring. Complete denitrification would result in no net accumulation of nitrite. Further research, in which the end-products of denitrification are measured, is required to test this hypothesis.

The results of the nitrite respiration experiments in the isolated bacteria and the whole bivalve demonstrate that the symbionts have access to sulphide *in vivo*. Although the toxic effects of sulphide suggest that host oxidation of sulphide, to non-toxic thiosulphate, would be advantageous when oxygen is available, the ability of the intact symbiosis to respire

nitrite when incubated in oxic conditions suggests that host oxidation mechanisms may not be 100% efficient. Some sulphide undoubtedly reaches the symbionts *in vivo*, possibly by diffusion or by hemoglobin-binding.

In contrast to the low or undetectable nitrate respiration rates for *Codakia orbiculata* isolated bacteria, or the whole animal, when incubated anoxically with sulphide, the nitrate respiration rates of the intact symbiosis incubated in oxic conditions with sulphide were high. It is suggested that sulphide is oxidized to thiosulphate, probably by host mitochondria, prior to reaching the symbionts. The net accumulation of nitrite in the incubation medium is a result, therefore, of thiosulphate-stimulated nitrate respiration. Thiosulphate did not stimulate nitrite respiration. Because some sulphide will reach the symbionts, it is probable that denitrification, and respiration of nitrite produced by thiosulphate-stimulated nitrate respiration, also will occur. The measurable net nitrite accumulation suggests, however, that thiosulphate-stimulated nitrate respiration is most significant.

The ability to utilize sulphide and alternate electron acceptors may have aided these bacteria-bivalve symbioses in colonizing habitats unavailable to other organisms. Lucinid bivalves have often been noted as the dominant infaunal species (Allen, 1958; Jackson, 1972; Monnat, 1970; Chapter 2) in their habitat. The sediment chemistry of the *Thalassia testudinum* sediment habitats in Bermuda support the hypothesis that a supply of oxygen, nitrate and sulphide may be optimal for the symbiosis. Oxygen is available from the sediment-

surface water via the clams' inhalant tubes, yet sediments are sulphide-rich with a high sulphate reduction rate. Gaseous H_2S in the interstitial water, unlike thiosulphate, can diffuse freely into host cells, resulting in a ready supply of internal reduced sulphur (Holmes and Donaldson, 1969; Wilmot and Vetter, 1992). Available oxygen would allow host oxidation of sulphide to non-toxic thiosulphate for transport to the bacterial symbionts. The bacterial symbionts, utilizing oxygen or nitrate as an electron acceptor, can further oxidize thiosulphate. Nitrate is available in interstitial waters in concentrations x5 greater than those in the sediment-surface water.

In conclusion, this study has demonstrated aerobic respiration, utilizing sulphide, thiosulphate or stored globular elemental sulphur, in the isolated symbionts of *Codakia orbiculata*. Nitrate respiration, in the presence of thiosulphate or SGES, and nitrite respiration, in the presence of sulphide, have also been demonstrated for the isolated and *in vivo* symbionts. Hypotheses regarding sulphide stimulation of denitrification, host oxidation of sulphide to thiosulphate and co-respiration of nitrate and oxygen by the symbionts have been put forward. However, the conditions to which the symbionts are exposed *in vivo* remain unknown, leaving the roles of aerobic and anaerobic respiration in the *in vivo* physiology of the symbionts open to controversy and in need of further research. In addition, the relative contributions of sulphide and thiosulphate to the *in vivo* sulphur metabolism of the bacterial symbionts remain to be quantified.

APPENDIX 1

COMPARISON OF METHODS FOR THE EXTRACTION OF INTERSTITIAL WATER

INTRODUCTION

Interstitial water from Bailey's Bay was collected in August 1990 using three methods: lysimeter, syringe and centrifugal filtration. Because dissolved sulphide reacts spontaneously with oxygen (Chen and Morris, 1972), methods for the collection of interstitial water for sulphide analyses must prevent oxygen contamination of the sample. The following comparison of methods determined which method resulted in the least oxygen contamination and in the most representative dissolved sulphide concentrations. The sample volumes obtained by each method are also compared

Because of the nature of the sampling methods, interstitial water could not be collected from exactly the same location within the bed using each of the 3 methods. As a result, spatial heterogeneity of dissolved sulphide levels within the seagrass sediments is inherent in the data. Numerous replicates were taken using each technique, however, and the data was then reduced to the mean ($\pm$ 1 s.d.) for each technique and each sediment depth. The means were compared and sampling techniques which resulted in consistently lower dissolved sulphide levels, suggestive of contamination with oxygen during sampling, were considered inferior.

METHODS AND MATERIALS

Lysimeters

The lysimeters are TFE teflon tubes which were inserted into the sediment and which collected interstitial water through polycarbonate frits in the sides of the tubes. At the top of the lysimeter is a stop-cock to which a 60-ml syringe was attached. The syringe was used to apply a vacuum to pull water across the frits into a collecting chamber, up a Tygon tube and into the syringe. Individual lysimeters collected interstitial water from either 5, 10, 20, or 30 cm sediment depth, determined by the distance between the frit and the top of the lysimeter. The lysimeters were filled with deoxygenated, FSSW immediately prior to installation. This water was extracted using the syringe and discarded prior to sample collection. Lysimeters were inserted 24 hours prior to sample extraction to allow equilibration. The average volume of sample collected was 50-60 ml. A detailed description of sample collection using lysimeters is given in Methods and Materials in Chapter 2 and is not repeated here.

Three sets of lysimeters were inserted randomly into the sediments, with one member of each set collecting interstitial water from either 5, 10, 20, or 30 cm sediment depth. Hence, 3 replicate samples were collected from each sediment depth. Syringes were placed in an Argon-filled, air-tight container immediately after sampling and transferred to an Argon-filled glove bag on return to the laboratory. The sample was filtered through 0.22-μm Nuclepore filter and 5 replicate 1-ml samples were taken from each sample.

All interstitial water samples were preserved using 20 µl of 2 M zinc acetate and were analyzed within 5 days for dissolved sulphide using the colourimetric assay of Cline (1969) (refer to Materials and Methods in Chapter 2 for detailed methodology).

Syringes

Three cores were taken from the seagrass bed sediment using Plexiglass core tubes (7.5 cm inside diameter). The core tubes had holes drilled every 5 cm down the length of the tube for syringe insertion and extraction of interstitial water. Holes were covered with duct tape during sampling. Core tubes were capped immediately after sampling, caps were taped to prevent water loss, and tubes are kept vertical for return to the laboratory. For a detailed description of the method of core collection, see Methods and Materials in Chapter 2.

The cores were processed immediately upon return to the laboratory. Sediment cores were kept vertical, using ring stands, for processing in an Argon-filled glove bag. The syringe needle was pushed through the duct tape and interstitial water was syringed from the sediments at 5 cm sediment depth. Due to blockages in the needle (a variety of gauge needles were tried with similar results), this procedure had to be repeated several times to obtain 5 ml of intersitial water. As per the lysimeter samples, all syringe samples were filtered through a 0.22-µm Nuclepore filter into acid-washed scintillation vials. The filtered sample was then subdivided

into 5 replicate 1-ml samples which were preserved using 20 µl of 2 M zinc acetate. Analysis of dissolved sulphide followed the procedure of Cline (1969), as presented in Chapter 2.

Centrifugation

Three cores were removed from the lucinid sediment habitat using plexiglass core tubes (7.5 cm diameter). These cores were capped immediately upon retrieval, caps were taped to ensure no leakage and were kept vertical for return to the laboratory. Once again, refer to the methods section in Chapter 2 for detailed methodology.

Cores were placed in the jack apparatus below the Argon-filled glove bag and sediment samples were extruded into the glove bag following the procedure outlined in Materials and Methods in Chapter 2. Cores were sectioned as follows: 0-5 cm, 5-10 cm and 10-15 cm. Interstitial water was extracted using modified 50-ml plastic centrifuge tubes with caps. The tubes had been cut into two sections which could be re-connected by a custom-designed, O-ring sealed, PVC connector. A 0.22-µm Nuclepore filter was placed on top of the connector (in the top section of the tube) and held in place with another O-ring pushed down the tube. Sediment (approximately 35 ml) was packed into the top of the centrifuge tube and the tube was capped and centrifuged at 5000 rpm for 10 min using an IEC Clinical centrifuge (in the glove bag). This method yielded 5-9 ml of filtered, interstitial water per tube, depending on sediment water content. The interstitial water in the bottom section of the tube was re-filtered through a 0.22 µm filter

into an acid-washed scintillation vial prior to subdivision into 5 replicate 1-ml samples. Samples were preserved with 20 µl of 2 M zinc acetate and analyzed using the colourimetric assay of Cline (1969), as reported in Chapter 2.

RESULTS

Dissolved sulphide concentrations in interstitial water collected using lysimeters, syringes and by centrifugation are presented in Table I. The large standard deviations around the means are indicative of the spatial heterogeneity at a given depth. The number of replicates (n) refers to the number of individual lysimeter or core samples analyzed; each replicate represents a different location within the *Thalassia testudinum* sediments. The maximum depth at which sulphide was measured with the syringe and centrifugation techniques was 10 cm, since the cores did not penetrate sufficiently to take a 20 cm sample for comparison with the lysimeter samples. While heterogeneity of the sulphide concentrations in the sediments must be taken into account, the sulphide levels in the centrifuged samples were consistently at least one order of magnitude lower than those in lysimeter and syringe samples. Dissolved sulphide in the lysimeter and syringe samples were comparable.

Table I. Dissolved sulphide (μM) in interstitial water collected using lysimeters, syringe and centrifugal extraction. Data are presented as means ± 1 s.d..

Method	Sediment Depth (cm)	n	Sulphide (μM)
Lysimeter	5	3	219.85 ± 110.48
	10	3	236.64 ± 143.54
	20	3	88.73 ± 31.03
	30	3	14.21 ± 6.96
Syringe	5	3	181.11 ± 169.99
	10	3	137.73 ± 46.01
Centrifugal Extraction	5	3	4.52 ± 0.32
	10	3	13.13 ± 8.40

DISCUSSION

Results indicate that the centrifugation method of extraction of interstitial water resulted in the lowest concentrations of dissolved sulphide. This suggests that, despite using the Argon-filled glove bag, oxidation of sulphide was occurring. It is possible that fragments of seagrass roots or rhizomes released compounds which oxidized the sulphide. Although the variances around the means were large, indicative of the heterogeneity of dissolved sulphide in the interstitial waters, the syringe extraction and lysimeter methods yielded comparable levels of dissolved sulphide.

Although dissolved sulphide concentrations of samples collected using the lysimeter and the syringe techniques were comparable, the practical advantages of the lysimeter technique were numerous. Most importantly, 50-60 ml of interstitial water could be collected from each lysimeter. This was sufficient volume for replicate samples for analysis

of dissolved sulphide, ammonia, nitrate, nitrite and dissolved iron. Syringe extraction yielded small volumes (<6 ml) of interstitial water. Lysimeters were easy to install and interstitial water could be collected to 30 cm sediment depth. In contrast, the core tubes rarely penetrated beyond 10 cm sediment depth. Collection of the interstitial water samples was relatively rapid and simple using the lysimeters, in comparison with extraction of interstitial water from sediment cores. Extracting water from the sediment using a syringe was problematical due to the needle blocking with sediment. The advantage of the syringe method was that a sample could be collected from a specific location in the sediment core. However, these samples were always <1 ml and the syringe needle had to moved to several locations to obtain adequate interstitial water volume to run replicate samples.

In summary, the results of this comparative study suggest lysimeters are suitable for the collection of interstitial water for analysis of dissolved sulphide and are a preferable method of sampling to syringe and centrifugal extraction.

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