ECOLOGY AND PHYSIOLOGY OF BACTERIAL ACTIVITY IN A TEMPERATE SALTMARSH LAGOON, WITH AN EMPHASIS ON NITROGEN FIXATION

by

Brian Jonathan Tibbles

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Supervisors: Dr. Michael I. Lucas
Prof. Douglas E. Rawlings
Prof. George M. Branch

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DECLARATION

This thesis reports the results of original research which I have carried out in the Marine Biology Research Institute, University of Cape Town, and has not been submitted for degree at any other university. Any technical and other assistance which I have received is fully acknowledged.

Signed by candidate

Brian Jonathan Tibbles

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ABSTRACT

Heterotrophic bacterial activity and nitrogen fixation are fundamental to nutrient regeneration and nitrogen cycling in saltmarsh ecosystems. Ecological and physiological aspects of bacterial production and nitrogenase activity in marine sediments and water were examined in Langebaan Lagoon, a temperate saltmarsh ecosystem. Emphasis was placed on factors modulating rates and patterns of nitrogen fixation.

Nitrogen fixation appeared to be dominated by heterotrophic bacteria. Rates of nitrogen fixation (estimated by the acetylene reduction technique), and bacterial production (estimated by tritiated thymidine incorporation, TTI) were higher in fine, muddy sediments near the head of the lagoon (Geelbek) than in coarser, sandy sediments near the mouth of the lagoon (Oesterwal). These comparisons (between sites) reflected the higher bacterial abundance and organic content of sediments from Geelbek. Examinations of five sedimentary microhabitats at each site (including those associated with beds of the seagrass *Zostera capensis*, burrows of the sandprawn *Callianassa kraussi* at Oesterwal, and burrows of the mudprawn *Upogebia africana* at Geelbek) showed that bacterial activity was higher in surface sediments than in subsurface sediments. Highest rates of nitrogen fixation (annual mean, 0.28 $\pm$ 0.07 nmol C_2H_4 g^{-1} dry sediment h^{-1}) were measured in *Zostera* bed sediments at Geelbek. Thymidine incorporation activity and nitrogenase activity were higher in burrow linings than in adjacent subsurface sediments, suggesting that burrow linings provided an improved subsurface environment for bacterial activity. Burrow linings also had a higher organic content than subsurface sediments away from burrows. Nitrogenase activity was not detected in lagoon water.

Bacterial production and nitrogen fixation in sediments varied seasonally. Rates were generally low in winter and gradually increased during spring to reach peak rates during summer/autumn. Seasonality of bacterial production reflected a similar seasonal pattern in the organic contents of sediments available to bacteria (largely seagrass and saltmarsh detrital material). However, rates of nitrogen fixation, measured during seasons different to the TTI studies, could not be correlated with the level of organic contents. Seasonality of nitrogenase activity did reflect numbers of diazotrophic bacteria (estimated by the most probable number technique), which were higher in

summer than in winter. Furthermore, nitrogen-fixing bacteria formed a significantly greater proportion of general heterotrophic isolates in summer than in winter.

Additions of glucose (ca. 15mM) stimulated nitrogenase activity in all sediments by 1 to 3 orders of magnitude. The degree of glucose-stimulation was significantly negatively correlated with the rate of nitrogen fixation in sediments that were not amended with glucose, indicating that nitrogenase activity in Langebaan sediments was limited by the availability of easily utilizable carbon sources. Additions of complex plant polysaccharides on the other hand stimulated nitrogenase activity differently according to the substrate type. Storage polysaccharides (glycogen and laminarin) were more effective at stimulating nitrogenase activity than structural polysaccharides (cellulose, carrageenan, xylan, and alginate). These findings indicate that nitrogen fixation may contribute to microbially-mediated nitrogen-enrichment of detrital materials, which would benefit the nutritional requirements of detritivores in these environments. Ammonium did not appear to have a significant role in the regulation of nitrogenase activity in sediments, as *in situ* concentrations (2.5 ± 2.3 μM) were lower than those required to inhibit activity, and methionine-DL-sulfoximine did not stimulate rates. Nitrate may have some influence on nitrogen fixation (one nitrogen-fixing isolate could reduce nitrate to nitrite), as nitrate availability varies seasonally in a manner correlated with upwelling along this coast. Modulation of nitrogenase activity by physical factors indicated that temperature and oxygen, but not light, have a significant effect on rates. Oxygen stimulated nitrogenase activity in surface sediments, but not in subsurface sediments, suggesting that aerobic or microaerophilic respiration is an important mechanism generating energy for nitrogenase activity in surface sediments.

In the absence of molybdenum, two isolates of nitrogen-fixing bacteria (a *Photobacterium* spp. and a *Vibrio* spp.) from Langebaan Lagoon reduced acetylene to ethylene and ethane, a property usually considered to be characteristic of the activity of alternative nitrogenases. Southern hybridization of the chromosomes of these strains with *anfH*, *vnfH*, and *nifH* genes as probes, indicated the presence of only one nitrogenase in these isolates. This is particularly interesting because all bacteria found to possess alternative nitrogenases to date, also possess the conventional molybdenum nitrogenase. Assays of nitrogenase activity in sediments did not indicate that molybdenum was limiting nitrogenase activity at *in situ* concentrations.

RATIONALE OF THESIS

My ecological and physiological studies in Langebaan Lagoon, a temperate saltmarsh ecosystem on the southwest coast of southern Africa, are aimed at elucidating the pathways of carbon and nitrogen fixation and their flows into microbial and higher trophic levels (see Mazure and Branch, 1979; Fielding *et al.*, 1988; Harris, 1993). The aim of this thesis is to examine bacterial activity and nitrogen fixation in the sediment and water of Langebaan Lagoon. More specifically, this thesis aims to elucidate the mechanisms controlling rates of bacterial production and nitrogen fixation at two differing, but representative, sites in the lagoon. Questions that are posed include: do macrofaunal-microbial or macrophytic-microbial interactions influence bacterial production or nitrogen fixation in Langebaan Lagoon; what are the *in situ* rates of bacterial production and nitrogen fixation, and how do these compare with those of other systems; what temporal and spatial variability occurs in nitrogenase activity; what ecological factors dominate the regulation of nitrogen fixation; and, do any diazotrophic bacteria isolated from Langebaan Lagoon have unique characteristics with respect to nitrogen fixation?

This thesis is presented in two sections. In the first section, Chapter 1 reviews the ecology and physiology of nitrogen fixation in marine environments, and thus provides a general background to the thesis. Chapter 2 presents a characterization of Langebaan Lagoon in terms of physico-chemical and biological properties which are relevant to microbial activity in general, and nitrogen fixation in particular. Thus, Chapter 2 serves as an ecological backdrop against which the other chapters are considered. Chapter 3 examines bacterial activity in Langebaan Lagoon, and furthers the basis for studies of nitrogen fixation in the second section. The second section examines the physiology and ecology of nitrogen fixation in Langebaan Lagoon. Spatial and temporal variations of nitrogen fixation are examined in Chapter 4, while the relative importance of different ecological factors in the control of nitrogenase activity is addressed in Chapter 5. Diazotrophic isolates obtained during the course of these studies were physiologically characterized, and the relevance of their characteristics to nitrogen fixation in general, and to Langebaan Lagoon in particular, is discussed in Chapter 6. In Chapter 7, a

synthesis of physiological and ecological studies from both sections is presented, incorporating possible future directions for research that the present work has introduced.

The chapters are presented in the form of separate manuscripts, linked by the common concern of elucidating mechanisms controlling rates of nitrogen fixation in Langebaan Lagoon. Chapter 3 has been published (Tibbles *et al.*, 1992), Chapter 5 is submitted for publication, and Chapter 6 is in press (Tibbles and Rawlings, 1994). Although I am co-authored on these papers, I am the senior author and was responsible for the work; my co-authors played a supervisory role. Because I was encouraged to write the thesis as papers, it is recognised that there is some duplication from chapter to chapter.

CHAPTER 1

General Introduction

The Significance of Nitrogen Fixation in the Marine Environment

Nitrogen is the nutrient most frequently cited by biologists as a crucial regulator of primary production in the marine environment. Deficiencies of inorganic nitrogen (NH_4^+, NO_3^-, and NO_2^-) have been shown to control the growth of phytoplankton in many pelagic and coastal waters (Ryther and Dunstan, 1971; Eppley *et al.*, 1973; Mann, 1982). Deficits of nitrogen available to primary producers may be so severe in some near-shore and off-shore waters that phytoplankton growth is stimulated following rainfall enriched with nitrogen. Paerl (1985b) showed that acid rain produced greater stimulation of primary production than oceanic-derived precipitation. Moreover, several workers have noted that NH_4^+ regenerated in the sediments and diffused into the overlying water, forms an important source of nitrogen for phytoplankton growth in coastal waters (Thorstensen and MacKenzie, 1974; Rowe *et al.*, 1975; Billen, 1978; Harrison, 1978; Nixon, 1981) .

The productivity of seagrass and saltmarsh macrophytes in benthic marine habitats has also been shown to be nitrogen-limited, in coastal localities as geographically diverse as Alaska (Iizumi, *et al.*, 1982 Short, 1983), the West Indies (Patriquin, 1972), the north-eastern United States (Sullivan and Daiber, 1974), and south-eastern Australia (Bulthuis and Woelkerling, 1981; Moriarty *et al.*, 1985). This evidence for nitrogen-limited primary production of marine macrophytes comes from several experimental approaches. Patriquin (1972) showed that turnover rates for nitrogen pools in the sediment were much higher than those for phosphorus, suggesting a role for nitrogen in the regulation of seagrass (*Thalassia testudinum*) production. Calculations based on NH_4^+ uptake rates, nitrogen requirements of seagrass growth, and ambient NH_4^+ concentrations also led Iizumi *et al.* (1982) and Short (1983) to conclude nitrogen-limitation of *Zostera marina* growth. In another approach, *in situ* enrichment of the

water column (Harlin and Thorn-Miller, 1981) or sediments (Bulthuis and Woelkerling, 1981) with nutrients (NH_4^+ and PO_4^{2+}) stimulated seagrass production, with the greatest effects measured in response to sediment enrichment with ammonium. Provision of excess nitrogen also affects the species composition and nitrogen content of marsh floras (Bulthuis and Woelkerling, 1981; Harlin and Thorn-Miller, 1981). The low nitrogen content of many marine plants (median C:N:P ratio of 550:30:1) underscores the severe deficiency of nitrogen present in marine ecosystems (Atkinson and Smith, 1983). Few studies of marine ecosystems have not concluded, or indicated, nitrogen-limitation of primary production (Smith, 1984; Dennison *et al.*, 1987).

Notwithstanding the low concentrations of utilizable nitrogen, saltmarsh and seagrass macrophytes exhibit high growth rates and rank among the most productive ecosystems known (Valiela *et al.*, 1976; McRoy and McMillan, 1977; Schubauer and Hopkinson, 1984). The ecological and economic importance of these ubiquitous ecosystems has been widely acclaimed; they function as nurseries and habitats for many migratory and indigenous fish, with obvious economic benefits to fishing and aquaculture industries. Since primary production in saltmarsh lagoons also supports large populations of invertebrates, further ecological benefits are derived by animals (e.g. birds) from higher trophic levels. Indeed, seagrass ecosystems are well known for their species richness (Ogden, 1980). They may also contribute nutrients to adjacent deeper-water food chains (Wolff, 1980). The source of nutrients for primary production in saltmarsh and seagrass ecosystems is therefore important for the ecology of these, and possibly other, marine communities. Consequently, considerable attention has been given to factors regulating nitrogen availability in the marine environment.

High rates of primary production in saltmarsh and seagrass ecosystems may be sustained to a large extent by the internal recycling of reduced nitrogen, but alternative sources of reduced nitrogen are essential to replenish that lost from the system by tidal exchange (Valiela *et al.*, 1978; Nixon, 1980; Whiting *et al.*, 1987) and denitrification (Sorensen, 1978; Seitzinger *et al.*, 1984; Seitzinger, 1988). Although nitrogen is usually the nutrient limiting phytoplankton production in marine waters, the mechanisms controlling its supply are still not well understood. Patriquin (1972)

suggested that, because the availability of nitrogen in the sediments of *Thalassia* communities is limited, bacterial fixation of dinitrogen probably maintains the interstitial pool of NH_4^+. Since then it has been shown that nitrogen fixation can indeed make substantial inputs of reduced nitrogen to seagrass and saltmarsh ecosystems (Goering and Parker, 1972; Patriquin and Knowles, 1972; Capone *et al.*, 1979; Teal *et al.*, 1979; Capone, 1982; O'Donohue *et al.*, 1991).

Rates of nitrogen fixation associated with *in situ* heterotrophic activity have been reviewed by Howarth *et al.* (1988a). Data available for saltmarshes, seagrass beds, and mangroves, show moderate to high rates rates of nitrogen fixation. Teal *et al.* (1979) calculated that heterotrophic nitrogen fixation was 10-14 times greater than algal fixation in the Great Sippewissett Marsh in Cape Cod, Massachusetts. Valiela and Teal (1979) calculated that nitrogen fixation in the rhizosphere of saltmarsh macrophytes at Great Sippewissett contributed 7% of the total nitrogen input to that system. Nitrogen fixation by heterotrophs associated with macrophytes in other ecosystems may also support a significant proportion of the nitrogen requirements of plant growth. O'Donohue *et al.* (1991) calculated that nitrogen fixation could provide between a third and a half of the nitrogen required by *Zostera capricorni* production in Moreton Bay, Australia. Capone (1982) estimated that 3-28% of the nitrogen required by *Zostera marina* production in Long Island, United States, could be supplied by nitrogen fixation, while Capone *et al.* (1979) found that 21-48% of the nitrogen demand of *Thalassia testudinum* growth in the Bahamas could be met by nitrogen fixation. True values could be significantly higher if calculations were based on data from experiments that excluded aerobic and microaerophilic diazotrophy (Capone *et al.*, 1979). Thus, it appears that nitrogen fixation is a quantitatively important source of nitrogen in some marine microhabitats, especially when associations of plants and heterotrophic bacteria are of mutualistic advantage. While such a source of reduced nitrogen provides an attractive solution to the problem, the mechanisms which control nitrogen fixation *in situ* remain an elusive subject. Concurrent recognition of diazotrophy (nitrogen fixation) as a source of nitrogen in the marine environment and the considerable spatial and temporal variability in bacterial activity and nitrogen

fixation, has stimulated interest in the regulation of nitrogen fixation (Capone, 1988; Paerl, 1990).

The Distribution of Nitrogen-Fixing Organisms

The ability to fix nitrogen appears to be confined to the prokaryotes. The occurrence of diazotrophy among physiologically primitive groups of prokaryotes, its association with 'ancient' enzymes such as hydrogenase, and its sensitivity to oxygen, support the view that it is a 'primitive' enzyme system (Postgate, 1982). Diazotrophs have been identified in taxonomically diverse bacterial genera and are represented in most physiological groups, including photoautotrophs, chemoautotrophs, and heterotrophs, in both free-living and symbiotic relationships (Postgate, 1982; Capone, 1988; Paerl, 1990). Diazotrophy appears to be scattered among unrelated bacterial genera with no clear evolutionary linkage. Postgate (1981) showed that this distribution pattern, or lack thereof, was present even within certain genera, and proposed that nitrogen fixation has been distributed in a disunited fashion among prokaryotes during its evolutionary history; this process is probably ongoing although its mechanisms remain uncertain. Mechanisms which could contribute to the lateral transfer of diazotrophy include transformation, transduction, or transmission by conjugative plasmids (Postgate, 1982).

Nitrogen fixation by cyanobacteria has received considerable attention for several reasons. From an evolutionary viewpoint, cyanobacterial fossils represent some of the oldest records of the history of life, and hence their physiology may provide clues as to how biological processes coped with the inhospitable climate of the pre-Cambrian period. From a physiological viewpoint, studies of cyanobacteria have yielded much information on the regulation of nitrogen fixation, which may be common to diazotrophs in general or characteristic of particular phototrophs. And from an ecological viewpoint, the interaction of cyanobacteria as primary producers and nitrogen fixers with other trophic levels has received some interest (Wilkinson and Sammarco, 1983).

Cyanobacteria appear to be responsible for most nitrogen fixation in planktonic communities (Howarth *et al.*, 1988a). Pelagic blooms of the free-living diazotrophic cyanobacterium *Trichodesmium* (*Oscillatoria* spp.) are often visually obvious in tropical and subtropical seas (Carpenter, 1983). However, nitrogen fixation in oceanic waters contributes $<<$ 1% of the total nitrogen inputs to these systems (Howarth *et al.* 1988a). Benthic cyanobacterial mats are common in some coastal regions (Stal *et al.*, 1985) and may exhibit high rates of nitrogen fixation (Jones, 1974; Wiebe *et al.*, 1975; Hansen and Gundersen, 1977). However, the areal cover of cyanobacterial mats is usually fairly limited, and while nitrogen fixation may be important to the mat communities themselves, the significance of this supply of nitrogen diminishes beyond the mat environment. In saltmarsh ecosystems, cyanobacterial nitrogen fixation is probably less important as a source of nitrogen than is nitrogen fixation by heterotrophic bacteria (Carpenter *et al.*, 1978; Teal *et al.*, 1979; Howarth *et al.*, 1988a).

Eubacterial diazotrophy is potentially an important source of reduced nitrogen for marine ecosystems (Paerl, 1990). Diazotrophs from photosynthetic, chemolithotrophic, and heterotrophic eubacterial genera occupy a wide range of marine microhabitats of diverse physicochemical properties. In addition to nitrogen fixation, these bacteria (particularly the heterotrophs) play fundamentally important roles in nutrient cycling. Recognition of the potential importance of heterotrophic diazotrophs in the marine environment has occurred relatively recently, considering that the biogeochemical importance of diazotroph-plant interactions in terrestrial environments has been recognized for well over a century (reviewed by Paerl, 1990).

The distribution of nitrogen fixation among natural assemblages has been examined in terms of active diazotrophy, as well as the potential for diazotrophy (the latter approach by enrichment and isolation of diazotrophs, or by probing for the presence of nitrogen fixation genes in bacterial DNA). Potential or active nitrogen fixation has been detected in natural assemblages from diverse marine habitats, ranging from oligotrophic pelagic waters (Guerinot and Colwell, 1985) to mangrove sediments (Zuberer and Silver, 1978), and from the intestinal tract of marine invertebrates (Guerinot and Patriquin,

1981; Waterbury *et al.*, 1983) to *Prochloron* symbionts (Paerl, 1984b). Both Guerinot and Colwell (1985) and Paerl (1990) noted that nitrogen-fixing bacteria could be isolated from most seawater samples they collected from oceanic and estuarine sources. Capone (1988) has reviewed the marine habitats of nitrogen-fixing bacteria more fully. Heterotrophic genera common to marine habitats and known to include species that fix nitrogen include *Azotobacter, Bacillus, Clostridium, Enterobacter, Klebsiella, Pseudomonas, Azospirillum,* and *Vibrio* (Capone, 1988; Paerl, 1990).

Enzymology of Conventional Nitrogenase

An introduction to the enzymology of nitrogen fixation is necessary for an understanding of the regulation of the process. Bacterial nitrogen fixation is mediated by the structurally and mechanistically complex enzyme, nitrogenase. Nitrogenase is composed of two major proteins, dinitrogenase and dinitrogenase reductase, which are both required to form a functional nitrogenase enzyme (Bulen and Le Comte, 1966). A remarkable characteristic of the conventional nitrogenase is its highly conserved nature (both molecularly and phylogenetically); enzyme components from different organisms can often be combined to produce active nitrogenase (Eady, 1986). Dinitrogenase and dinitrogenase reductase are each composed of polypeptide subunits. Dinitrogenase reductase has two identical subunits of about 30 kilodaltons each, whereas dinitrogenase has an $\alpha_2ß_2$ structure with four 61-kilodalton subunits. In *Klebsiella pneumoniae*, dinitrogenase reductase is encoded by the *nifH* gene, the α-subunit of dinitrogenase reductase by *nifD*, and the ß-subunit by *nifK*. In the conventional nitrogenase, dinitrogenase reductase contains iron, whereas dinitrogenase contains molybdenum and iron. Consequently, the two proteins have been referred to by many workers as the Fe-protein and the MoFe-protein respectively.

The molybdenum and about 50% of the iron content of dinitrogenase is present as a unique prosthetic group (iron-molybdenum cofactor, or FeMoco), which also contains sulfur (Shah and Brill, 1977) and homocitrate (Hoover *et al.*, 1989). The view that FeMoco forms at least part of the active site at which dinitrogen is reduced, is supported by evidence that it restores the activity of certain Nif$^-$ mutants (Shah and

Brill, 1973; Roberts *et al.*, 1978), and can catalyze the reduction of acetylene to ethylene (Shah *et al.*, 1978). Of the 17 genes required for nitrogen fixation in *K. pneumoniae* (Postgate, 1982), six of them (*nifE, nifN, nifB, nifV, nifQ,* and *nifH*) are involved in the biosynthesis of FeMoco (Roberts *et al.*, 1978; Hawkes *et al.*, 1984; Imperial *et al.*, 1984; Ugalde *et al.*, 1984; Filler *et al.*, 1986; Robinson *et al.*, 1987). Howarth *et al.* (1988b) have suggested that part of the high energetic cost of nitrogen fixation might be associated with the assimilation of elements required for nitrogenase biosynthesis.

The Requirement for Molybdenum

The presence of Mo in the MoFe-protein of nitrogenase explains a long-known requirement for Mo in nitrogen fixation by some bacteria (Bortels, 1930). Studies of Mo-regulation of nitrogenase activity have not, however, established a uniform pattern of response to Mo-deprivation. Under Mo-starvation *in vitro*, *Plectonema boryanum* synthesized both dinitrogenase and dinitrogenase reductase (Nagatani and Hazelkorn, 1978), *Azotobacter vinelandii* synthesized only dinitrogenase reductase (Nagatani and Brill, 1974), whereas *Clostridium pasteurianum* synthesized neither component (Cardenas and Mortensen, 1975).

Regulation of nitrogen fixation by the availability of molybdenum in natural environments is poorly understood, although it has been proposed to potentially limit nitrogen fixation in soils (Anderson and Spencer, 1949), estuaries (Brattberg, 1977), and oceans (Capone and Carpenter, 1982). In oxygenated seawater Mo is most stable as the molybdate ion, a structural analogue of sulfate. Howarth and Cole (1985) argued that the high concentration of sulfate in seawater (ca. 28mM) could restrict the availability of Mo to marine bacteria by competing with molybdate uptake, and thus hinder nitrogen fixation. The mean concentration of molybdate in seawater is 110nM (Collier, 1985). Howarth and Cole (1985) showed that additions of SO_4^{2-} depressed molybdate uptake by phytoplankton (see also Cole *et al.*, 1986), and restricted the growth of bacteria growing with nitrate as the sole nitrogen source; nitrate reductase is a molybdoenzyme, as is nitrogenase. They also presented evidence that variation of the

molar ratio of sulfate:molybdate in mesohaline Baltic Sea water correlated with changes in nitrogenase activity (acetylene reduction), which were consistent with their hypothesis. However, direct evidence that restrictions on the uptake of Mo limits nitrogenase activity in fully saline seawater was not presented, and as Paerl (1990) has noted, sulfate competition with molybdate uptake is not equivocal evidence for molybdenum limitation of nitrogen fixation.

In contrast to the findings of Howarth and Cole (1985), Paerl *et al.* (1987) and Paulsen *et al.* (1991) found that additions of Mo did not stimulate nitrogenase activity in North Carolina coastal seawater. Furthermore, additions of sulfate had no effect on existing or carbohydrate-stimulated nitrogenase activity. This led Paulsen *et al.* to conclude that Mo concentration in fully saline seawater is sufficient to meet the demands of nitrogen fixation, and that *in situ* SO_4^{2-} concentrations do not adversely affect the availability of Mo to bacteria for nitrogen fixation. In this context it is noteworthy that although molybdate is the most common form of Mo in seawater, in reduced environments such as sediments, molybdate is unstable, and Mo occurs in reduced form. Hence, SO_4^{2-} interference with Mo availability in sediments should generally not be significant.

Alternative Nitrogenases

The role of molybdenum in regulating nitrogenase activity has a further interesting aspect. Until recently, all nitrogenases which had been isolated and characterized contained molybdenum, and molybdenum was believed to be an absolute requirement for nitrogenase biosynthesis and hence nitrogen fixation. This is now known not to be the case. The existence of Mo-independent nitrogenases was first proposed by Bishop *et al.* (1980) who found that Nif$^-$ mutants of *A. vinelandii* were able to fix nitrogen in the absence of Mo. These findings were supported when Robson *et al.* (1986) isolated an alternative nitrogenase, composed of a vanadium-iron (VFe) protein and an Fe protein, from the related species *Azotobacter chroococcum*. Subsequently, two alternative nitrogenases were isolated from *A. vinelandii*. One of these is similar to the alternative nitrogenase of *A. chroococcum* in that it contains V. The second alternative nitrogenase from *A. vinelandii* contains Fe, but apparently no Mo or V. All three nitrogenases of

A. vinelandii, the conventional MoFe-nitrogenase ('nitrogenase-1'), the alternative VFe-nitrogenase ('nitrogenase-2'), and the alternative Fe-nitrogenase ('nitrogenase-3') were shown to be genetically distinct (Jacobson *et al.*, 1989; Joerger *et al.*, 1989; Joerger *et al.*, 1990). The genes for the structural proteins have been designated as *nifHDK* (Mo-dependent nitrogenase), *vnfHDGK* (V-dependent nitrogenase), and *anfHDGK* (alternative nitrogenase). The *vnfG* and *anfG* genes apparently encode a third subunit, δ, for the dinitrogenase components of the alternative systems (Robson *et al.*, 1989). Thus, in contrast to the Mo-dinitrogenase, alternative dinitrogenase proteins probably have a hexameric subunit structure of $\alpha_2\beta_2\delta_2$.

Expression of alternative nitrogenases in *A. vinelandii* and *A. chroococcum* is responsive to the presence or absence of Mo and V (Joerger and Bishop, 1988; Bishop and Joerger, 1990); the presence of 50nM Mo prevents synthesis of nitrogenase-2 in *A. chroococcum* and prevents the synthesis of both alternative nitrogenases in *A. vinelandii* (Dilworth *et al.*, 1987; Joerger and Bishop, 1988; Bishop and Joerger, 1990). Nitrogenase-2 is expressed in cells deprived of Mo but not V, whereas nitrogenase-3 is found in cells grown in the absence of both Mo and V. Fe is required by all three nitrogenases. The molecular basis for metal-regulation of nitrogenase activity in *A. vinelandii* is not yet fully understood. The regulatory genes *nifA*, *vnfA*, and *anfA* have been implicated: NifA binds to an upstream activator sequence and activates transcription of *nif* (nitrogenase-1) genes (Buck *et al.*, 1986); VnfA is required for expression of nitrogenase-2 while it represses the synthesis of nitrogenase-1, and similarly AnfH is required for diazotrophic growth in Mo- and V-deficient medium (Joerger *et al.*, 1989). The factors which regulate the relative expression of *nifA*, *vnfA*, and *anfA* in *A. vinelandii* are not known. Additional regulatory genes designated ntrC and nfrX have also been described, but the mechanism by which these genes regulate the expression of the three nitrogenases in *A. vinelandii* is not known (Toukdarian and Kennedy, 1986; Santero *et al.*, 1988). Furthermore, VnfH (dinitrogenase reductase-2) seems to be required for the transcription of the *anfHDGK* (nitrogenase-3) operon in *A. vinelandii*, although it appears that dinitrogenase reductase-1 can act as a substitute for this function in VnfH$^-$

mutants (Joerger *et al.*, 1991). Clearly, metal regulation of the expression of alternative nitrogenases is complex and presently not well understood.

Significance of Alternative Nitrogenases

The ecological and physiological significance of multiple nitrogenases in one organism is not clear. Application of natural selection theory would invoke benefits to the organism in environments where the availability of Mo or V is limited, such as some tropical soils (Bishop and Joerger, 1990). Indeed, such an explanation seems plausible, and is supported by our present understanding of metal regulation (which is consistent with the lower efficiency of N_2 reduction by alternative nitrogenases relative to that of Mo-nitrogenase), as well as the fact that *Azotobacter* is a soil-inhabitant. Although such a role for alternative nitrogenases may not be significant in seawater (Paerl *et al.*, 1987; Paulsen *et al.*, 1991), the relevance of this argument still holds for other environments. The catalytic properties of nitrogenases 2 and 3 include the reduction of acetylene not only to ethylene, but also partly to ethane (Dilworth *et al.*, 1987). In *A. vinelandii* and *A. chroococcum* this characteristic distinguishes alternative nitrogenase activity from that of Mo-nitrogenase, which reduces acetylene only to ethylene. Thus acetylene reduction to ethylene and ethane has been considered to indicate the likely presence of alternative nitrogenases (Dilworth *et al.*, 1987).

Unravelling the significance of alternative nitrogenases in nitrogen fixation could be assisted by knowledge of the distribution of alternative nitrogenases among diazotrophs. There has been much interest recently in the occurrence of alternative nitrogenases among diazotrophs from phylogenetically diverse genera. The modulation of ethane production by Mo and/or V has been presented as evidence of V-nitrogenase in *Clostridium pasteurianum*, *Anabaena variabilis*, and *Heliobacter gestii* (Dilworth *et al.*, 1987; Kentemich *et al.*, 1988; Kimble and Madigan, 1992). Other approaches have also been used in investigations of alternative nitrogenases. Schneider *et al.* (1991) showed that a *nifHDK*$^-$ mutant of the photosynthetic bacterium *Rhodobacter capsulatus* could grow diazotrophically in the absence of Mo and V. Scherer (1989) showed that the methanogen *Methanosarcina barkai* could grow diazotrophically in the presence of

V instead of Mo, and suggested that this organism may also possess a V-nitrogenase. More convincing evidence comes from the use of alternative nitrogenase gene probes in Southern hybridizations to identify genetic potential for alternative nitrogenases. Using Southern hybridizations, Fallik *et al.* (1991) showed that *Azotobacter beijerinkii* and *Azotobacter nigricans* may possess nitrogenases 1 and 2, whereas *Azotobacter paspali* and *Azomonas agilis* may possess nitrogenases 1, 2, and 3. Lehman and Roberts (1991) used Southern hybridizations to identify the genetic potential for an alternative nitrogenase in the phototroph *Rhodospirillum rubrum*. They followed this with Western hybridizations to show metal-regulated synthesis of nitrogenase proteins. Together with Mo and/or V modulation of ethane production, such evidence represents the most comprehensive and most convincing study of the presence of alternative nitrogenases in an organism. Greater homology with *anf* probes than *vnf* probes suggested that the alternative nitrogenase of *R. rubrum* is an Fe-nitrogenase (Lehman and Roberts, 1991). Thus, it would follow that an organism need not possess nitrogenase-2 in order to possess nitrogenase-3. Furthermore, although all diazotrophs studied to date have nitrogenase-1, the possibility exists for an organism to have nitrogenase-2 and/or 3 without Mo-nitrogenase. Such an organism would be biologically unique and interesting.

Mechanism of Nitrogenase Activity

Biological nitrogen fixation, the reduction of dinitrogen to ammonia by bacterial nitrogenase, can be represented as follows:

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i \qquad (1)$$

Glutamine synthetase then catalyses the assimilation of NH_3 with glutamate to produce glutamine, an important intermediate in the biosynthesis of many cellular compounds (eg. purines, carbomoyl phosphate, tryptophan). H_2 is an obligatory byproduct of the reaction. Furthermore, it is clear that nitrogen fixation is an energy expensive process (equation 1). This large energy requirement arises partly because N_2 is an extremely stable molecule due to a triple bond between the two nitrogen atoms. Consequently, the

activation energy required to break this bond and reduce nitrogen is very high. Therefore, besides N_2, nitrogenase also requires a source of reductant (ferredoxin or flavodoxin) and MgATP as substrates. The exact amount of ATP required to reduce N_2 varies according to the environment of the reaction; Burris (1991) has noted that 16 moles of ATP per mole N_2 fixed is the minimum requirement under ideal (*in vitro*) conditions. Within the physiological environment of the cell, however, this requirement is around 20-30 moles MgATP per mole N_2 fixed.

A model for nitrogenase activity has been developed through studies of *K. pneumoniae* (reviewed by Postgate, 1982; Paerl, 1990; Burris, 1991). Reduced dinitrogenase reductase ($Kp2_{red}$), binds 2MgATP which lowers its redox potential to around -400mV. At that potential it can transfer electrons to dinitrogenase (Kp1). $Kp2_{red}$ combines with Kp1, ATP is hydrolysed, and electrons are passed to Kp1. The complex then dissociates to $Kp2_{ox}$, 2MgADP, and $Kp1_{red}$. At least 2MgATP are required for each electron transferred, and the cycle must be repeated until the dinitrogenase has accumulated enough electrons to reduce nitrogen. The electrons are thought to be channelled to the FeMoco site on dinitrogenase, which is believed to be the active site for N_2 reduction. Nitrogenase activity is inhibited by MgADP, but despite its high ATP requirement, it is not inhibited by NH_3 or glutamine (Gottschalk, 1986).

Physiological and Ecological Regulation of Nitrogen Fixation in the Marine Environment

Factors which regulate nitrogen fixation in the marine environment include temperature, salinity, light, the availability of trace metals, inorganic nitrogen, carbohydrates, and oxygen. The relative importance of each of these factors varies in accordance with the particular physiology of each diazotroph. For bacterial activity, the 'environment' of importance is the microenvironment or 'microzone' local to the bacterial cell (Paerl, 1990). The effects of oxygen, inorganic nitrogen, and energy availability have been well documented for many marine environments, and are usually considered to be the major determinants of nitrogen fixation in most systems.

Oxygen

Nitrogenase is extremely oxygen-labile, and thus oxygen is a major determinant of nitrogenase activity. Both the MoFe-protein and particularly the Fe-protein are irreversibly inactivated upon contact with oxygen in cell-free extracts (Postgate, 1982). Nitrogenase therefore requires reduced conditions if it is to remain fully functional. However, apart from the difficulty of always excluding oxygen from the immediate environment of the cell, many bacteria require O_2 to generate ATP for growth and diazotrophy. Considering the distribution of functional *nif* among genera from diverse physiological groups, it is not surprising therefore that the degree of O_2-sensitivity of nitrogen fixation *in vivo* (intact cells) differs markedly between organisms. The presence of O_2-protection mechanisms appears to be at least partly responsible for O_2-tolerance in some diazotrophs. Whether the different mechanisms that protect nitrogenase from damage by O_2 were developed specifically for that function, or are fortuitous secondary-function benefits, is not clear in many cases. Such protective mechanisms include hydrogenase-coupled O_2 respiration (Bothe *et al.*, 1978), superoxide dismutase, catalase, and other free-radical scavenging systems (Di Guiseppi and Fridovich, 1984). Mechanisms which may have evolved more specifically to protect nitrogenase are found in obligate aerobes, such as azotobacters. Two features of azotobacter diazotrophy are worthy of note: (i) although purified nitrogenase from azotobacters is just as sensitive to O_2 as those from any other diazotrophs, crude nitrogenase extracts from azotobacters can be handled in air; (ii) azotobacter diazotrophy is sensitive to O_2 under conditions where the medium is highly aerated. Dalton and Postgate (1969) proposed that two mechanisms dominate O_2-protection of nitrogenase in azotobacters: (i) although functional nitrogenase is sensitive to oxygen, the enzyme can also exist in a conformationally-protected, inactive state, where protein-protein interaction screens O_2-sensitive sites on the enzyme; and (ii) that functional nitrogenase is actively protected by elevated respiration. The latter proposal is supported by evidence that carbon-limited cultures were hypersensitive to O_2. Mucilage formation might be another mechanism for restricting O_2 access to sites of nitrogenase activity in some bacteria including azotobacters (Postgate, 1982).

Diazotrophic cyanobacteria must deal with an incompatability between their O_2-generating photosynthetic activity and O_2-labile nitrogenase activity. Some filamentous cyanobacteria have overcome this problem by restricting nitrogen fixation to specialized cells, or heterocysts, and thereby spatially separating these two processes. The reduced environment within heterocysts is maintained by their lack of O_2-evolving photosystem II, elevated rates of respiration, and a thickened cell wall, which apparently acts as an O_2-diffusion barrier (Donze *et al.*, 1971; Postgate, 1982; Murry *et al.*, 1984; Paerl, 1990). Non-heterocystous cyanobacteria may spatially separate nitrogen fixation and oxygenic photosynthesis in other ways. For example, nitrogenase activity in several non-heterocystous cyanobacteria is correlated with the ability of filaments to form aggregates. Nitrogen fixation takes place in the internal regions of these aggregates where O_2 minima occur; oxygenic photosynthesis is restricted to cells at the periphery of the aggregates (Carpenter and Price, 1976; Paerl and Bland, 1982; Paerl and Bebout, 1988; Paerl and Carlton, 1988). Physical disruption of these aggregates, for example by turbulence during storms, would break down the internal O_2 gradients and potentially lead to inhibition of nitrogenase activity (Paerl, 1985a). Moreover, nitrogen fixation in *Trichodesmium* is strongly light-dependent, with higher rates of nitrogen fixation occurring during the day than at night (Saino and Hattori, 1978). This diel pattern suggests that nitrogenase activity in *Trichodesmium* is directly dependent upon photosynthetic products.

Non-heterocystous cyanobacteria may also overcome incompatability between photosynthetic and nitrogenase systems by temporal control of these processes with respect to photic conditions; nitrogen fixation is limited to periods of low light intensity when photosynthesis is not evolving O_2 (Millineaux *et al.*, 1981; Pearson *et al.*, 1981; Stal and Krumbein, 1985; Grobbelaar *et al.*, 1986; Mitsui *et al.*, 1986; Villbrandt *et al.*, 1991). Bebout *et al.* (1993) showed that night-time fixation of dinitrogen by cyanobacteria-dominated microbial mats was dependent on preceding oxygenic photosynthesis, suggesting that night-time nitrogen fixation was dependent on storage products derived from oxygenic photosynthesis during the daytime.

Despite the extreme sensitivity of nitrogenase to O_2, it is clear that diazotrophs have at their disposal a number of mechanisms which enable them to overcome this problem. This is also reflected in numerous reports of O_2-effects, ranging from inhibition to stimulation of nitrogenase activity in natural assemblages (reviewed by Capone, 1988).

Inorganic Nitrogen

Ammonium is another well known regulator of nitrogen fixation, although it does not act alone to accomplish this. It appears to act in unison with the glutamine synthetase system to regulate nitrogenase synthesis and/or exert allosteric control of nitrogenase activity (Postgate, 1982). Ammonium "switch-off" of nitrogenase activity is rapid and mostly reversible; ammonium inhibition involves some destruction of pre-existing enzyme. However, following depletion of NH_4^+ most of the nitrogenase activity is restored and *nif* expression is derepressed (Postgate, 1982).

Ammonium-regulation of nitrogenase activity *in situ* has been examined by workers using several approaches. The concentration of NH_4^+ required to inhibit nitrogen fixation *in situ* appears to vary between different ecosystems, and consequently the importance of ammonium control in the ecology of nitrogen fixation seems uncertain. Bebout *et al.* (1987) reported that nitrogenase activity associated with a cyanobacterial mat in an oligotrophic marine environment (North Carolina coast) was unlikely to be ammonium-regulated as ambient NH_4^+ concentrations were far lower than those required to inhibit nitrogenase activity. In contrast, Diaz *et al.* (1990) found that nitrogenase activity associated with *Microcoleus lyngbyaceus* mats was closely regulated by NH_4^+ concentrations typical of their habitat in Puerto Rico. Inverse correlations between ambient NH_4^+ concentrations and nitrogenase activity have been noted for saltmarsh sediments. Carpenter *et al.* (1978) observed an inverse correlation between nitrogenase activity and NH_4^+ concentrations in interstitial waters of saltmarsh sediments, but noted the decrease in activity was gradual with no clear threshold; nitrogenase activity appeared to be unaffected at NH_4^+ concentrations below 100μM. In studies of the same marsh, Teal *et al.* (1979) concluded that regulation of nitrogen fixation by NH_4^+ seemed unlikely, since ambient NH_4^+ concentrations

seldom exceeded 50μM, which was four times lower than the concentration required to inhibit nitrogenase activity in those sediments. Dicker and Smith (1980a) found temporal variations in the response of nitrogenase activity to NH_4^+ in a Delaware saltmarsh; ammonium additions had little effect on nitrogenase activity during early summer, but resulted in inhibition of activity during late summer and early fall. Hanson (1977a) found spatial variation of NH_4^+-effects; ammonium additions had no effect on nitrogenase activity associated with tall *Spartina* marsh, but resulted in 50% lower activity in short *Spartina* marsh.

Capone and Carpenter (1982) used a perfusion method to assay nitrogenase activity, wherein C_2H_2-saturated seawater was passaged through sediment cores from saltmarsh and seagrass communities. They found that increased nitrogenase activity during their assays correlated with depletion of interstitial NH_4^+; amendment of the seawater (before perfusion) with increasing concentrations of NH_4^+ gradually inhibited this stimulation, although some stimulation still occurred at 500μM NH_4^+. Yoch and Whiting (1986) and Capone (1988) used L-methionine-D,L-sulfoximine (MSX) to inhibit glutamine synthetase activity and thus interfere with ammonium-control of nitrogen fixation. Both reported stimulation of nitrogenase activity by additions of MSX to saltmarsh and seagrass sediments, indicating that ambient NH_4^+ was possibly responsible for submaximal activity. However, additions of MSX had no consistent effect on nitrogenase activity in tropical carbonate marine sediments, and even inhibited activity in some cases (O'Neil and Capone, 1989). Thus it appears that, although NH_4^+ inhibition of nitrogen fixation *in vitro* is well known, its effect on this process in natural populations shows no consistent trend, making precise predictions difficult at present.

The effect of nitrate on nitrogenase activity has also received some interest. The mechanism by which nitrate represses nitrogenase activity is not clear. Assimilatory nitrate reduction may produce ammonium, which then inhibits nitrogen fixation (Hattori, 1983). Another possible mechanism is that nitrate reductase, induced by nitrate, successfully competes with nitrogenase for reducing power (Dicker and Smith, 1980a). Whatever the exact mechanism of repression, additions of nitrate have been

reported to repress nitrogenase activity *in situ* (Hanson, 1977a; Dicker and Smith, 1980a; Capone, 1982). However, the significance of nitrate repression is disputable for many marine environments, since denitrification generally ensures that nitrate does not accumulate (Capone, 1988). An exception may be coastal environments adjacent to upwelling systems where nutrient-rich water containing nitrate ($\pm 20\mu M$) is regularly introduced to these environments (Andrews and Hutchings, 1980).

Energy Source

The requirement by nitrogen fixation for a large and steady supply of ATP and reductant potentially represents a major limitation for this process. The interaction of nitrogen fixation and photosynthesis in photoautotrophic diazotrophs has been discussed above. Heterotrophic nitrogen fixation on the other hand relies on energy and reductant from the oxidation or fermentation of organic subtrates. Evidence for the limitation of nitrogen fixation by a lack of easily-utilizable organic substrate in saltmarsh and seagrass ecosystems, is provided by a large number of reports of stimulation of nitrogenase activity by additions of simple mono- and disaccharides (reviewed by Capone, 1988). Stimulation of nitrogenase activity by such carbon sources (eg. glucose, mannitol) can be profound. However, the majority of organic material available to bacteria in saltmarsh ecosystems consists of complex polysaccharides of plant or animal origin. Much of this material is not readily metabolized. Few studies of nitrogen fixation have considered its modulation by the availability of polysaccharides. Paerl *et al.* (1987) examined the relative importance of inorganic nutrients and organic matter availability, including the effect of saltmarsh and seagrass detritus on nitrogenase activity in coastal seawater. They concluded that ambient concentrations of molybdenum, iron, and phosphorus exceeded the demands of nitrogen fixation. However, enrichment with sucrose, fructose, glucose, maltose, mannitol, *Spartina* and *Zostera* detritus all elicited or stimulated nitrogenase activity. Whereas the sugars and mannitol may have provided an energy source for bacterial metabolism, Paerl *et al.* attributed the effect of detrital particles to their function as a surface for O_2-depleted microzone formation, and thus as suitable sites for O_2-sensitive nitrogen fixation (see also Paerl, 1984a; Paerl and Carlton, 1988). While this may be largely true, the

possibility that detritus is also a potentially useful energy source for nitrogen fixation cannot be discounted.

The production and use of detritus in marine ecosystems have been the subjects of a large number of studies (Harrison and Mann, 1975; Tenore *et al.*, 1979; Hanson and Tenore, 1981; Stuart *et al.*, 1981; Stuart *et al.*, 1982; Azam *et al.*, 1983; Newell *et al.*, 1983; Mann, 1988; Newell *et al.*, 1988). Heterotrophic bacteria are not only important in the remineralization of organic matter in these ecosystems, but also play an important role in increasing the nutritional (nitrogen) value of detritus for consumers (Newell, 1965; Mann, 1988). Although a large amount of work has been done on various aspects of biological activity in saltmarsh ecosystems, I am not aware of any studies which have examined the modulation of nitrogen fixation by polysaccharides as potential energy sources. This seems quite surprising, especially as the bulk of organic material available for bacterial use in saltmarsh and seagrass ecosystems originates as complex polysaccharide based materials. In these systems the supply of such material is unlikely to be static; seasonal die-back of macrophyte beds, and damage due to storms are two processes that contribute to a dynamic supply of detritus. The dynamics of detritus availability should be important to nitrogen fixation if resident diazotrophic microflora are able to utilize it. Furthermore, several different polysaccharides constitute plant material, and some may be better substrates than others in terms of supporting diazotrophy. The relative contributions of the different components of material available for use by diazotrophs as energy sources for supporting nitrogen fixation could assist our understanding of interactions between carbon and nitrogen cycling, and energy flow in these nearshore marine communities.

Trace Metals

Biogeochemical controls of nitrogen fixation, including the bioavailability of micronutrients, have been reviewed by Howarth *et al.* (1988b). The MoFe proteins contain 2Mo and about 33Fe atoms per tetramer, with slightly less sulfur than iron (Eady, 1986). The role of molybdenum in the regulation of nitrogen fixation has been discussed above. Very little work has been done on the role of iron in regulating

nitrogen fixation. Wurtsbaugh and Horne (1983) reported that additions of iron to eutrophic lake water frequently stimulated rates of nitrogen fixation during a cyanobacterial bloom. Typically, oceanic waters have iron concentrations ca. 10^2 to 10^3-fold lower than those of the lake waters studied by Wurtsbaugh and Horne (Gordon *et al.*, 1982; Collier and Edmond, 1984). Therefore, iron could conceivably limit nitrogen fixation in marine waters. However, factors influencing the availability of iron for nitrogen fixation are poorly understood. More information is needed on the influence of other metals, such as copper (Horne and Goldman, 1972), the effect of pH (Howarth *et al.*, 1988b), the effect of salinity (Sharp *et al.*, 1984), and the role of organically-complexed iron in the availability of iron for nitrogen fixation (Morel, 1983).

Temperature

Seasonal variations in nitrogenase activity have been recorded for several saltmarsh and seagrass ecosystems (Dicker and Smith, 1980b; Teal *et al.*, 1979; Jones, 1982; Capone, 1982) concurrent with temperature changes. Temperature may also affect nitrogenase activity over shorter time periods (e.g. diel) as it affects microbial metabolism in general (Jones, 1982; Smith and Hayasaka, 1982a, 1982b). The importance of temperature and seasonality should not be neglected in extrapolations of point data to annual estimates (Howarth *et al.*, 1988a).

Salinity and pH

Salinity fluctuations in fully saline seawater are usually minor and thus salinity is unlikely to have major influence on nitrogenase activity. However, salinity may have more influence on nitrogen fixation in estuaries where freshwater inputs and tidal movements of seawater cause salinity gradients (Capone, 1988). In such environments, diazotrophic bacteria with salt-dependent or -independent metabolism are likely to be found. The influence of pH on nitrogenase activity is also considered to be minor in the well-buffered medium of seawater (Capone, 1988).

Significance of Nitrogen Fixation to Heterotrophs

Although the relationship between nitrogen fixation and the requirements of primary production has been studied extensively in some areas, the significance of nitrogen fixation for heterotrophs is poorly understood. To date, there have been no investigations of nitrogen fixation in relation to overall heterotrophic bacterial activity. Technical developments over the past decade have made it possible to quantify heterotrophic bacterial productivity using isotopic labelling techniques (Moriarty, 1986). In particular, the incorporation of tritiated thymidine (Fuhrman and Azam, 1980) and leucine (Chin-Leo and Kirchman, 1988) into bacterial DNA and protein respectively, have gained widespread acceptance in microbial ecology as reliable methods for measuring bacterial productivity. Bacterial productivity and nitrogen fixation have both been linked to primary productivity in seagrass beds (Moriarty and Pollard, 1982; O'Donohue *et al.*, 1991). These relationships suggest that heterotrophic bacterial activity and nitrogen fixation are also linked. The impact of nitrogen fixation on bacterial productivity is also important for bacterivores and detritivores, since bacteria are a potentially important source of nitrogen for consumers in nitrogen-depleted marine environments (Newell, 1965; Mann, 1988). The nitrogen requirements of bacterial growth on detritus can be supplemented with inorganic nitrogen taken from the environment and nitrogen fixation.

Biotic Interactions

The community structure of saltmarsh lagoons supports a 'mosaic' of microhabitats with different physicochemical and biological properties. Benthic invertebrates and macrophytes are two components of saltmarsh communities which together probably represent the bulk of organic material in the system. The benthic macrofauna of many estuaries in southern Africa is dominated by the thalassinidean prawns, *Callianassa kraussi* and *Upogebia africana*, which reach densities of 350 and 400 individuals m^{-2} sediment respectively (Hanekom, 1980). Thalassinidean prawns exert considerable influence on ecosystem processes through their activities while burrowing and feeding (Bird, 1982; Branch and Pringle, 1987). For instance, the burrowing activities of

thalassinids result in profound bioturbation, which has been shown to contribute to sediment transportation (Roberts *et al.*, 1981), increase in oxygenation (Dye, 1978), alteration of the depth-distribution of microalgae (Branch and Pringle, 1987), and has been correlated with enhanced numbers of bacteria (Yingst and Rhoads, 1980; Branch and Pringle, 1987). Bioturbation by thalassinids may also modulate cover by seagrass beds (Suchanek, 1983). Thalassinids often line their burrows with organically rich mucus to preserve the structure, while certain species may also line their burrows with seagrass detritus (Dobbs and Guckert, 1988). Organic deposits such as these form a particular microenvironment for microbial populations, and may influence the biomass, composition, and physiologic state of associated microbial communities (Dobbs and Guckert, 1988). Thus, the activities of thalassinids could directly, and potentially indirectly through effects on nitrogen fixation, affect primary production of diatoms and macrophytes, which provide the food source of these invertebrates as well as many other consumers.

CHAPTER 2

Seasonal and Spatial Variability in Physico-Chemical Parameters in Relation to Bacterial Activity and Nitrogen Fixation in a Temperate Saltmarsh Lagoon

Abstract. Two sites in a temperate saltmarsh lagoon were compared in terms of parameters important to primary production, bacterial production, and nitrogen fixation in saltmarsh ecosystems. Sheltered, muddy sediments at Geelbek, near the lagoon head, were more anaerobic than sandy sediments exposed to tidal currents at Oesterwal near the lagoon mouth. Organic contents of sediments and water from Geelbek were greater in quantity and quality (C:N ratio) than those from Oesterwal. Concentrations of porewater ammonium were low at both sites (2.51 ± 2.32 μM). Concentrations of porewater ammonium, exchangeable ammonium, nitrate, and nitrite in sediments and water were not significantly different between sites. However, concentrations of phosphate were significantly higher at Geelbek. Concentrations of phosphate at both sites were in excess relative to the N:P requirements of marine macrophytes and algae. General heterotrophic isolates were represented by significantly higher numbers of nitrogen fixers, denitrifiers, and nitrate reducers in summer than in winter. The most probable number technique also indicated that densities of nitrogen fixers were greater in summer than in winter.

INTRODUCTION

Saltmarsh and seagrass ecosystems are ecologically important marine environments. They exhibit high rates of primary production (McRoy and McMillan, 1977), support rich a biodiversity (Ogden, 1980), and may contribute significant quantities of nutrients to the food-chains of adjacent ecosystems (Valiela and Teal, 1979; Wolff, 1980; Whiting *et al.*, 1987). High rates of primary production in these ecosystems are sustained notwithstanding generally low concentrations of biologically utilizable nitrogen, which is usually considered to be the limiting nutrient. Consequently,

considerable attention has been given to factors regulating nitrogen availability in these marine ecosystems.

As in other aquatic environments, heterotrophic bacteria in saltmarsh and seagrass ecosystems play key roles in organic matter decomposition and nutrient cycling (Fenchel, 1977; Hanson, 1977a; Howarth and Teal, 1979; Capone and Taylor, 1980; Capone, 1982; Kaspar, 1983; Howes *et al.*, 1984; Aziz and Nedwell, 1986; Boon *et al.*, 1986a, 1986b; Boon and Cain, 1988). They are also basic components of the food webs of these ecosystems (Tenore, 1976; Fenchel, 1978; Hanson and Tenore, 1981; Mann, 1988). Interactions between microbiota and their environment determine their function and activity. The parameters of an ecosystem with which the microbiota interact obviously influence the dynamics of microbiologically-mediated processes. In nature, such factors could be physico-chemical (eg. temperature, salinity, availability of nutrients for bacterial growth, availability of oxygen and other terminal electron acceptors), or biological (eg. bacterial abundance and/or activity, and the abundance of macro- and meiofauna).

The community structure of saltmarsh lagoons includes a mosaic of microhabitats, each with different properties that may influence bacterial abundance and activity. For example, seagrasses can have a marked effect on the chemical and microbiological characteristics of rhizosphere sediments through their production of detritus, exudation of organic matter, and release of oxygen (Moriarty and Boon, 1989). Similarly, some benthic invertebrates construct and maintain burrows, and thereby modify chemical and microbiological properties of their environment. Burrows form a particular microenvironment for bacteria, and may influence the biomass (Yingst and Rhoads, 1980; Branch and Pringle, 1987), composition, and physiological state of associated microbial communities (Dobbs and Guckert, 1988). Physicochemical and microbiological studies of saltmarsh environments should consider such spatial variability, as well as temporal variability due to seasonality.

Langebaan Lagoon is a saltmarsh ecosystem situated between 33°00' to 30°13'S and 17°57' to 18°08'E on the south west coast of South Africa (Fig. 1). This lagoon is a

partially enclosed marine system with no riverine input, and receives very little fresh water from precipitation or runoff. Consequently, salinity values are relatively constant and approximate those of seawater, or may become hypersaline in the summer months (Shannon and Stander, 1977). Physicochemical characteristics of seawater from Saldanha Bay and Langebaan Lagoon were reported by Shannon and Stander (1977), and their data for surface water are summarised in Table 1.

Tidal exchange of water occurs between Langebaan Lagoon and Saldanha Bay, which opens to the southern Benguela region of the Atlantic Ocean (Fig. 1). Upwelling occurs along this coast predominantly during the summer months when strong south-easterly winds and Eckmann forcing drive surface waters offshore, with the subsequent influx of nutrient-rich ($[NO_3^-]$ = ca.20μM) South Atlantic Central Water (Andrews and Hutchings, 1980). Thus, the availability of nutrients to bacteria and plants in this region increases during summer. Seasonal fluctuations of the concentrations of certain nutrients have also been measured inside Langebaan Lagoon (Henry *et al.*, 1977), indicating that coastal upwelling and tidal exchange influences this system too.

The volume of the lagoon is small when compared to that of Saldanha Bay, and at spring low tides approximately half the lagoon's water passes into the bay, leaving exposed expansive intertidal flats in the lagoon (Shannon and Stander, 1977). Tidal currents at the mouth of the lagoon may reach 1 m s^{-1} during spring tides. Interchange of water between Saldanha Bay and the open sea is limited, with turnover times for water in the bay estimated to be at least 20 days.

Langebaan Lagoon is a tidally controlled regime and water circulation is not significantly influenced by wind (Flemming, 1977). Sediments in the lagoon are composed mostly of SiO_2 (mean = 90%) and $CaCO_3$ (Willis *et al.*, 1977). Willis *et al.* also examined the trace element composition of Langebaan sediments, and reported values for V (ca. 5ppm) but did not report values for Mo (Table 2). According to Flemming (1977), the sediments of Langebaan Lagoon can be separated into four physiographic units: (i) tidal channels, (ii) subtidal flats and sandbanks, (iii) intertidal

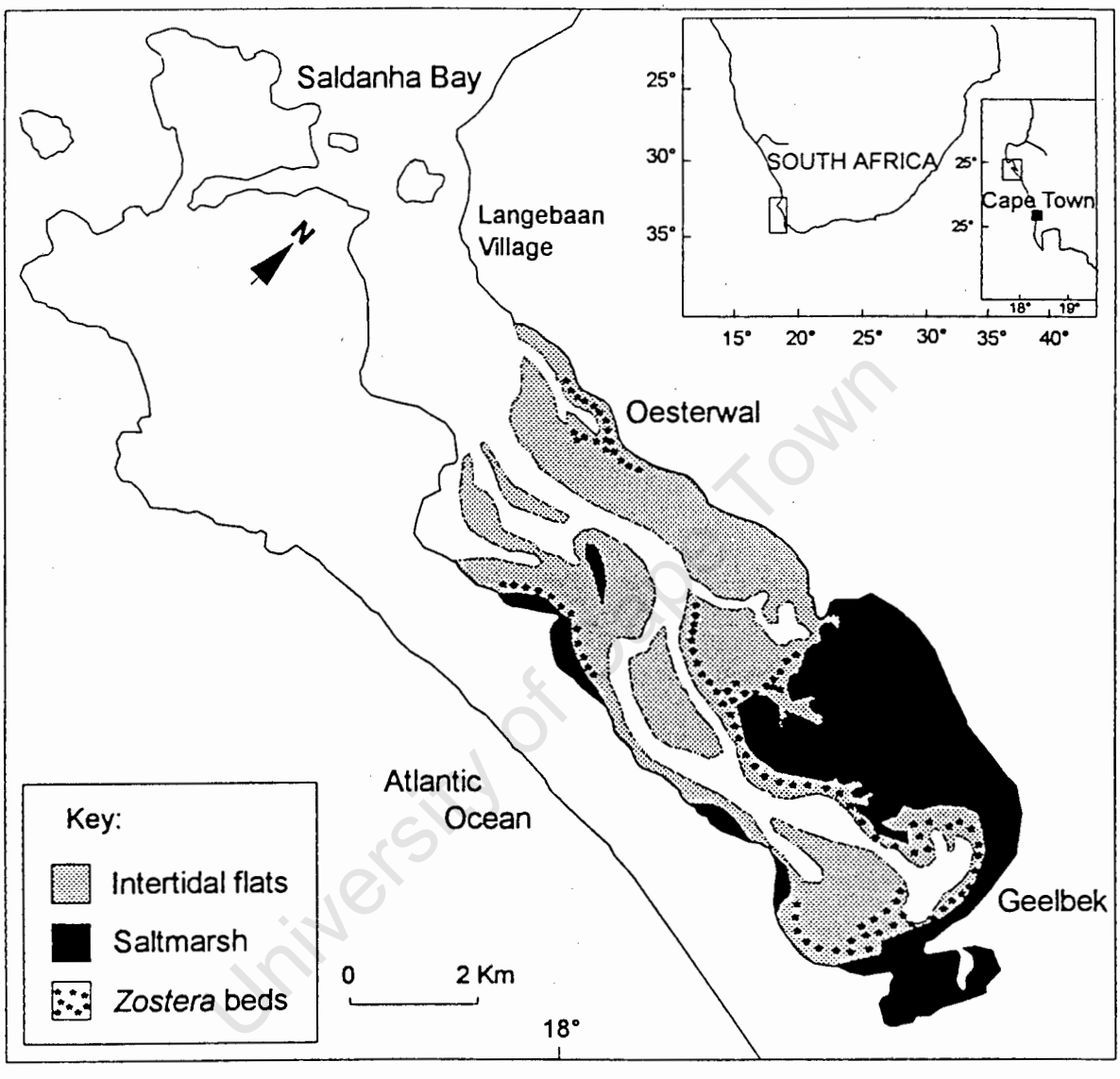

Figure 1. Location of Langebaan Lagoon, showing sampling sites, extent of intertidal mud- and sandflats, and the distribution of macrophytes.

TABLE 1. Physical and chemical properties of seawater from Saldanha Bay and Langebaan Lagoon (from Shannon and Stander, 1977)[a].

Parameter	Value[b]
Temperature (°C)	15.66 ± 1.86 (251)
Salinity (‰)	34.97 ± 0.25 (236)
Dissolved O_2 (ml l^{-1})	6.02 ± 1.30 (196)
pH	8.26 ± 0.14 (181)
Nitrate (μM)	5.60 ± 5.10 (190)
Phosphate (μM)	1.40 ± 1.00 (191)
Silica (μM)	19.20 ± 15.40 (192)
Total chlorophyll (μg l^{-1})	11.50 ± 9.50 (156)

[a] Measurements were reported for surface seawater samples taken from stations in Langebaan Lagoon and Saldanha Bay during April 1974 to October 1975.

[b] values are means ± SD for all samples, with number of replicates in parentheses.

flats, and (iv) the saltmarshes. These sedimentary units reflect the major energy levels of the tidal system, and distinguish between the sheltered southern reaches and sediments that are exposed to currents and limited wave action near the lagoon mouth (Fig. 1).

Two sites in the lagoon were selected for study: Oesterwal, near the lagoon mouth has sandflats which are exposed to tidal currents and limited wave action, while Geelbek, in the sheltered, southern reaches has anaerobic mudflats. Both sites have extensive intertidal flats. Seagrass (*Zostera capensis*) and saltmarsh macrophytes (including *Sarcocornia pillansii, Chenolea diffusa, Juncus krausii, Phragmites australis, Salicornia meyeriana, Sarcocornia perennis, Spartina maritima,* and *Typha capensis*) constitute the bulk of plant cover at both sites (Fig. 1). The deposit-feeding sandprawn *Callianassa kraussi* and the filter-feeding, detritivorous mudprawn *Upogebia africana* dominate the benthic macrofaunal biomass at Oesterwal and Geelbek respectively. At Oesterwal *C. kraussi* occurs at densities of approximately 175 prawns m^{-2}, whereas at Geelbek *U. africana* occurs at densities of about 110 prawns m^{-2} (Wynberg, 1991).

TABLE 2. Chemical composition of sediments from Langebaan Lagoon (from Willis *et al.*, 1977).

Elements	Oesterwal[a]	Geelbek[b]	Lagoon[c]
Major chemical elements[d]			
SiO_2	61.27	60.79	90.35
TiO_2	0.05	0.10	0.07
Al_2O_3	2.34	1.71	1.36
Fe_2O_3	0.30	0.63	0.33
MnO	0.001	0.003	0.001
MgO	0.31	3.14	0.21
CaO	18.20	12.08	3.03
Na_2O	0.59	1.11	0.36
K_2O	1.14	0.35	0.62
P_2O_5	0.32	0.21	0.13
S	0.19	0.49	0.09
H_2O	1.14	10.34	1.28
CO_2	14.28	9.48	2.39
Trace chemical elements[e]			
Ba	190	64	91
Sr	864	754	149
Rb	48	27	26
Y	10	8	6
Zr	36	101	62
Nb	<2	<2	<2
Zn	8	13	6
Cu	5	3	<1
Ni	<2	3	<2
Co	<3	<3	<3
Cr	15	19	13
V	4	10	5
Br	10	64	9

[a] Samples (n=6) collected at the mouth of the lagoon, near Oesterwal.
[b] Sample collected in the saltmarsh at the head of the lagoon, near Geelbek.
[c] Samples (n=16) collected in lagoon between Oesterwal and Geelbek.
[d] Value units are percentages of chemical composition.
[e] Value units are parts per million by mass.

Since macrofauna with different feeding strategies are likely to establish different microenvironments in their burrows, it is reasonable to anticipate that associated

microfloral characteristics may also differ. Macrophytic and macrofaunal affects on sediment characteristics were therefore examined by including different sedimentary microhabitats in a microscale comparison at each site.

Both sites were characterized in terms of environmental parameters important to bacterial activity and particularly to nitrogen fixation. This characterization is intended to be an introduction to the system and thus serves as a backdrop against which further physiological and process studies are considered in other chapters. More specifically, at each site, and for different microhabitats at each site, measurements were made of pH, temperature, salinity, redox potential, oxygen concentration, nutrient (NH_4^+, NO_3^-, NO_2^-, and PO_4^{2+}) concentrations, organic matter content, C:N ratio, colony forming units on different media, isolate characterizations, and enumeration of diazotrophs.

MATERIALS AND METHODS

General Sampling Procedures

Samples of sediment and water were collected from Oesterwal and Geelbek (Fig. 1). Water samples were collected either in plastic bottles or in sterile glass containers from < 1m below the water surface. To examine macrophytic and macrofaunal influence on chemical and microbiological properties of sediments, five sediment microhabitats were selected for study at each site (Fig. 2): (1) surface sediment to a depth of 5mm, away from the seagrass beds and between prawn burrow openings; (2) sediment to a depth of 5mm from within the *Zostera* beds; (3) sediment from the rims of prawn burrow openings; (4) sediment from burrow linings; and (5) sediment from a depth of 10cm below the surface (extracted with a PVC corer and subsampled using a 10ml syringe with the luer end removed). Samples were always collected on the morning of spring tides, at low tide when the sediment flats were exposed, and were either processed (see below) on site, or transported to the laboratory in an insulated container within 2 hours of collection. In addition to spatial comparisons of chemical and microbiological characteristics between sites and between microhabitats at each site, seasonal variation

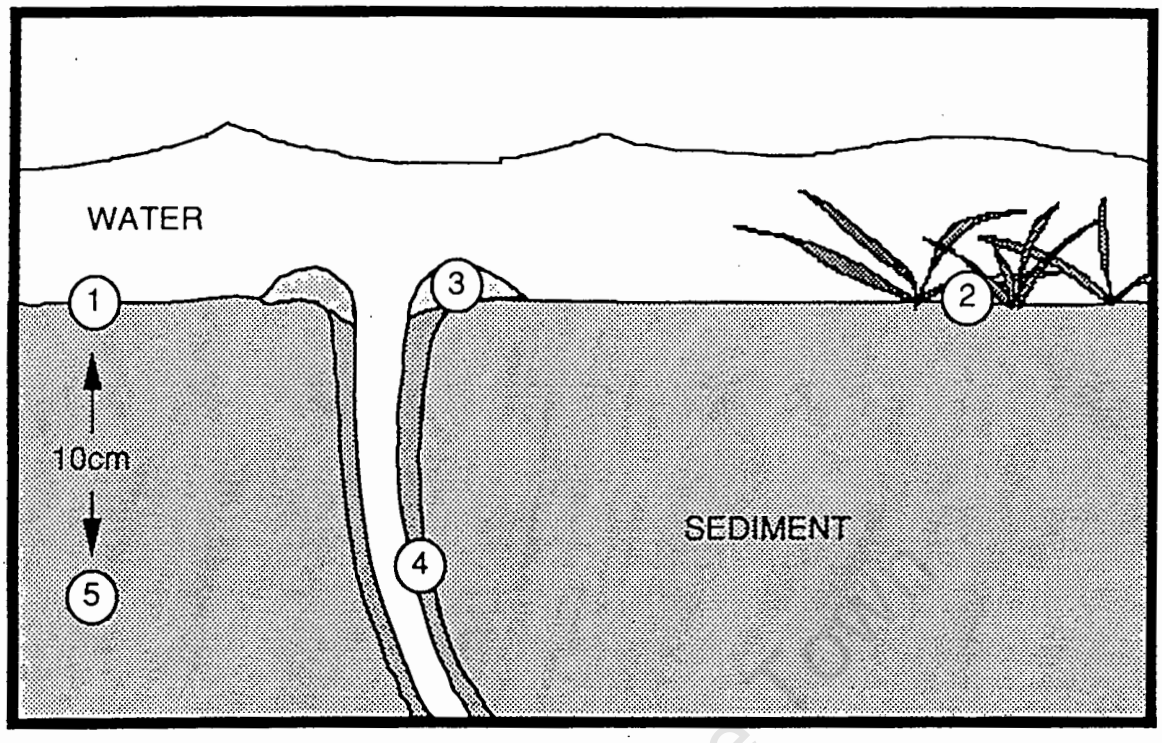

Figure 2. Location of different microhabitats: 1, surface sediment between burrows; 2, *Zostera* bed sediment; 3, burrow opening sediment; 4, burrow lining; 5, subsurface sediment from 10cm depth.

of some of these characteristics was also examined. Seasonal sampling was initiated during September 1989, and determinations were made in January 1990, April 1990, July 1990, October 1990, and in January 1991.

Measurement of Physical Parameters

Measurements of temperature, pH, salinity, dissolved oxygen, and redox potential were carried out in the field. With the exception of salinity, all measurements were made with meter instrumentation (Hanna Instruments; YSI Technology). Salinity was determined using a salinometer.

Determination of Nutrient Concentrations

Samples of interstitial water were collected from sediments by centrifugation for determinations of porewater ammonium. Extractable ammonium, nitrate, nitrite, and

phosphate were collected by mixing $5cm^3$ of freshly collected sediment with 10ml 1M KCl for 30 minutes, and then centrifuging the samples at 3000 x *g* for 10 minutes. Supernatants from centrifuged samples were collected for nutrient analyses. Concentrations of ammonium were determined according to the phenol-hypochlorite method of Koroleff (1983), while nitrate, nitrite, and phosphate were determined on a Technicon autoanalyzer using the methods of Armstrong *et al.* (1967) and Chan and Riley (1970).

Determination of Organic Content, Organic Carbon and Total Nitrogen

The particulate matter in replicate (n=3) samples of seawater (500 to 2000ml) from each site was collected by filtering these samples through pre-ashed Whatman GF/F filters in the field. Filters were then wrapped in tin foil and transported on ice back to the laboratory, where they were stored at -20°C. Filters were later oven-dried (60°C for 48 hours), and organic content determined by weight loss after combustion at 450°C for 4 hours. Total organic carbon and total nitrogen were determined on a Carlo-Erba CHN elemental analyzer.

Replicate (n=3) sediment samples were collected from each microhabitat at each site, transported to the laboratory on ice, and stored at -20°C. Samples were later oven-dried (60°C for 48 hours), and organic content determined by weight-loss after combustion at 450°C for 4-6 hours. Total organic carbon in sediments was determined by the dichromate oxidation method (Walkley and Black, 1934), while total nitrogen was determined by the phenate method (Nicholson, 1984).

Total Counts of Bacteria, Diatoms, Protozoa, and Meiofauna

Replicate (n=3) $1cm^3$ samples of sediment were collected using a 10ml syringe with the luer end removed. Sediment samples were placed into acid-washed plastic containers, fixed with 5ml 4% (w/v) formaldehyde in 0.2μm-filtered seawater, and stored in the dark at 4°C. Replicate (n=3) 100ml water samples were fixed with formalin to a final concentration of 4% (w/v) formaldehyde and stored as for sediment

samples. Enumeration of bacteria (by fluorescence microscopy using the DNA intercalating stain, 4'6-diamidino-2-phenylindole), diatoms, protozoa and meiofauna (by light microscopy using the tissue stain, Rose Bengal), in these samples is described by Harris (1993).

Plate Counts and Isolations of General Heterotrophic Bacteria

Appropriate dilutions of sediment and water samples were made in SM buffer (0.37M NaCl, 9mM KCl, 25mM $MgSO_4$, 23mM $MgCl_2$, 10mM Tris, pH 7.8) to be used as inocula producing plate counts of 30-300 colonies. Aliquots (100μl) of the appropriate dilutions were plated out on different media (see below) in duplicate, and incubated at 20°C under different conditions (see below) for up to 4 weeks. The dilution of samples and plating onto anaerobic media were carried out in an anaerobic chamber (Forma Scientific), before the dilutions were removed from the chamber for plating onto aerobic media.

Most Probable Number Technique and Enrichments

Nitrogen-fixing bacteria in sediment and water samples were enumerated by the most probable number (MPN) technique (Wright, 1984). Aliquots (100μl for sediment and 1ml for water samples) of appropriate dilutions (see above) were inoculated in triplicate into 10ml nitrogen-free glucose broth (NFGB, Tibbles and Rawlings, 1994) or nitrogen-deficient glucose broth (NDGB, Tibbles and Rawlings, 1994) in Hungate tubes. Tubes were sealed under N_2 in the anaerobic chamber, removed from the chamber and incubated in the dark at 20°C. Tubes were checked each day for bacterial growth according to visible turbidity. Acetylene (0.05 atm.) was added to those tubes in which turbidity occurred, and ethylene production was assayed by gas chromatography (see below) after a further 2-7 days' incubation. Those tubes in which ethylene was detected were scored as positive for the purposes of MPN calculations, and also served as enrichments for subsequent isolations of nitrogen-fixing bacteria (see Chapter 6). Plate counts of general heterotrophs were compared with estimates of viable counts obtained from the MPN technique. Medium for MPN tubes was low

strength seawater broth (LSSWB: 0.75g peptone, 0.15g yeast extract, 0.2g glucose, 1000ml 75% filtered seawater (see below)).

Characterization of Heterotrophic Isolates

Different strains of heterotrophic bacteria were isolated from sediment and water samples during winter (July) 1990 and summer (January) 1991. Plates from viable count experiments (see below for media) were examined for different colony morphologies. At least 29, and on average 35, different strains (by colony morphology) were isolated from water and each sediment microhabitat at each site. These isolates were maintained on medium strength seawater agar (MSSWA: 2.5g peptone, 1g yeast extract, 1g glucose, 1000ml 75% filtered seawater (see below)) or Marine Agar (Difco) and characterized according to standard biochemical tests (Smibert and Krieg, 1981).

Acetylene Reduction Assays

Gas samples (100μl) were withdrawn from the headspace with a gas-tight syringe at appropriate time intervals for determination of ethylene and ethane content in a Hewlett Packard 5880A gas chromatograph. Gases were separated on a Porapak N column (3m x 3mm) at 80°C, with nitrogen as the carrier gas at a flow rate of 20ml min^{-1}. The injector and detector temperatures were 200°C and 250°C respectively, and the flow rates of hydrogen and air were 30ml min^{-1} and 400ml min^{-1} respectively. Peak areas were integrated on a Hewlett Packard 5880A Series integrator and expressed as nmol product by comparison with peak area standard curves of known quantity.

Statistical Analyses

Analysis of variance (ANOVA) was used where Bartlett's test for homogeneity of variance indicated homoscedasticity. Statistical comparisons of means were carried out either by the Student's t-test or the Wilcoxon paired t-test by rank. Significance levels for all tests were set at 95%.

RESULTS

Physical Parameters

The temperature of exposed surface sediment and water at Oesterwal and Geelbek was measured seasonally from July 1989 to January 1991, during the morning (06h00 - 12h00) of spring tides. There was a difference of at least 10°C between seasons for water and sediment at both sites. Temperatures measured during the mornings ranged from about 11.5°C in winter to 25.5°C in summer (Fig. 3), whereas on some summer afternoons temperatures > 28°C were measured for exposed surface sediments at Geelbek. The mean annual temperatures at Geelbek (sediment, 19.7°C; water, 19.4°C) and Oesterwal (sediment, 19.5°C; water, 17.6°C) were not significantly different

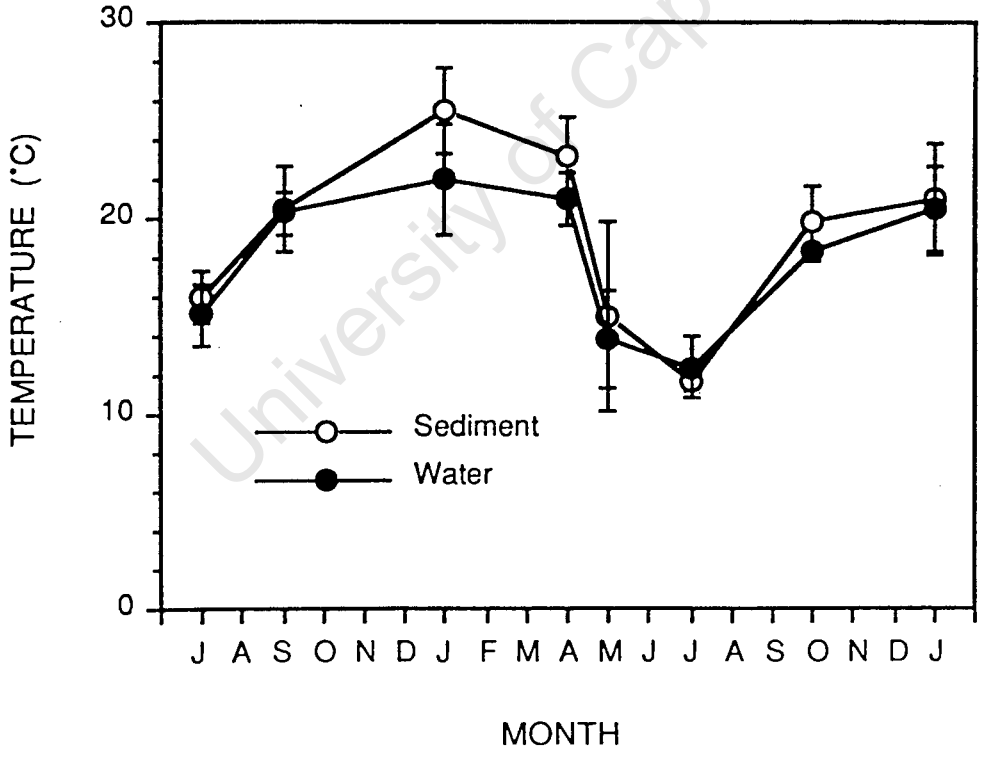

Figure 3. Seasonal fluctuations of temperature of surface sediment and water from Langebaan Lagoon. Values represent means of measurements for Oesterwal and Geelbek, during July 1989 - January 1991.

TABLE 3. pH, salinity, dissolved oxygen concentration, and redox potential of different habitats at Oesterwal and Geelbek.

Habitat	pH[a]	Salinity (‰)[b]	O_2 (ml l^{-1})[c]	Redox (mV)[c]
Oesterwal				
Surface sediment	8.24 ± 0.51	36.33 ± 1.53	2.55 ± 1.02	-25.5 ± 43.8
Zostera sediment	8.78 ± 0.24	n.d.	2.23 ± 1.31	-92.0 ± 46.7
10cm subsurf.	7.62 ± 0.13	n.d.	1.10 ± 0.98	-29.3 ± 14.1
Burrow water	7.77 ± 0.15	n.d.	3.50 ± 0.50	n.d.
Water column	7.84 ± 0.05	35.33 ± 0.58	9.87 ± 0.21	n.d.
Geelbek				
Surface sediment	8.76 ± 0.04	37.67 ± 6.43	0.10 ± 0.00	-65.3 ± 24.5
Zostera sediment	8.17 ± 0.47	n.d.	0.20 ± 0.20	-147.0 ± 39.7
10cm subsurf.	7.87 ± 0.36	n.d.	0.10 ± 0.00	-79.0 ± 37.3
Burrow water	8.00 ± 0.33	n.d.	3.50 ± 1.35	n.d.
Water column	8.37 ± 0.03	36.00 ± 3.61	10.17 ± 0.15	n.d.

[a] To determine pH, dissolved O_2, and redox potential, probes of respective meters were pushed into the sediment to depths of either 1cm (surface and *Zostera* sediments) or 10cm (subsurface sediment) and allowed to equilibrate before readings were taken. pH values represent means ± SD of seasonal determinations (n=4).

[b] Salinities were determined using a salinometer; values represent means ± SD of seasonal determinations (n=7).

[c] Redox potential and dissolved O_2 concentration were determined during November 1992 and during March 1993. Values represent means ± SD (n=4, except for redox measurements in Geelbek *Zostera* sediment where n=11).

n.d., not determined.

($p > 0.05$). Diel variations in temperature were measured at Oesterwal on three occasions: once during winter and twice during late spring/early summer. There was a 4-6°C diel variation in the temperatures of water, exposed surface sediment, and subsurface sediment on these occasions. The pH, redox potential, and concentration of dissolved oxygen for different habitats showed no seasonal pattern. However, salinity tended towards higher values (maximum, 45‰ measured for interstitial water from surface sediment at Geelbek) during summer, due to evaporation. Dissolved O_2 and redox potential were lower in Geelbek sediments than those at Oesterwal (Table 3).

Nutrient Concentrations

The concentration of ammonium dissolved in interstial water (porewater ammonium) from different sediments was determined on two occasions: once in spring 1992 and once in late summer 1993. Concentrations were similar on both occasions, and thus average values are presented (Table 4). Porewater ammonium concentrations were higher for Geelbek than for Oesterwal. The highest concentration of porewater ammonium was measured in *Zostera* bed sediment from Geelbek. At Oesterwal highest porewater ammonium concentrations were measured in *Zostera* bed sediment, while the concentration of ammonium in burrow water was also high. Difficulties were encountered in collecting interstitial water from burrow lining and burrow head sediments, and so burrow water was assumed to reflect conditions in sediments of the

TABLE 4. Ammonium concentrations in interstitial and burrow waters[a].

Source	NH_4^+ concentration (μM) Oesterwal	Geelbek
Surface sediment	0.59 ± 0.76	1.25 ± 3.12
Zostera bed sediment	2.68 ± 2.32	7.18 ± 15.8
Burrow water	2.38 ± 6.95	1.09 ± 2.74
10cm depth sediment	0.41 ± 0.81	4.51 ± 13.2

[a] values represent means ± SD of replicates (n=12) measured during spring and late summer.

burrow environment. Concentrations of nutrients in KCl-extractions of sediments were examined three times during late spring/early summer 1993. Highest concentrations of KCl-extracted ammonium were measured in subsurface sediments (10cm depth) and *Zostera* bed sediment at Geelbek (Table 5). At Oesterwal, highest concentrations of KCl-extracted ammonium occurred in burrow-associated and subsurface sediments. Concentrations of KCl-extracted nitrate reflected the spatial pattern of ammonium, except that nitrate concentrations in *Zostera* bed sediment at Geelbek were not high (Table 5). Conversely, nitrite and phosphate concentrations at each site were highest

TABLE 5. Concentrations of ammonium, nitrate, nitrite, and phosphate in sediments and water from Langebaan Lagoon[a].

Habitat[a]	Ammonium	Nitrate	Nitrite	Phosphate[b]
Oesterwal				
Surface sediment	< 0.1	6.1 ± 1.0	1.1 ± 0.1	44.0
Zostera sediment	0.5 ± 0.9	2.4 ± 0.5	1.6 ± 0.3	116.0
Burrow opening	5.8 ± 7.0	15.0 ± 1.9	1.0 ± 0.1	62.0
Burrow lining	n.d.	58.7 ± 3.9	0.4 ± 0.1	110.0
10cm depth	39.1 ± 24.5	13.6 ± 0.4	0.5 ± 0.3	18.0
Water	< 0.1	0.9 ± 0.0	0.1 ± 0.0	3.0 ± 0.9
Geelbek				
Surface sediment	< 0.1	4.4 ± 1.2	0.6 ± 0.2	n.d.
Zostera sediment	61.7 ± 11.3	4.9 ± 1.2	1.4 ± 0.2	436.0
Burrow opening	< 0.1	4.5 ± 0.6	0.7 ± 0.1	266.0
Burrow lining	n.d.	59.8 ± 0.0	1.1 ± 0.0	128.0
10cm depth	87.2 ± 52.0	16.8 ± 5.7	0.3 ± 0.1	110.0
Water	< 0.1	0.4 ± 0.1	0.1 ± 0.0	3.9 ± 0.9

[a] Samples were collected on three occasions during ebbing tides, late October, 1993. Phosphate analyses for sediments were carried out on one set of samples only.

[b] Sediment nutrients were extracted with 1M KCl.

[c] Units for sediment nutrients in nmol cm^{-3}, and for water nutrients in μM.

n.d. Not determined.

in *Zostera* bed sediment. Ammonium, nitrate and nitrite concentrations were not significantly different between Oesterwal and Geelbek. However, phosphate concentrations were significantly higher ($p < 0.05$) at Geelbek than at Oesterwal (Table 5). Insufficient data were available to analyse temporal/seasonal variations in nutrient concentrations.

Composition of Organic Matter Content of Sediments and Water

The organic content of sediments and water was examined seasonally (October, spring; January, summer; April, autumn; July, winter) for organic carbon and total nitrogen content, and C:N ratio. Strong seasonality was not evident in these data. Therefore,

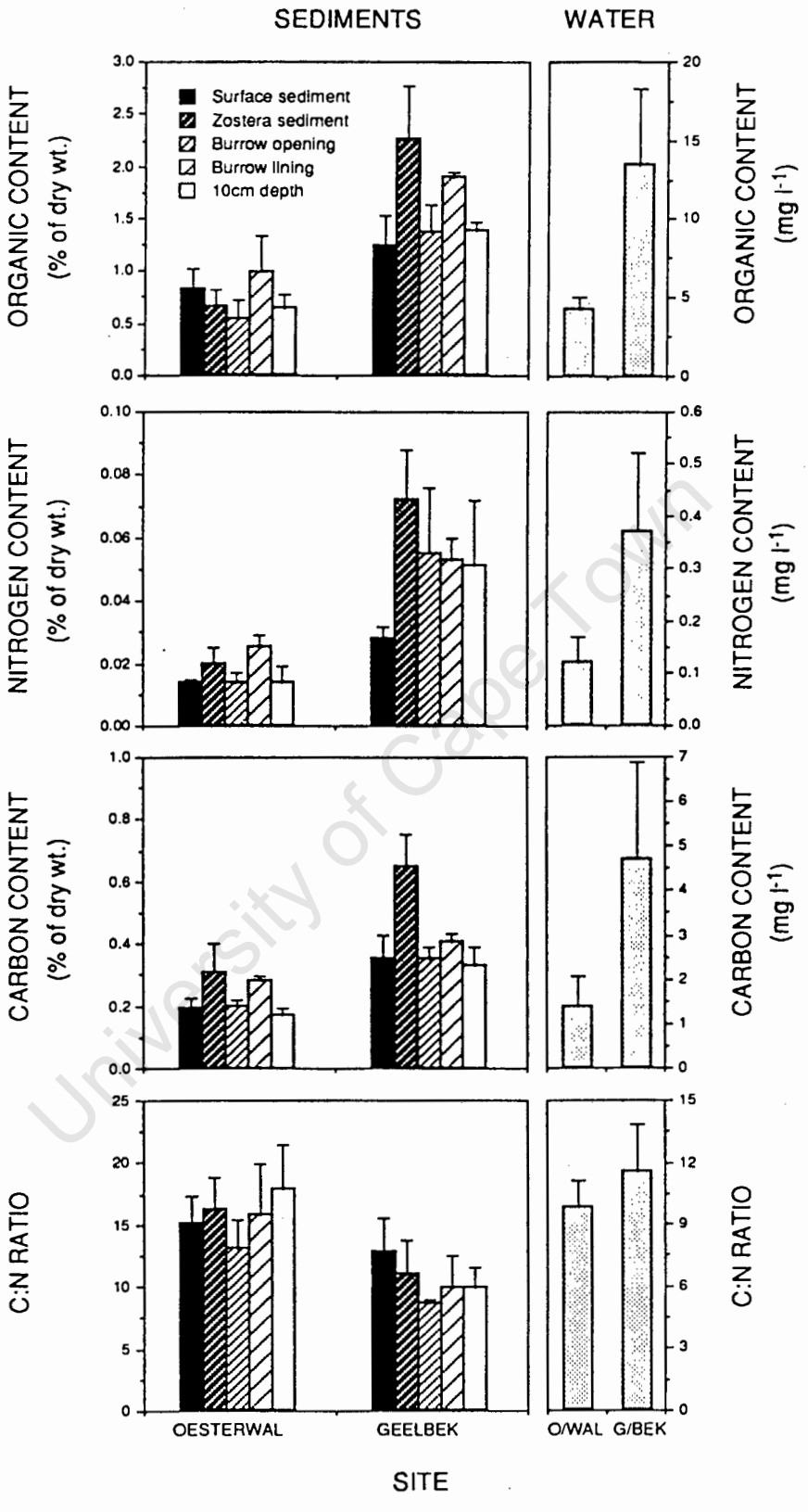

Figure 4. Organic content, total nitrogen content, organic carbon content, and the C:N ratio of organic matter in sediments and water from Langebaan Lagoon. Values represent annual means ± SE of four seasonal determinations. Each seasonal determination was the mean of triplicate samples collected on one day.

annual means ± SE of seasonal data for different habitats are presented in Fig. 4. The organic content of all sediments from Geelbek was significantly higher ($p<0.05$) than that from Oesterwal. The organic content of water from Geelbek was also higher than that from Oesterwal, although this comparison was not significant ($p>0.05$) due to large variation in the data for Geelbek. At Oesterwal sediment from *Callianassa* burrow linings had the highest organic content (9.82 ± 3.60 mg g^{-1} dry sed.), whereas at Geelbek sediment from *Zostera* beds had the highest organic content (22.65 ± 2.89 mg g^{-1} dry sed.) (Fig. 4).

Total nitrogen contents of all sediments from Geelbek were higher than those from Oesterwal (significant for surface sediment, burrow lining, and *Zostera* bed sediment, $p<0.05$) (Fig. 4). Water from Geelbek also had a higher nitrogen content than that from Oesterwal (not significant, $p>0.05$). Comparison of nitrogen content between microhabitats at each site reflected the pattern of organics data, ie. highest nitrogen contents were recorded for burrow lining (0.025 ± 0.004 % of dry wt.) and *Zostera* bed sediment (0.072 ± 0.016 % of dry wt.) at Oesterwal and Geelbek respectively.

Organic carbon contents of all sediments from Geelbek were higher than those from Oesterwal (significant for surface sediment, burrow lining, *Zostera* bed sediment, and 10cm subsurface sediment, $p<0.05$) (Fig. 4). Geelbek water also had a higher organic carbon content than that from Oesterwal, although this comparison was not statistically significant ($p>0.05$). Comparison of organic carbon content between sediment microhabitats at each site reflected the patterns already discussed for organic content and total nitrogen (see above).

Sediments from Geelbek had lower C:N ratios than sediments from Oesterwal, indicating that organic material in Geelbek sediments had a higher nitrogen content relative to organic carbon, than Oesterwal sediments (Fig. 4). In contrast, organic material in Oesterwal water had a lower C:N ratio than Geelbek water, although this comparison was not statistically significant ($p>0.05$).

Harris (1993) quantified the biomass of diatoms, bacteria, protozoa, and meiofauna in sediments and water from Oesterwal and Geelbek collected concurrently with the samples analysed here for organic carbon and total nitrogen. These data were used to calculate the annual mean contribution of various biota and detritus to the organic carbon and total nitrogen contents of sediments and water (Table 6). Detritus constituted the bulk of the organic material in both sediments and water from Oesterwal and Geelbek (Table 6). At Oesterwal, detritus constituted on average 88% of the organic carbon in water and sediments, whereas at Geelbek detritus constituted on average 75% and 99% of the organic carbon in sediments and water respectively. In terms of nitrogen, detritus constituted on average 77% and 79% of the total nitrogen content in Oesterwal sediments and water respectively, whereas at Geelbek 65% and 81% of the nitrogen in sediments and water respectively was detrital. Although the biomass of bacteria, protozoa and meiofauna at Geelbek was higher than at Oesterwal (Harris, 1993), these biota constituted similar proportions of the organic carbon and total nitrogen contents of sediments and water at both sites (Table 6). Diatoms constituted a greater proportion of organic carbon and total nitrogen in sediments from Geelbek than in sediments from Oesterwal. This reflects their greater numbers at Geelbek (Harris, 1993).

Plate Counts and Isolations of General Heterotrophic Bacteria

Preliminary studies sought to optimize media composition for the growth of bacteria from Langebaan Lagoon. Different compositions of media were tested in isolations of aerobic and anaerobic heterotrophs and scored according to colony forming units (CFU) on agar plates. Trials indicated that unpolluted, natural seawater (filtered through Whatman No1 filter paper and diluted to 75% seawater, 25% distilled water by volume) was a better base for media than artificial seawater.

Numbers of CFU on aerobically incubated plates decreased with increasing richness of the medium. When appropriate dilutions of lagoonal sediments and water were plated onto different media, numbers of CFU on full strength seawater agar (FSSWA: 5g

TABLE 6. Biotic contributions to total organic nitrogen and carbon pools in sediments and water[a].

Habitat	Detritus	Diatoms	Bacteria	Protozoa	Meiofauna
CARBON					
Oesterwal					
Surface sed.	89	3	5	2	1
Zostera sed.	81	3	12	3	1
Burrow open.	90	3	5	3	0
Burrow lining	92	4	4	0	0
10cm depth	87	6	6	<1	<1
Water	88	<1	6	5	<1
Geelbek					
Surface sed.	74	20	6	4	0
Zostera sed.	72	13	13	1	1
Burrow open.	75	14	7	4	0
Burrow lining	79	13	6	2	0
10cm depth	74	14	10	1	1
Water	99	4	3	4	0
NITROGEN					
Oesterwal					
Surface sed.	75	7	7	7	3
Zostera sed.	64	7	18	9	2
Burrow open.	77	6	7	8	2
Burrow lining	86	7	5	2	0
10cm depth	81	9	6	2	2
Water	79	2	7	13	0
Geelbek					
Surface sed.	45	33	9	12	1
Zostera sed.	65	19	12	3	1
Burrow open.	67	19	6	7	1
Burrow lining	76	16	5	2	1
10cm depth	74	15	7	3	1
Water	81	6	3	10	0

[a] Values represent annual mean percentages of organic carbon or total nitrogen, calculated using data from each of the four seasons. Each seasonal value is based on triplicate samples on one day. Abundance data and organic carbon and nitrogen contents for different biota were derived from Harris (1993). Some variability due to the methods used to determine these values is reflected where values for certain microhabitats do not total to exactly 100%.

peptone, 1g yeast extract, 15g agar, 1000ml 75% filtered seawater (see above)) or Marine Agar (MA, Difco) were lower than low strength seawater agar. Highest numbers of CFU were recorded on ultra low strength seawater agar (ULSSWA: 10mg yeast extract, 15g agar, 1000ml 75% filtered seawater (see above)). However, colony growth on the latter medium was slow, requiring 2-4 weeks' incubation to develop visible pin-head sized colonies, and most colonies on ULSSWA could not be subcultured. In contrast, most colonies isolated on LSSWA could be subcultured and viability could be maintained. Hence, two media were selected for aerobic plate counts and isolations: LSSWA and ULSSWA. Growth (CFU) under anaerobic conditions was optimal on anaerobic LSSWA (AnLSSWA: LSSWA with 10mM $NaHCO_3$); anaerobic growth on ULSSWA was not detectable after 4 weeks' incubation (results not shown).

Isolated strains grew better and were less likely to lose viability when subcultured onto a slightly richer medium than the isolation media LSSWA and ULSSWA. Hence, pure cultures of general heterotrophic bacteria were maintained on MSSWA or on MA, depending on the specific requirements of individual strains.

General heterotrophic bacteria were isolated from sediments and water seasonally. Counts of CFU (LSSWA) from sediments and water samples followed no consistent seasonal trend (Fig. 5). Seasonal fluctuations of CFU did not reflect seasonal fluctuations in the abundance of bacteria in sediments. However, for water samples, seasonal fluctuations of CFU and bacterial numbers did correspond at both sites.

Annual means derived from four seasonal determinations of CFU (LSSWA) and bacterial abundance are shown in Fig. 6. There was a greater abundance of bacteria in all sediments and water from Geelbek than those from Oesterwal (Harris, 1993). However, CFU isolated from sediments did not reflect this trend; a larger number of CFU were isolated from Oesterwal sediments on average than from Geelbek sediments. Furthermore, recovery of CFU from different sedimentary microhabitats at each site did not reflect the bacterial abundance between microhabitats. More CFU were recovered from surface sediment between burrows and *Zostera* bed sediment

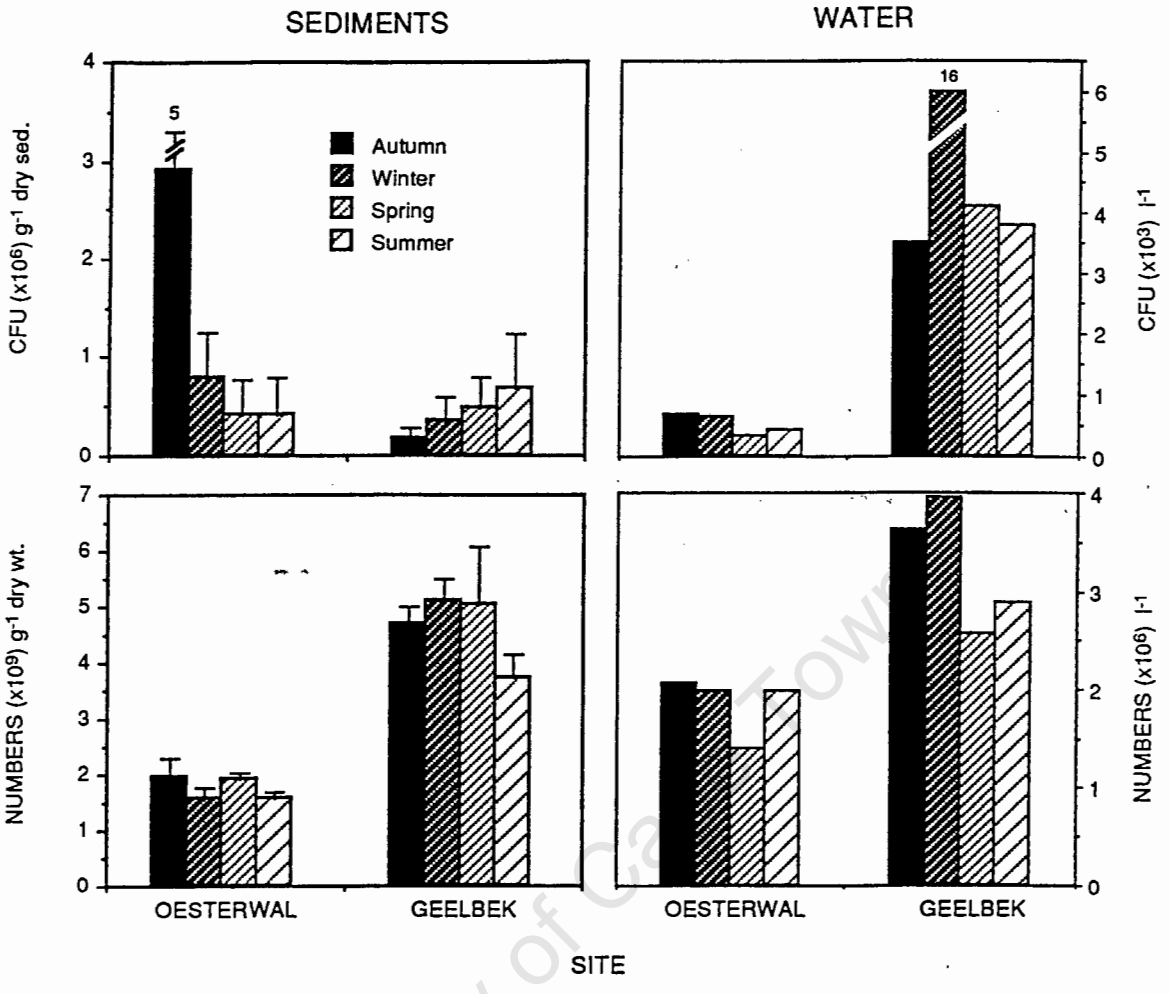

Figure 5. Seasonal variation in colony forming units and bacterial abundance. Values for sediments represent means ± SE of all microhabitats. Values for water have no error bars as only one type of sample was taken. Data for bacterial abundance after Harris (1993).

than from subsurface sediments and burrow openings. Bacterial abundance reflected the trends of CFU isolated from water samples (Fig. 6).

The proportions of bacterial abundance recovered on different heterotrophic media used in this study are presented in Table 7. Generally, less than 1% of the total bacterial numbers in sediment samples were recovered as CFU on different heterotrophic media. Up to 4% of the total count was recovered as CFU from water samples. In this regard, the effectiveness of media used for plating ranked as follows: ULSSWA > LSSWA > AnO_2-LSSWA. The MPN technique recorded between 2.5% and 32% of the bacterial

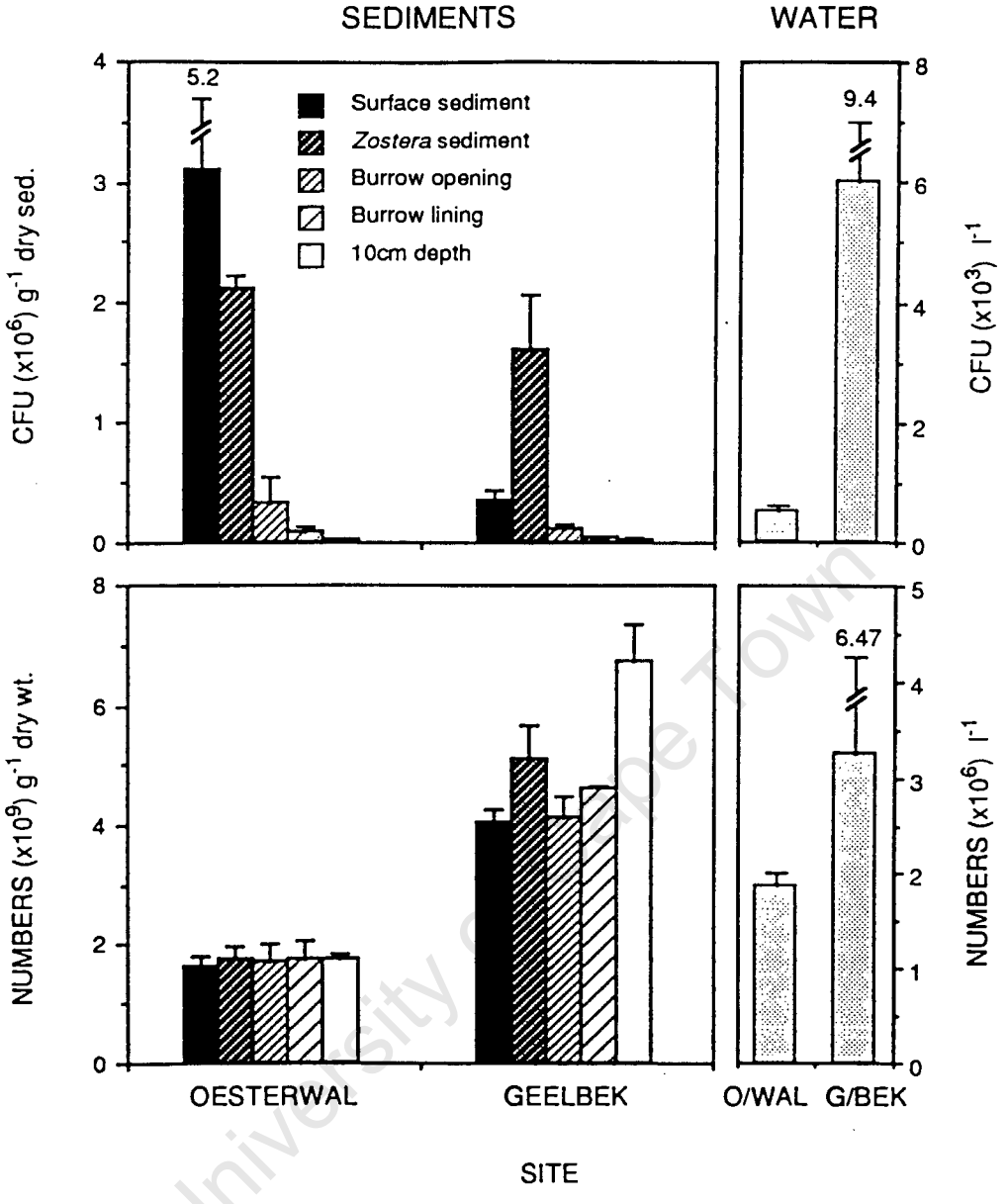

Figure 6. Annual means of CFU and bacterial abundance for sediments and water from Langebaan Lagoon. Values represent means ± SE of four seasonal determinations. Each seasonal determination was the mean of duplicate samples collected from each microhabitat. Data for bacterial abundance after Harris (1993).

abundance in sediment and water samples, indicating that MPN may provide more realistic estimates of the active (viable) proportion of total cell numbers (Table 7). The purpose of plate counts in the present study was 2-fold: (1) to examine the plateable population for possible seasonal fluctuations in numbers of CFU, and (2) to isolate a 'spread' of strains for biochemical characterization of the platable population and to examine possible seasonal changes in population physiologies.

TABLE 7. Percentages of total bacterial cell numbers grown on different heterotrophic media[a].

Medium	Type	Oesterwal		Geelbek	
		Sediments	Water	Sediments	Water
ULSSWA	CFU	0.746	0.396	0.559	4.081
LSSWA	CFU	0.064	0.028	0.006	0.168
AnO_2-LSSWA	CFU	0.009	0.011	0.003	0.030
LSSWB	MPN	2.574	15.063	30.549	32.855

[a] Values are the ratio (x 100%) of 'viable' counts (by CFU or MPN methods) to total count (by microscopy), and are therefore unitless. Values represent annual averages (using data for four seasons) for all sediment microhabitats at each site.

Strains isolated on LSSWA were examined for selected characteristics: (A) the ability to produce acid from glucose which indicates the proportion of isolates with a fermentative metabolism; (B) grow at 37°C relative to growth at 20°C indicates the proportion of strains with a growth preference for higher temperatures; (C) the ability of aerobic isolates to grow anaerobically would provide information about the proportion of the isolates that are facultative (none of the isolates which survived subculturing in this study were strict anaerobes); (D) agarase production was used as an indicator of the ability of isolates to produce complex extracellular enzymes; (E) urease production, (F) nitrate reduction, (G) denitrification, and (H) nitrogen fixation relate to the isolates' use of nitrogen, which is central to this thesis. These characteristics for strains isolated during winter and summer are presented in Tables 8 and 9 respectively. The occurrence of these characteristics was fairly evenly distributed amongst strains isolated from different microhabitats, with little indication of any strong selectivity by particular microhabitats for certain characteristics. Comparison of data between sites also indicated little selectivity for characteristics on a spatial scale. Comparison of data on a temporal scale (ie. seasonally) showed significant ($p < 0.05$) summer increases in the proportions of isolates that were able to produce acid from glucose, reduce nitrate to nitrite, denitrify, and fix nitrogen.

TABLE 8. Characteristics of heterotrophic bacteria isolated from lagoonal sediments and water during winter (July 1990).

Habitat	n	Percentage of isolates positive for [a] : A	B	C	D	E	F	G	H
Oesterwal									
Surface sed.	40	12.5	17.5	55.0	7.5	10.0	27.5	2.5	0
Zostera sed.	39	12.8	17.9	25.6	2.6	2.6	15.4	0	0
Burrow opening	31	22.6	12.9	38.7	3.2	6.5	16.1	0	0
Burrow lining	40	7.5	32.5	62.5	7.5	7.5	50.0	0	0
10cm subsurf.	33	24.2	54.5	60.6	0	3.0	33.3	3.0	0
Water	40	2.5	10.0	47.5	5.0	10.0	50.0	0	0
Average		13.7	24.2	48.3	4.3	6.6	32.1	0.9	0
Geelbek									
Surface sed.	40	7.5	5.0	20.0	0	10.0	17.5	2.5	0
Zostera sed.	33	30.3	15.2	45.5	0	15.2	9.1	0	0
Burrow opening	32	12.5	21.9	25.0	0	6.3	21.9	0	0
Burrow lining	29	27.6	24.1	31.0	3.4	13.8	3.4	3.4	0
10cm subsurf.	32	25.0	28.1	53.1	0	18.8	15.6	6.3	0
Water	30	20.0	33.3	60.0	0	13.3	3.3	0	0
Average		20.5	21.3	39.1	0.6	12.9	11.8	2.0	0
Average overall		17.1	22.1	43.7	2.4	9.8	21.9	1.5	0

[a] Characteristic A, acid from glucose; B, grow at 37°C relative to growth at 20°C; C, anaerobic growth; D, agarase positive; E, urease positive; F, nitrate reduction to nitrite; G, denitrification; H, nitrogen fixation

TABLE 9. Characteristics of heterotrophic bacteria isolated from lagoonal sediments and water during summer (January 1991).

Habitat	n	Percentage of isolates positive for [a]: A	B	C	D	E	F	G	H
Oesterwal									
Surface sed.	40	20.0	20.0	35.0	0	12.5	30.0	5.0	2.5
Zostera sed.	32	28.0	34.3	37.5	12.5	15.6	40.6	34.4	18.8
Burrow opening	40	45.0	12.5	67.5	0	17.5	45.0	0	0
Burrow lining	40	37.5	25.0	60.0	12.5	2.5	57.5	0	0
10cm subsurf.	40	65.0	27.5	72.5	5.0	15.0	62.5	5.0	2.5
Water	32	34.4	31.3	25.0	3.1	9.4	50.0	0	3.1
Average		38.3	25.1	49.6	5.5	12.1	47.6	7.4	4.5
Geelbek									
Surface sed.	40	45.0	75.0	37.5	0	12.5	30.0	5.0	0
Zostera sed.	33	37.5	43.8	28.1	9.4	21.9	43.8	12.5	3.1
Burrow opening	32	46.9	9.4	59.4	0	6.3	34.4	0	0
Burrow lining	29	55.0	7.5	65.0	0	5.0	27.5	12.5	0
10cm subsurf.	32	62.5	31.3	68.8	6.3	12.5	40.6	9.4	0
Water	30	56.3	31.3	28.1	0	15.6	40.6	3.1	0
Average		50.5	33.1	47.8	2.6	12.3	36.2	7.1	0.5
Average overall		**44.4**	29.1	48.7	4.1	12.2	**41.9**	**7.2**	**2.5**

[a] Characteristic A, acid from glucose; B, grow at 37°C relative to growth at 20°C; C, anaerobic growth; D, agarase positive; E, urease positive ; F, nitrate reduction to nitrite; G, denitrification; H, nitrogen fixation. Figures in bold type signify overall means for summer isolates that were significantly ($p<0.05$) higher than the corresponding values for winter isolates (Table 8), according to the Wilcoxon paired t-test by rank.

Isolation and Enumeration of Nitrogen-Fixing Bacteria

Extensive efforts at selecting for nitrogen-fixing bacteria on agar-containing media were unsuccessful. Aliquots (100μl) of appropriate dilutions were plated onto NFGA and NDGA (Tibbles and Rawlings, 1994), and incubated either anaerobically or aerobically until visible pin-head sized colonies had developed (usually 2-4 weeks). Strains which survived subculturing onto NFGA, NDGA, or anaerobic LSSWA were tested for acetylene reduction, which is indicative of nitrogenase activity (Hardy *et al.*, 1968). None of the strains isolated directly onto nitrogen-free or nitrogen-deficient plates were found to reduce acetylene. Moreover, substitution of the glucose component of these media with mannitol, sucrose, succinate, or citrate, or substitution of the artificial seawater base with natural seawater (aged for > 6 months before filtering as above), did not improve the effectiveness of these media at isolating nitrogen-fixing bacteria. Incubation under a gaseous phase purified of nitrogenous compounds by passage through concentrated sulphuric acid also failed to improve selection for nitrogen-fixing bacteria. Growth of non-acetylene reducing strains on plates was therefore ascribed to the probable scavenging of contaminating nitrogenous material in the bacteriological agar (Biolab; Merck) component of these media.

Attempts to isolate and enumerate nitrogen-fixing bacteria subsequently utilised enrichment and MPN techniques. Table 10 shows the numbers of heterotrophic nitrogen fixers in sediment and water samples as estimated by MPN. Surface sediment and *Zostera* bed sediment were examined since preliminary studies had indicated that acetylene reduction activity was highest in these microhabitats. Water was also examined for comparison with the sediment microhabitats. Higher numbers of diazotrophs were detected in summer than in winter. Sediments contained more diazotrophs than water by volume. Highest densities of diazotrophs were detected in *Zostera* bed sediment. MPN tubes which were positive for nitrogenase activity (acetylene reduction) were subsequently used as enrichments for isolating strains, which were added to a strain collection for characterisation studies (see Chapter 6).

TABLE 10. Seasonal variation of abundance of heterotrophic, nitrogen-fixing bacteria in sediments and water from Langebaan Lagoon.

Habitat	Autumn	Winter	Spring	Summer
Oesterwal				
Surface sed.	$< 5 \times 10^2$	$< 5 \times 10^2$	$< 5 \times 10^2$	$< 5 \times 10^2$
Zostera sed.	$< 5 \times 10^2$	$< 5 \times 10^2$	$< 5 \times 10^2$	1.1×10^4
Water	$< 5 \times 10^0$	$< 5 \times 10^0$	$< 5 \times 10^0$	4.0×10^2
Geelbek				
Surface sed.	$< 5 \times 10^2$	$< 5 \times 10^2$	$< 5 \times 10^2$	$< 5 \times 10^2$
Zostera sed.	$< 5 \times 10^2$	$< 5 \times 10^2$	4.0×10^4	1.5×10^5
Water	4.0×10^2	$< 5 \times 10^0$	$< 5 \times 10^0$	$< 5 \times 10^0$

[a] Units for numbers in sediments are cells g^{-1} dry sed., and for numbers in water are cells l^{-1}.

DISCUSSION

Measurements of physico-chemical parameters in relation to bacterial activity in Langebaan Lagoon were made to serve as a backdrop against which further physiological and process studies are considered in the following chapters. The temperatures of sediments and the overlying water column from Langebaan Lagoon showed differences of at least 10°C between seasons, and diel fluctuations of 4-6°C. Temperature fluctuations such as these are likely to influence bacterial activity in a temperate saltmarsh ecosystem, as temperature influences bacterial metabolism in general (Gottschalk, 1986). Since the two study sites, Oesterwal and Geelbek, are at opposite ends of a partially enclosed system (ca. 15km apart), thermal variability may be significant in a comparison of these sites. When measured at the same time during the day, temperatures at each site were often different (by 0.5°C - 7.0°C), but no evidence was obtained to indicate that one site was consistently warmer than the other. Seasonal variation in bacterial production and nitrogen fixation, and the effects of temperature on nitrogenase activity are addressed in Chapters 3, 5 and 6 respectively.

Seasonal fluctuations were not apparent in O_2 concentration, redox potential, or pH data for sediments and water, but O_2 concentration and redox potential did indicate that sediments at Geelbek were more anaerobic than those at Oesterwal. This comparison

corresponds with other characteristics of the two sites such as the higher organic content of Geelbek sediments, which is expected to enhance anaerobiosis, and the location of these sites in the lagoon with respect to features of the water circulation. Such features include tidal current velocites and wave action, which have more impact at Oesterwal than at Geelbek.

The major pools of nitrogen in sediments include organic N, ammonium dissolved in the interstitial water (porewater ammonium), ammonium adsorbed to sediment particles (exchangeable ammonium), dissolved nitrate, and dinitrogen. The mineralization of organic matter is the principle source of ammonium. Newly produced ammonium in sediments has the following possible fates: it may be incorporated into microbiota or taken up by macrophytes, it may diffuse from the sediments into the overlying water, it may be oxidized to NO_3^-, or it may enter the general sediment NH_4^+ pool. Blackburn and Henriksen (1983) calculated that 44-66% of the net production of nitrogen (NH_4^+, NO_3^-, and N_2) from mineralization diffused from the sediment into the overlying water, and that this (NH_4^+ and NO_3^-) could supply 30-82% of the N requirement of the planktonic primary producers. Blackburn and Henriksen (1983) also showed that benthic infauna could increase the flux of NH_4^+ from the sediment by 50%. The concept of "benthic-pelagic" coupling has been a focus of ecological research as workers attempt to unravel the interrelationships between sediments and overlying waters in shallow, coastal ecosystems (Rowe *et al.*, 1975; Nixon, 1981; Jorgensen *et al.*, 1983). The role of NH_4^+ in the regulation of nitrogen fixation in saltmarsh and seagrass ecosystems has also received attention. Ammonium is a powerful repressor of nitrogenase activity, and, at *in situ* concentrations of 100-200μM, inhibits nitrogen fixation in saltmarsh ecosystems on the North American east coast (Carpenter *et al.*, 1978; Teal *et al.*, 1979).

The concentrations of porewater ammonium from sediments (range 0 - 53μM) and in the overlying water column ($<0.1\mu$M) were lower in Langebaan Lagoon than those reported for other seagrass ecosystems (Short, 1983; Horrigan and Capone, 1985; Moriarty *et al.*, 1985). Large populations of burrowing invertebrates are present at Oesterwal and Geelbek. These animals pump a significant volume of water through

their burrows while feeding, causing subsurface sediments to be well ventilated (Dye, 1978). Benthic infauna have been shown to increase the flux of nutrients from sediments (Aller, 1978; McCaffrey *et al.*, 1980; Blackburn and Henricksen, 1983; Pomroy *et al.*, 1983), which could account for the unusually low ammonium concentrations in porewater from Oesterwal and Geelbek. Limited seasonal data were available for porewater ammonium concentrations in Langebaan Lagoon, and so temporal trends were difficult to assess. Overall, porewater ammonium concentrations at Geelbek were higher than at Oesterwal, but this difference was not significant ($p > 0.05$). Concentrations of porewater ammonium were highest in *Zostera* bed sediment and subsurface sediment from Geelbek, where prawn densities, and thus ventilation rates, are lowest.

The concentrations of exchangeable ammonium in sediments followed the trend of porewater ammonium concentrations: highest concentrations of exchangeable ammonium were measured in *Zostera* bed sediment and subsurface sediment from Geelbek, although Oesterwal and Geelbek were not significantly different ($p > 0.05$) in this respect. Complete seasonal data were not available for NO_3^-, NO_2^-, and PO_4^{2+}, and so temporal trends in these data were difficult to analyse. Spatial comparisons showed that concentrations of NO_3^- and NO_2^- were not significantly different between Oesterwal and Geelbek. Highest concentrations of NO_3^- and NO_2^- occurred in burrow lining and *Zostera* bed sediment respectively from both sites. Concentrations of phosphate in sediments were significantly ($p < 0.05$) higher at Geelbek than at Oesterwal, while highest concentrations of PO_4^{2+} at both sites occurred in *Zostera* bed sediment. There has been some debate as to whether it is nitrogen or phosphorus availability that most often limits primary production in marine ecosystems (Ryther and Dunstan, 1971; Eppley *et al.*, 1973; Mann, 1982; Smith, 1984; Smith, 1990). The median C:N:P atomic ratio of marine macrophytes is about 550:30:1 (Atkinson and Smith, 1983). In Langebaan Lagoon, PO_4^{2+} concentrations exceed those of NH_4^+, NO_3^-, and NO_2^-. Thus, it appears that nitrogen, and not phosphorus, is more likely to limit primary production in Langebaan Lagoon.

The organic matter content, total nitrogen content, and organic carbon content of all sediments and water were higher at Geelbek than at Oesterwal. This comparison corresponds with the findings of previous studies that bacterial and microalgal productivities were higher at Geelbek than at Oesterwal (Fielding *et al.*, 1988; Tibbles *et al.*, 1992). At Geelbek, *Zostera* bed sediment contained more organic matter, total nitrogen, and organic carbon than the other four sedimentary microhabitats. This pattern at the microhabitat level was expected, as *Zostera* bed sediment is in closer proximity, relative to the other microhabitats, to a major source of primary production. However, at Oesterwal, burrow lining sediment contained more organic matter and total nitrogen than the other microhabitats. This is consistent with the behaviour of *Callianassa* which often line their burrows with organically rich mucus and possibly seagrass detritus (Dobbs and Guckert, 1988). Such behaviour has implications for the microenvironment of the burrow and may thus influence the absolute and relative abundances of microbiota, and their physiological state in the burrow lining (Branch and Pringle, 1987; Dobbs and Guckert, 1988).

Although the quantity of available organic material is generally considered to be a major determinant of bacterial abundance in sediments, the quality of this material may also influence bacterial abundance (Schallenberg and Kalff, 1993). One parameter that is often considered indicative of the quality of organic material for consumers is the C:N ratio. The rate of decomposition of detritus in marine sediments is generally inversely correlated with the C:N ratio of the substrate (Linley and Newell, 1984). In marine ecosystems, where plant material is commonly deficient in nitrogen content, lower C:N ratios represent more nutritious food sources for consumers. Some heterotrophic bacteria are able to supplement their nitrogen requirement with nutrients taken from the surrounding water or by nitrogen fixation; the latter method is at high energy cost. Bacterial growth on detritus in the marine environment may also lead to N-enrichment of that material, and thus improve its quality for detritivores (Newell, 1965; Pomeroy, 1980). C:N ratios of the organic contents of sediments from Geelbek were lower than those from Oesterwal, indicating that organic material in Geelbek sediments was relatively richer in nitrogen that at Oesterwal.

Besides the C:N ratio, the quality of organic material in sediments and water can also be considered in terms of its biotic constitution, or the origins of its constituents. Quality may be reflected in such information, since the biochemical characteristics of different components of organic matter may influence how bacteria use them. For instance, lignocellulosic materials in seagrass and saltmarsh plants are relatively resistant to degradation whereas materials high in organic nitrogen and other hydrolyzable compounds are more easily utilized (Gunnison and Alexander, 1975; Rice and Tenore, 1981). Saltmarsh and seagrass detritus was the major constituent of the organic content of sediments at both sites. At Oesterwal, detritus formed a larger proportion of the organic pool (organic carbon and total nitrogen) than at Geelbek. Diatoms, bacteria, and protozoa were more abundant at Geelbek than at Oesterwal (Harris, 1993), and also constituted a greater proportion of the organic pool at Geelbek than at Oesterwal. The sheltered mudflats at Geelbek would provide a better environment for the growth of diatoms than the sandflats at Oesterwal, which are exposed to tidal currents and wave action. Materials of scenescing diatoms, bacteria, and protozoa are likely to be more easily utilized by heterotrophic bacteria than detritus relatively rich in complex structural polysaccharides such as microcrystalline cellulose and lignin. Hence, such material can be described as being of "better quality" for bacterial use. Therefore, sediments from Geelbek were "richer" than those at Oesterwal in terms of both the quantity and quality (C:N ratio and biotic constitution) of organic material. This difference is likely to have influence on both the abundance of bacteria and on the physiology of their assemblages.

Optimization of the plate-count technique established appropriate compositions of media for the isolation of heterotrophic bacteria from Langebaan Lagoon. Colony counts on ultra-low strength nutrient media, containing only the residual nutrients in bacteriological agar, filtered, natural seawater, and 10mg yeast extract per litre, were ca. 10- to 100-fold higher than counts on media richer in nutrients. Similar findings have been reported for the isolation of bacteria from other natural populations (Buck, 1974; Martin, 1975; Hattori, 1981; Olsen and Bakken, 1987), and were attributed to the dominance of oligotrophic bacteria, which are only able to grow at very low nutrient concentrations at first isolation (Kuznetzov *et al.*, 1979). The plateability

(defined here as colony counts/microscopical counts) of bacteria from Langebaan sediments was less than 1%, while that from water samples was less than 4%. Plateability of bacteria from Geelbek was lower than for Oesterwal samples. Similar rates of recovery have been reported for other marine environments (Jannasch and Jones, 1959). The presence of dead or moribund cells may account for some of the descrepancy between colony (viable) counts and microscopic counts (Xu *et al.*, 1982), but low plateabilities of natural populations on most media have elicited criticism of the plate count technique for its selectivity (Brock, 1987). The plate-counting technique may be considered an inadequate method for counting all viable cells in natural populations and for confidence in isolating the dominant strains or even a representative selection of the population. However, it is often the method of choice for a "first-look" at an ecosystem for its simplicity and economy, and is still the only method available for the viable isolation of bacteria from the environment. Molecular techniques and process measurements (eg. tritiated thymidine incorporation, nitrogenase activity, gene probing, 16SrRNA analysis) are more acceptable methods for examining natural populations as they are sensitive and have relatively low bias, but ultimately such technology relies on information gained from pure culture studies and wherever possible the assumptions based in such technology should be tested for validity.

Colony counts of oligotrophs, general heterotrophic aerobes and anaerobes showed no consistent seasonal trends for isolations from either sediment or water samples. Temporal fluctuations of CFU reflected those of bacterial abundance in water, but not in sediments, possibly due to a greater patchiness of bacterial activity in sediments. Whereas both CFU and bacterial abundance were higher for Geelbek water than for Oesterwal water, CFU for Oesterwal sediments were higher than those for Geelbek sediments due to higher plateability of bacteria from Oesterwal. Plateabilities were higher for surface sediments (sediment between and at burrows, and *Zostera* bed sediment) than for subsurface sediment (burrow lining and 10cm depth) (Fig. 7). If one assumes that the proportion of non-viable cells in the population is small and relatively consistent between habitats, then these comparisons suggest that different plateabilities of bacteria from sediments may be linked to the proportion of anaerobic bacteria in the sample. However, this reasoning (at least between sites) is contradicted by the higher

plateability of bacteria from Oesterwal on anaerobic media (Table 5), since Oesterwal is relatively aerobic compared to Geelbek. Furthermore, as Geelbek can be considered a richer environment than Oesterwal in terms of both the quantity and quality of growth substrates available to bacteria, one would not expect there to be a higher proportion of oligotrophs at Geelbek than at Oesterwal. Thus the different plateabilities of bacteria from the two sites cannot be attributed to different proportions of oligotrophs. Rather, this effect is probably due to a higher number of fastidious strains at Geelbek whose growth requirements were not met by the media and conditions used in these isolations.

The biochemical and physiological profiles of strain collections isolated during winter and summer were different in certain characteristics. Proportions of strains positive for acid production from glucose, nitrate reduction, denitrification, and nitrogen fixation were higher for summer isolates than for winter isolates. The latter three characteristics refer to the use of nitrogen by these marine isolates. Denitrification and nitrogen fixation respectively represent loss of nitrogen from, and gain of nitrogen to, a system. Loss of nitrogen due to denitrification is important relative to nitrogen inputs in many freshwater and marine ecosystems, and may account for the low concentrations of combined nitrogen in the latter (Seitzinger, 1988). Nitrogen fixation has frequently been reported to be an important source of combined nitrogen for marine macrophytes (Patriquin and Knowles, 1972; Capone *et al.*, 1979; Capone, 1982; O'Donohue *et al.*, 1991). Moreover, seasonality of nitrogen fixation and denitrification is common in many marine ecosystems (Teal *et al.*, 1979; Jones, 1982; Seitzinger *et al.*, 1984; Law *et al.*, 1991), supporting the indications of seasonality in these processes by the characterization studies of the present work. Seasonality of denitrification and nitrate reductase activity is interesting in that this pattern coincides with the seasonality of upwelling on the west coast of South Africa. An adequate supply of nitrate is essential to drive these processes. Upwelling occurs during spring and summer months on this coast and introduces nutrient-rich water (including nitrate at typically 10-16μM) to Saldanha Bay and Langebaan Lagoon (Henry *et al.*, 1977). Variations in these physico-chemical parameters are likely to affect bacterial activity in Langebaan Lagoon.

CONCLUSIONS

In terms of pH and temperature, Oesterwal and Geelbek are similar. Higher salinities at Geelbek during summer are possibly due to evaporation. Geelbek sediments are more anaerobic than those at Oesterwal. Sediments and water at Geelbek are 'richer' in terms of both quantity and quality (C:N ratio and biotic constitution) of organic matter (mostly seagrass and saltmarsh detritus) available for bacterial use, and this reflects a greater abundance of bacteria at Geelbek than at Oesterwal. Ammonium concentrations in sediments and water from Langebaan Lagoon were lower than those reported for other saltmarsh and seagrass ecosystems (Moriarty and Boon, 1989; Carpenter *et al.*, 1978). Highest concentrations of nutrients were generally measured in sediments from Geelbek. Furthermore, at each site highest concentrations of nutrients were measured in *Zostera* bed sediment, burrow lining, and subsurface sediment. In terms of the nutrient requirements of primary production of benthic marine macrophytes, concentrations of phosphate were in excess relative to those of ammonium and nitrate. Bacterial abundance and the number of CFU on different microbiological media followed no consistent seasonal trend. However, the proportions of isolates that were positive for nitrate reductase, denitrification and nitrogen fixation were higher in summer than in winter. Numbers of nitrogen-fixing bacteria in sediments and water were also higher in summer than in winter.

CHAPTER 3

Estimates of Bacterial Productivity in Marine Sediments and Water from a Temperate Saltmarsh Lagoon

Abstract. Tritiated thymidine incorporation (TTI) into DNA was used to estimate bacterial productivity in sediment and water samples from two sites in Langebaan Lagoon. Routine analysis of isotope dilution showed seasonal variations of approximately 3-fold in the thymidine precursor pool sizes for bacterial assemblages from each site. Dual label incorporation of ^{3}H-thymidine and ^{14}C-leucine, into DNA and protein respectively, showed that pelagic, but not sediment assemblages were in a balanced state of growth during TTI. This, to my knowledge, is the first report of dual label measurements of bacterial production in sediments. Sediments supported bacterial productivities that exceeded those in the water column by factors from 5- to 950-fold, while bacterial abundance supported by sediments exceeded that in the water column by more than 3 orders of magnitude. Estimates of bacterial production in sediments were coincident with levels of organic content in sediments, but not with bacterial abundance. Measurements of TTI activity for 5 different benthic microhabitats at one lagoon site showed highest activity associated with seagrass beds (2.11 $\pm$ 0.84 nmol thymidine h^{-1} g^{-1} DW), while activity decreased with depth (0.46 $\pm$ 0.21 nmol thymidine h^{-1} g^{-1} DW) below sediment surface.

INTRODUCTION

Coastal marine ecosystems with saltmarsh components are highly productive communities based on high rates of primary production, frequently dominated by *Spartina* spp., *Zostera* spp., and other halophytes. Benthic diatoms may also make substantial contributions to overall primary production (Mann, 1988). However, owing to the refractory nature of much of this material, most of the primary production enters higher trophic levels through heterotrophic bacteria (Phillips and McRoy, 1980; Mann, 1988). One consequence of this is that typically nitrogen-impoverished material

becomes nitrogen-enriched, with clear benefits to consumer communities. The N-requirements of bacterial growth on N-deficient detritus may be supplemented with inorganic nitrogen obtained from the environment, or by nitrogen fixation. Thus, bacterial growth (production) on detritus may also include their use of plant polysaccharides as carbon/energy sources to support nitrogenase activity (see Chapter 5). Estimates of bacterial productivity are therefore essential in quantifying biogeochemical flows, and for determining the ecological role of bacteria in the trophic dynamics of saltmarsh systems. In particular, ecologists have sought to measure bacterial production in terms of carbon or nitrogen for comparison with autotrophic production and consumer requirements.

Measurements of bacterial productivity in natural samples can be problematic. Direct observations of changes in bacterial abundance with time are difficult, particularly for sediments. However, the close association between rates of macromolecular synthesis and cell division provides a means of estimating bacterial productivity if the rate of incorporation of labeled precursors into bacterial macromolecules can be accurately measured. The use of adenine as a suitable precursor of macromolecular synthesis has been proposed (Karl, 1979), but results obtained from adenine incorporation are difficult to interpret as both it and ATP are involved in many different biochemical processes in all organisms (Fuhrman *et al.*, 1986). Other precursors that have been proposed to measure bacterial productivity include thymidine (Fuhrman and Azam, 1980) and leucine (Chin-Leo and Kirchman, 1988).

There is a direct correlation between the rates of cell division and DNA synthesis (Moriarty, 1986), which enables the technique of [*methyl*-^{3}H]thymidine (^{3}H-Tdr) incorporation (TTI) into DNA to be used to estimate the productivity of bacteria in natural systems (Fuhrman and Azam, 1980). TTI into DNA has gained widespread acceptance in microbial ecology, because it is specific to heterotrophic bacteria, and most of the label is incorporated into DNA during short incubation periods. However, several potential problems with this method have been identified, and results may be interpreted incorrectly if these potential sources of error are not recognized.

Contrary to the assumptions of earlier procedures (Fuhrman and Azam, 1980, 1982), the lack of specificity of ^{3}H-Tdr in the labeling of DNA and the variability of DNA content in cold trichloroacetic acid (TCA) precipitate have been demonstrated for freshwater (Karl, 1982; Robarts *et al.*, 1986; Robarts and Wicks, 1989), seawater (Servais *et al.*, 1987; Hollibaugh, 1988; Simon and Azam, 1989), and sediment (Ducklow *et al.*, 1985; Brittain and Karl, 1990; Moriarty and Pollard, 1990) bacterial assemblages. These reports emphasize the the need to isolate labeled DNA from macromolecular extracts.

Isotope dilution of exogenously supplied ^{3}H-Tdr by intracellular and extracellular sources of unlabeled precursor in the final pool of ribosyl-thymine 5'-triphosphate (dTTP) may lead to underestimations of bacterial productivity. The final extent of dilution, and therefore the specific radioactivity of dTTP available for incorporation, can be measured by isotope dilution analysis (Pollard and Moriarty, 1984). If extracellular sources are insignificant, then isotope dilution can be limited by the addition of ^{3}H-Tdr at concentrations in excess of the precursor pool size measured by isotope dilution analysis (Moriarty, 1986). However, extracellular dilution has been reported to be occasionally significant (Jeffrey and Paul, 1988), and the variations of macromolecular labeling by ^{3}H-Tdr cited above suggest that isotope dilution may vary on spatial and temporal scales.

TTI measurements must represent balanced growth in bacterial assemblages if the data are to be translated into bacterial carbon production. Chin-Leo and Kirchman (1988) have shown that ^{3}H-Tdr and ^{14}C-leucine (^{14}C-Leu) can be used in dual label incorporation (DLI) experiments to simultaneously measure DNA and protein syntheses. Under conditions of balanced growth, the rates of macromolecular syntheses are coupled and the ratio of Leu:Tdr incorporation should remain relatively constant over progressive periods of incubation. Another assumption of TTI is that all growing bacteria in the sample are capable of incorporating exogenously supplied ^{3}H-Tdr into DNA. Although most bacteria probably do possess Tdr kinase as an essential enzyme of the salvage pathway (Moriarty and Pollard, 1981), the limitations of this assumption have been indicated by reports of a lack of TTI into DNA in some strains (Pollard and

Moriarty, 1984; Davis, 1989; Jeffrey and Paul, 1990). If the proportion of a population able to incorporate TdR is significantly lower than the proportion able to incorporate Leu, then protein synthesis may exceed DNA synthesis and the ratio of Leu:Tdr may vary over time. DLI can thus also be used to establish confidence that TTI is applicable to most growing bacteria in the sample.

Bacterial productivity is linked to seagrass productivity in temperate seagrass beds (Moriarty and Pollard, 1982), and is apparently also regulated primarily by phytoplankton production in pelagic systems (Lovell and Konopka, 1985a, 1985b; Murray and Hodson, 1985). Studies in a saltmarsh ecosystem in Southern Africa are aimed at elucidating the pathways of carbon and nitrogen fixation and their flows into microbial and higher benthic consumers. In this study, slightly modified TTI methods were used to estimate variations in bacterial productivity in sediment and water samples from Langebaan Lagoon. Spatial differences in TTI were measured at a macro-scale (two sites, Geelbek and Oesterwal) and at a micro-scale (different sediment microhabitats at Oesterwal). Temporal (seasonal) variations in measurements of isotope dilution and bacterial productivity were examined in both sediment and water column for each study site. Furthermore, DLI was used to assess whether bacterial assemblages were in balanced growth and to test the assumption that TTI can be used as a measure of bacterial carbon production.

METHODS

Sampling Procedure

The study sites, Oesterwal and Geelbek, in Langebaan Lagoon have previously described in Chapter 2. Samples of surface sediment and water column were taken from each site for the seasonal study (April 1990, autumn; July 1990, winter; October 1990, spring; January 1991, summer). Samples were always taken on the morning of spring tide, and the temperatures of the samples at time of collection were recorded. There was at least a 10°C difference in temperature between seasons for each study site. Temperatures of surface sediment ranged from 11.1°C in winter to 24.5°C in

summer, while water ranged from 11.2°C in winter to 22.0°C in summer. The temperatures of samples from Geelbek were similar to those for Oesterwal for each season. Samples were transported to the laboratory in an insulated container and processed within 2h of collection.

Measurement of TTI in Sediments

Surface sediment samples were taken intertidally to a depth of 5mm, away from the seagrass beds and between prawn burrow openings. Composite samples were collected over an area of approximately $5m^2$ on the sediment flats, and used to make a sediment slurry (approximately 200g) immediately before the experiment. All experiments were carried out in triplicate. 1μCi [*methyl*-^{3}H]thymidine (83 Ci $mmol^{-1}$, Amersham Corp.) was added to 500mg wet weight sediment slurry in microfuge tubes. Incubation at 20°C was terminated after 0 and 20 min by the addition of 1ml extraction reagents (0.3M NaOH, 25mM disodium EDTA, 0.1% (w/v) SDS). Macromolecules were extracted and RNA hydrolyzed at 25°C for 12 h. The sediment was pelleted by centrifugation at 8000x*g* for 60s, which was the standard centrifugation procedure used in all experiments. The sediment was washed with a further 1ml extraction reagents. Supernatants were removed to test tubes and neutralized with 3N HCl before TCA was added to a final concentration of 5% (w/v). 100μg of herring sperm DNA (Sigma Chemical Co.) was added as a carrier to aid precipitation of labeled DNA. Samples were cooled on ice for 45 min and then filtered through 1.2μm pore size cellulose acetate filters (Millipore Corp.). The tube and filter were rinsed with 5ml ice-cold 5% (w/v) TCA, and the filter was then washed with 5ml phenol-chloroform (50% w/v) followed by 5ml ice-cold 80% (v/v) ethanol. The filters were placed in 7ml Filter-Count (Packard Instrument Co., Inc.) and disintegrations per minute (dpm) determined by Packard TriCarb and Beckman LS 5000TD liquid scintillation counters.

Sediment samples for the comparison of TTI between microhabitats at Oesterwal were taken during early summer and midsummer from five locations as described in Chapter 2. Two sets of midsummer samples were taken from near low and high water marks on the sandflats. For the determination of TTI in different sediment microhabitats at

Oesterwal, [*methyl*-^{3}H]thymidine (28 Ci mmol^{-1}) was added at 0.15nmol per 500mg sediment slurry and incubated at 20°C for 20-30 min. TTI was then determined as described above.

Measurement of TTI in Water

Water column samples (500ml) were taken from 30cm below the surface. The method of Wicks and Robarts (1987) was used with the following modifications: samples were incubated with 2μCi (*methyl*-^{3}H)thymidine (83 Ci mmol^{-1}); extraction of macromolecules with NaOH was carried out at 4°C for 12h; 100μg herring sperm DNA was added, and precipitates were collected on 0.45μm pore size cellulose acetate filters (Millipore Corp.). Liquid scintillation counting of whole filters was performed as described above.

Determinations of Isotope Dilution, Calculations of Bacterial Productivity and Statistical Analysis of Data

Seasonal determinations of isotope dilution were made as follows. Crystalline thymidine (Sigma Chemical Co.) was added at concentrations of 0, 0.05, 0.15, and 0.35nmol per 500mg sediment slurry, and of 0, 10, 30, and 70nM for water samples. Methods for sediment and water TTI determinations as described above were then carried out. The isotope dilution plot described by Pollard and Moriarty (1984) was used to determine the extent of isotope dilution in each sample. The specific radioactivity of the dTTP pool was thus corrected and bacterial productivity was then calculated for the sample using a conversion factor of 1.1×10^{18} cells produced mol^{-1} thymidine incorporated (Riemann *et al.*, 1987). Analysis of variance (ANOVA) was performed on untransformed data sets where Bartlett's test indicated homogeneity of variance. Where Bartlett's test revealed that variances differed significantly, Kruskal-Wallis non-parametric analysis was substituted for ANOVA.

Comparison of Tdr and Leu Incorporation Rates

Rates of TTI were compared to rates of ^{14}C-Leu incorporation using DLI methods. [*Methyl*-^{3}H]thymidine (28 Ci mmol^{-1}) and ^{14}C-leucine (308 mCi mmol^{-1}, Amersham Corp.) were added at concentrations of 0.15 and 0.30 nmol respectively per 500mg sediment slurry or 10ml water sample. Water samples were processed using the filter method described above, except that a further wash with cold 5% TCA was substituted for the phenol-chloroform wash.

Sediment samples were processed according to a modification of the dialysis method of Moriarty and Pollard (1990), as this method had been previously applied to TTI studies in sediments. Sodium molybdate (20mM) was added to selected samples to inhibit the activity of sulfate reducing bacteria (Oremland and Capone, 1988). Time series incubations over 3h were stopped by the addition of 1.5ml ice-cold 80% (v/v) ethanol with 100mg l^{-1} unlabeled thymidine, and placed on ice for 1 hour. The sediment was centrifuged and washed with a further 1.5ml ethanol. 2ml of extraction reagents was then added to extract macromolecules from the sediment and to hydrolyze RNA at 25°C for 12 hours. The sediment was pelleted by centrifugation, and the supernatant was mixed with 36μl acetic acid and removed to dialysis tubing (12 000-14 000 Da. cut-off, Spectrum Medical Industries) using apparatus as described by Pollard (1987). The sample tubes were rinsed into the dialysis bags with 1ml distilled water. The samples were dialysed overnight against running tap water and then transferred to scintillation vials with 10ml Insta-gel (Packard Instrument Co., Inc.). Dual label dpm were determined by a Beckman LS 5000TD liquid scintillation counter. Energy spectrum spillover was corrected according to the method described in the manual of this instrument.

Determinations of Organic Carbon Content and Bacterial Abundance

Replicate 100ml water samples (n=3), collected for enumeration of bacteria, were fixed with 4% formalin (final concentration). Replicate 1cm^3 sediment samples (n=3) were taken using a 10cm^3 syringe with the luer end removed, and fixed with 4%

formalin (final concentration in 0.2μm filtered seawater). All samples were stored at 4°C in the dark and subsequently stained with 4',6-diamidino-phylindole (DAPI) for bacterial counts by the method of Porter and Feig (1980). Water samples (2ml) were stained at a concentration of 0.1μg ml^{-1} DAPI. Sediment samples were diluted (1:2500 v/v for Geelbek, and 1:1500 v/v for Oesterwal) in 0.01M tetrasodium pyrophosphate, sonicated (30-50 mHz) for 5 min, and stained at a concentration of 5μg ml^{-1} DAPI. Counts were corrected for sediment masking using the method of Ellery and Schleyer (1984). Randomly selected fields on each of duplicate filters per sample were counted at 1000x magnification on a Nikon compound microscope with a Neofluar objective until 20 fields or at least 400 bacteria were counted. Bacterial numbers were corrected to dry weight (DW) sediment after determination of the water content in samples (n=3) dried at 60°C for 48 h.

Particulate organic content in replicate (n=3) water samples (500-2000ml) was collected by filtering water onto precombusted (450°C for 6 h) Whatman GF/F filters. Replicate (n=3) 1cm^3 sediment samples for organic content were collected as described above. All samples were stored at -20°C and then dried at 60°C for 48 h. Total organic content was determined by weight loss by dried samples when combusted at 450°C for 6 h.

RESULTS

Determinations of Isotope Dilution and Estimates of Bacterial Production

Time course experiments over 4 h for both sediment and water samples showed that incubation periods of 20-30 min fell within the limits where TTI was linear with time. Isotope dilution analysis was performed on each sample to determine the variation of this parameter and to improve the accuracy of bacterial production estimates. The sizes of precursor pools in the samples (Table 1) measured by isotope dilution plots (Pollard and Moriarty, 1984) showed no uniform trend according to season. Table 1 shows that ^{3}H-Tdr should be added to samples from Langebaan Lagoon at concentrations above

TABLE 1. Isotope dilution analysis of ^{3}H-Tdr in sediment and water samples from Oesterwal and Geelbek[a].

Season	Sediment (nmol g^{-1} DW)		Water (nM)	
	Oesterwal	Geelbek	Oesterwal	Geelbek
Autumn	0.281	0.361	9.30	7.80
Winter	0.183	0.208	7.00	3.40
Spring	0.119	0.703	0.60	6.00
Summer	0.498	0.458	0.30	1.90

[a] Values represent effective pool sizes of thymidine prior to incorporation into DNA.

9.3nM for water or 0.7nmol g^{-1} DW for sediment, and that isotope dilution determined during one season may not necessarily be applicable as estimates in other seasons. An approximate variation of three fold in isotope dilution was measured between seasons; this variation was larger in Oesterwal water (Table 1).

Estimates of bacterial productivity for sediments showed similar trends for seasonal samples from Geelbek and Oesterwal, with minima in winter and maxima in summer (Fig. 1). However, there was no significant difference ($p>0.05$) between average annual productivities for sediment slurries from Oesterwal ($2.15 \pm 1.82 \times 10^{6}$ cells h^{1} g^{-1} DW) and Geelbek ($2.76 \pm 1.70 \times 10^{6}$ cells h^{-1} g^{-1} DW). Average sediment volume to wet weight ratios were approximately 0.6ml g^{-1} wet weight, and therefore sediments were more productive than the water column (by volume) by factors from 5 to 950 fold.

Bacterial productivity estimates for the water column showed that productivity at Geelbek exceeded that at Oesterwal during spring and summer (Fig. 1). Water column productivity minima and maxima were greater for Geelbek than for Oesterwal, as was the average productivity estimate ($1.40 \pm 0.70 \times 10^{5}$ cells h^{-1} ml^{-1}). The average water productivity estimate was more variable for Oesterwal samples ($8.26 \pm 8.93 \times 10^{4}$ cells h^{-1} ml^{-1}).

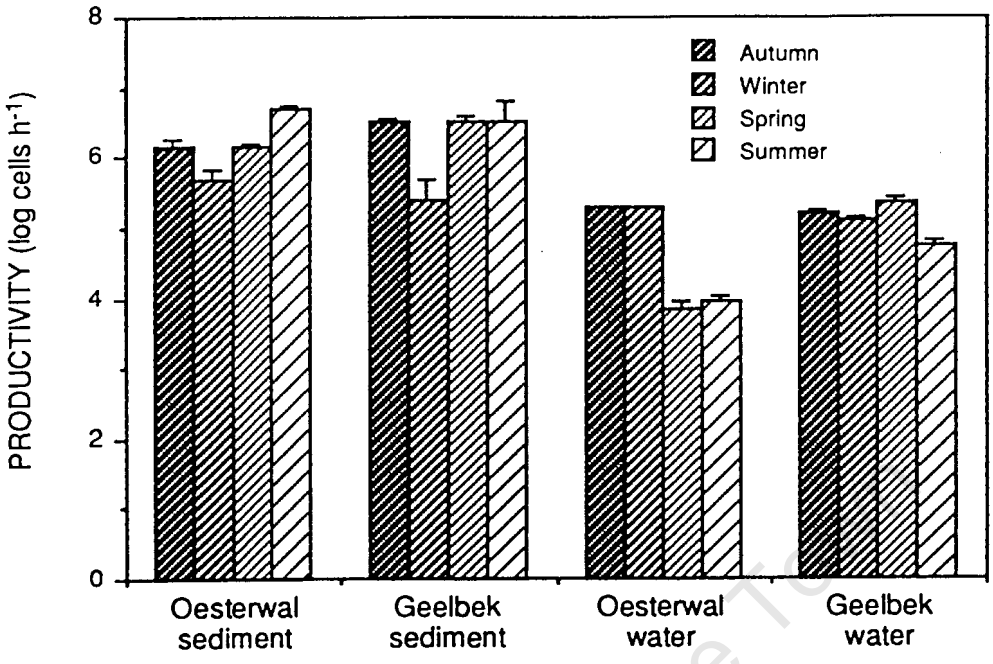

Figure 1. Estimates of bacterial productivity for seasonal samples of sediment and water from Geelbek and Oesterwal, Langebaan Lagoon. Values represent means ± standard deviations.

Total counts of bacteria by epifluorescence microscopy showed that cells were more abundant in samples from Geelbek than in samples from Oesterwal (Table 2). Similarly, samples from Geelbek had a higher organic content than those from Oesterwal (Table 2). Although sediment samples collected for CHN analyses did not show consistent seasonality of organic contents (Chapter 2), the organic contents of sediment samples collected for TTI assays did show seasonality (Table 2). The latter findings reflect those of Mazure and Branch (1979), and correspond with the predicted supply of organic material from primary production. Sediments contained a greater percentage of organic carbon by volume, and supported larger bacterial populations, than water samples (Table 2). The trends exhibited by productivity estimates for sediments (Fig. 1) were not evident in the data for bacterial abundance (Table 2). However, variations in the organic content of sediments did follow the trends of the bacterial productivity estimates.

TABLE 2. Bacterial abundance and organic content of water and sediment samples from Oesterwal and Geelbek.

Sample	Season	Bacterial abundance[a]	Organic content[b]
Oesterwal sediment	Autumn	2.32 ± 0.10	12.54 ± 0.06
	Winter	1.34 ± 0.79	4.27 ± 0.01
	Spring	1.90 ± 0.72	6.44 ± 0.03
	Summer	1.35 ± 2.39	8.20 ± 0.01
Geelbek sediment	Autumn	4.21 ± 0.78	15.96 ± 1.05
	Winter	4.22 ± 2.06	5.52 ± 0.33
	Spring	4.40 ± 1.09	4.98 ± 0.24
	Summer	3.56 ± 1.22	12.32 ± 1.14
Oesterwal water	Autumn	2.07 ± 0.50	5.80 ± 2.12
	Winter	1.99 ± 0.08	2.07 ± 0.21
	Spring	1.40 ± 0.29	3.85 ± 0.50
	Summer	1.98 ± 0.78	5.17 ± 1.76
Geelbek water	Autumn	3.62 ± 2.16	3.27 ± 0.47
	Winter	3.96 ± 2.39	24.93 ± 5.55
	Spring	2.57 ± 1.17	17.60 ± 0.57
	Summer	2.88 ± 2.39	7.87 ± 4.46

[a] units in cells x 10^9 g^{-1}DW for sediments, and cells x 10^6 ml^{-1} for water
[b] units in mg g^{-1}DW for sediments, and mg l^{-1} for water.
Values represent means ± standard deviations.

Variability of TTI Between Sediment Microhabitats

Thymidine incorporation rates for 5 different sediment microhabitats at Oesterwal are shown in Fig. 2. Highest rates of incorporation occurred in *Zostera* bed sediment, while rates were significantly lower ($p < 0.05$) in subsurface sediment. Incorporation rates for surface sediment and burrow opening were similar, while TTI in burrow lining was enhanced relative to that in burrow-free sediment from 10cm depth. Bacterial abundance and sediment organic content were determined for microhabitat samples taken during midsummer from the high water mark (Table 3). Highest organic

TABLE 3. Bacterial abundance and organic carbon content of sediment microhabitats at the high tide mark at Oesterwal during midsummer.

Microhabitat	Bacterial abundance (cells x 10^9 g^{-1}DW)	Organic content (mg g^{-1}DW)
Surface sed.	1.35 ± 0.44	8.04 ± 0.02
Zostera bed sed.	1.79 ± 0.16	8.91 ± 0.10
Burrow opening sed.	1.22 ± 0.41	5.64 ± 0.45
Burrow lining sed.	1.75 ± 0.30	11.53 ± 0.92
10cm depth sed.	1.73 ± 0.38	6.56 ± 0.59

Values represent means ± standard deviations.

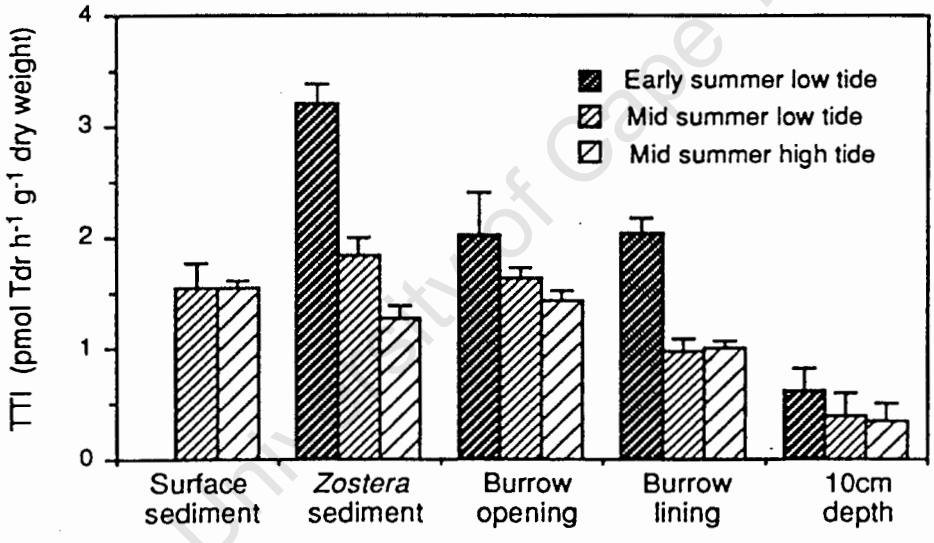

Figure 2. TTI activities in sediment samples from different benthic microhabitats at Oesterwal. Values represent means ± standard deviations. Average values of activity (pmol Tdr incorporated h^{-1} g^{-1} DW) for each microhabitat were 1.55 ± 0.17 in surface sediment, 2.11 ± 0.84 in *Zostera* bed sediment, 1.69 ± 0.34 in burrow opening sediment, 1.34 ± 0.52 in burrow lining, and 0.46 ± 0.21 in sediment from 10cm below the surface. No data are available for surface sediment in early summer.

contents and bacterial densities were recorded for sediments from burrow lining and *Zostera* beds.

Comparison of Leu and Tdr Incorporation Rates

Molar ratios of Leu:Tdr incorporation were calculated for incubation periods of 30, 60, and 180 min for sediment and water samples (Fig. 3). Leu:Tdr ratios for water samples remained more constant over time of incubation relative to those for sediment samples, where the Leu:Tdr ratio tended to decrease with time. These results indicate that Tdr and Leu incorporation are measuring balanced growth in bacterial populations in water samples, but not necessarily in sediment populations.

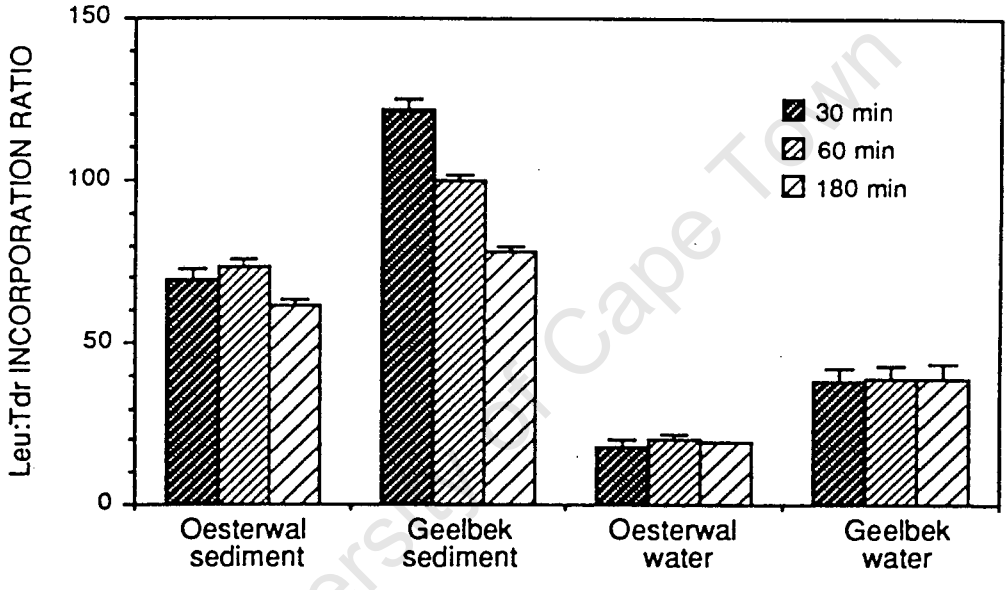

Figure 3. Time course molar incorporation ratios of Leu:Tdr in summer samples of sediment and water from Geelbek and Oesterwal, Langebaan Lagoon. Values represent means ± standard deviations for incubations terminated after 30, 60, and 180 min.

There is an apparent relationship between the ratio of Leu:Tdr incorporation (Fig. 3) and the organic status of the sample (Table 2). This relationship is also evident in Table 4. Leu:Tdr ratios and organic carbon contents for sediments exceeded those calculated for water, while ratios for samples from Geelbek generally exceeded those for Oesterwal (Fig. 3). There was little variation of Leu:Tdr incorporation ratios with sediment depth or with the addition of 20mM sodium molybdate (Table 4). It is therefore unlikely that variations in populations of sulfate reducing bacteria were responsible for spatial variations in the Leu:Tdr ratio in this study.

TABLE 4. Dual label incorporation of ^{3}H-Tdr and ^{14}C-Leu in sediment and water samples from Langebaan Lagoon[a].

Sample	Molybdate	Leu:Tdr ratio	Organic content[b]
0cm[c]	-	73.0 ± 5.2	5.5 ± 0.8
0cm[c]	+	79.7 ± 33.2	5.5 ± 0.8
5cm[c]	-	50.7 ± 12.9	5.8 ± 0.5
5cm[c]	+	68.7 ± 5.2	5.8 ± 0.5
10cm[c]	-	78.0 ± 20.6	6.8 ± 1.0
10cm[c]	+	71.4 ± 24.1	6.8 ± 1.0
20cm[c]	-	62.9 ± 20.7	7.5 ± 2.0
20cm[c]	+	77.2 ± 15.1	7.5 ± 2.0
OHWS[d]	-	113.2 ± 32.5	9.6 ± 1.4
OZS[e]	-	68.9 ± 11.0	10.5 ± 1.0
GHWS[f]	-	84.9 ± 28.2	24.0 ± 12.5
OW[g]	-	24.9 ± 0.1	12.5 ± 0.1
GW[h]	-	38.8 ± 3.8	48.6 ± 4.9

[a] Samples were collected during early summer 1991. Variations of the Leu:Tdr molar incorporation ratio were recorded with sediment depth and organic content of the sample. Samples were amended with 20mM molybdate as indicated and incubated with ^{3}H-Tdr and ^{14}C-Leu for 30 min. Values represent means ± standard deviations.

[b] units in mg g^{-1} DW for sediment and mg l^{-1} for water.

[c] sediment from different depths below surface at low water mark, Oesterwal.

[d] surface sediment from high water mark at Oesterwal.

[e] surface sediment from *Zostera* bed at Oesterwal.

[f] surface sediment from high water mark at Geelbek.

[g] water from Oesterwal.

[h] water from Geelbek.

DISCUSSION

The measurement of TTI in sediments for estimates of bacterial production is more difficult than in water. Established TTI methods were used for water, but an appropriate method for sediments was optimized for Langebaan Lagoon and modified to enable comparisons with water data. The TTI methods used here allowed the separation of DNA from general macromolecular extracts, and thus measured only label incorporated into DNA. The methodological conclusions reached here agree with those of Moriarty and Pollard (1990), who found that filtration and dialysis methods gave similar results, although variability between samples was greater for the filtration method. Despite this variability, however, regression coefficients of isotope dilution plots in the present work were consistently within the confidence limits required for a linear regression of 4 points.

Isotope dilution analysis showed that variations in precursor pool sizes did not follow a regular seasonal pattern. Estimated pool sizes in sediment and water samples from each site followed the same pattern, except for summer samples of sediment and water from Oesterwal. These data also illustrate the need to assess isotope dilution in different study sites, taking cognizance of spatial and temporal variation. The present study showed that isotope dilution in the sediment could be more than 100-fold greater than that in the water column, and that approximately a 3-fold variation in precursor pool size occurred over an annual cycle. Determinations of isotope dilution for sediment or water in one season may not, therefore, necessarily apply to other seasons. Variability in isotope dilution may be attributed to factors such as the differential participation of bacterial aggregates in TTI, or to the participation of extracellular thiamine nucleotide pools. Jeffrey and Paul (1988) differentiated intracellular from extracellular isotope dilution by inhibiting thymidylate synthase in the *de novo* synthesis pathway with 5-fluoro-2'-deoxyuridine. They found that extracellular isotope dilution was significant in 20% of the cases they studied. If extracellular isotope dilution is occasionally significant then the assumption, held in much of the TTI literature, that addition of thymidine at concentrations high enough to cause feedback inhibition of thymidylate synthase, becomes problematic. The accuracy of bacterial production estimates

subsequently becomes contingent on a routine analysis of isotope dilution. It is conceivable that greater variabilities in isotope dilution may be encountered in other environments.

Concurrent measurements of DNA and protein synthesis rates indicated that pelagic bacterial assemblages in summer samples from Langebaan Lagoon were in a balanced state of growth (Fig. 3). Other workers have also reported high correlations between Tdr and Leu incorporation for pelagic assemblages (Chin-Leo and Kirchman, 1988; Kirchman and Hoch; 1988). However, the ratio of Leu:Tdr incorporation for sediments decreased with time (Fig. 3), indicating that these populations were possibly in unbalanced growth. Bacteria respond rapidly to changes in their physical and chemical environments, and protein and DNA syntheses become uncoupled when bacteria shift from one growth rate to another. Disturbance effects caused by the use of slurries for sediment TTI determinations may have caused unbalanced growth in these studies. The condition of balanced versus unbalanced growth has important implications for the calculation of bacterial carbon production from TTI, as variations in DNA concentration and cellular sizes parallel changes in rates of DNA and protein syntheses (Chin-Leo and Kirchman, 1990).

This is, as far as I am aware, the first report of dual label measurements of bacterial production in sediments. Ratios of Leu:Tdr incorporation for sediments were much higher than those for water samples from Langebaan Lagoon. Saltmarsh ecosystems are known to support the growth of large populations of sulfate-reducing bacteria, which may lack the ability to incorporate exogenous Tdr into their DNA (Moriarty, 1986). The proportion of anaerobes in a community may be expected to increase with sediment depth as oxygen levels decrease. Although incorporation of Leu and Tdr decreased with sediment depth (in contrast with bacterial abundance, Table 2), little variation in the Leu:Tdr ratio was noted with respect to depth (Table 4). Moreover, the amendment of samples from different sediment depths with 20mM sodium molybdate, to inhibit the activity of sulfate reducers (Oremland and Capone, 1988), had little effect on the Leu:Tdr ratio (Table 4). The most likely explanation for the consistency of Leu:Tdr

ratios over sediment depth is, therefore, that oxygen-sensitive anaerobes were inactivated in the sediment slurries by the disturbance of oxygen gradients.

Trends in the variation of Leu:Tdr incorporation ratios of different lagoon samples correlated with similar trends in the organic status of different lagoon samples. These patterns are also reflected in the patterns of isotopic dilution (Table 1). Variations in the Leu:Tdr incorporation ratio may be explained as follows: in contrast to leucine, thymidine is a polar molecule and may readily adsorb to organic or clay particles, effectively lowering the concentration of ^{3}H-Tdr available to bacteria for incorporation. Secondly, isotope dilution may lower specific radioactivities of the thymidine pool available for incorporation into bacterial DNA, and thus result in higher Leu:Tdr incorporation ratios.

Primary production is frequently reported to stimulate bacterial productivity (Moriarty and Pollard, 1982; Lovell and Konopka, 1988a; Moriarty *et al.*, 1990), and maximum bacterial densities in temperate marine intertidal sediments usually correspond with increases in plant detritus in the late summer to early autumn (Alongi, 1988). In this study I measured TTI in seasonal samples from Langebaan Lagoon and found significant trends corresponding to expected seasonal patterns for sediment slurries ($p < 0.05$), where productivity maxima occurred in summer. Seasonal estimates of bacterial productivity for sediment slurries from Langebaan Lagoon were coincident with variations in the supply of organic material from primary production. Although the availability of organic material may have supported greater bacterial densities at Geelbek than at Oesterwal (see Chapter 2), bacterial abundance showed little seasonal variability with respect to particulate organic content. Average annual sediment productivity estimates were similar for Oesterwal and Geelbek, although bacterial densities were greater in the mudflats of Geelbek. However, DLI indicated the possibility that TTI did not include the growth of some populations (possibly the sulfate reducers), which may have been more abundant in the muds of Geelbek. The seasonal variability in sediment productivity was lower for Geelbek, where a larger organic pool in the mudflats probably sustained bacterial population sizes in periods of lower primary productivity.

Production estimates for water samples were more variable at Oesterwal than at Geelbek, while bacterial densities in the water column were more consistent between seasons. Oesterwal lies nearer the lagoon mouth than Geelbek and is thus more subject to the influence from tidal exchange of waters from different hydrographic origins and therefore containing bacterioplankton assemblages which exhibit differing physiological characteristics (Chapter 2). The water column at Geelbek was richer in particulate organic content, and supported greater bacterial densities and average annual productivity than Oesterwal. These results contrast with previous reports that bacterial concentrations in the water column were greater at Oesterwal than at Geelbek (Mazure and Branch, 1979).

The estimates of bacterial productivity for sediments reported here are up to two orders of magnitude lower than those reported for an Australian temperate saltmarsh (Moriarty and Pollard, 1981, 1982). This difference can be partly explained by the higher temperatures (up to 31°C) of the latter incubations, and the higher organic content of seagrass sediments relative to those from open flats as at Langebaan (Table 3).

Rates of TTI were measured in each of five different sediment microhabitats at Oesterwal (sediments from surface, *Zostera* beds, burrow heads, burrow lining, and 10cm depth). Comparisons of these data reflect the findings of other workers. Moriarty and Pollard (1982) reported that TTI in *Zostera* bed sediment was about 10-fold higher than that in sediment from adjacent open flats, owing to an enrichment with organic carbon originating from the macrophytes and associated microalgae. In samples from Oesterwal, TTI was greatest in *Zostera* bed sediment, and less in subsurface sediments. The reduction in bacterial productivity with depth in sediment has been previously documented (Karl and Novitsky, 1988; Thorn and Ventullo, 1988; Moriarty and Pollard, 1990). I also examined sediments from the burrow openings and burrow linings of the sandprawn *C. kraussi*, to determine if they contributed differently to overall bacterial productivity as a consequence of their exposure to possible gradients of benthic and pelagic organics and nutrients. TTI in burrow opening sediment was not significantly different ($p > 0.05$) from that in the open flats. However, TTI in burrow

lining was approximately 3-fold greater than that in sediment away from burrows, 10cm below the surface. While these results support the findings of Branch and Pringle (1987), who reported that more bacteria were present in the immediate vicinity of *C. kraussi* burrows in Langebaan Lagoon than in adjacent sediments, our bacterial abundance data does not correspond with this pattern. Variation within each sediment microhabitat was both temporal and spatial, as indicated by samples from early summer and midsummer, and from different locations on the sandflats. Differences in TTI between the seagrass beds, the open flats, and the subsurface sediments depend not only upon the organic content and bacterial abundance, but also upon species composition and the metabolically active proportion of the bacterial community.

The variability in TTI within and between sediment microhabitats, the differences in isotope dilution and productivity estimates from Geelbek and Oesterwal, and the variable ratios of Leu:Tdr incorporation for sediment samples emphasize the complications of converting TTI measurements into estimates of bacterial carbon production for an entire lagoon system. The calculation of bacterial carbon production from measurements of TTI assumes a constant relationship between the labeling of DNA and cellular carbon production (balanced growth). DLI measurements in pelagic assemblages showed that TTI could be used to estimate bacterial productivity in the water column, but not to obtain accurate estimates of bacterial carbon production for sediments. However, when applied to a seasonal survey, TTI indicated that bacterial production in sediment slurries from Langebaan Lagoon was coincident with variations in the supply of organic material from primary production. Bacterial growth (production) on detrital materials in saltmarsh ecosystems may also support heterotrophic nitrogen fixation, as this mechanism supplements the N-requirements of bacterial growth in these N-deficient environments. The ecology and physiology of nitrogen fixation are further explored in the next section of this thesis.

CHAPTER 4

Rates of Nitrogen Fixation in Saltmarsh and Seagrass Ecosystems: Langebaan Lagoon

Abstract. Rates of nitrogen fixation were measured (acetylene reduction) in sediments and water from Langebaan Lagoon, a temperate saltmarsh ecosystem on the west coast of South Africa. Nitrogenase activity was not detected in lagoon water samples. In sediments, rates of nitrogen fixation varied seasonally, with higher rates (by ca. 1- to 7-fold) in summer than in winter. Nitrogenase activity was ca. 5-fold higher in muddy sediments from Geelbek, in the southern reaches, than in sandy sediments from Oesterwal, near the lagoon mouth. At both sites, nitrogenase activity was higher in surface sediments than in subsurface sediments. Highest rates (0.28 ± 0.07 nmol C_2H_4 g^{-1} h^{-1}) were associated with beds of *Zostera capensis* at Geelbek, which also had the highest organic carbon content. Additions of glucose (ca. 15mM) stimulated nitrogenase activity in all sediments by 1 to 3 orders of magnitude. Evidence indicated a significant relationship between nitrogenase activity in sediments and the availability of easily utilizable carbon sources. The degree of glucose-stimulation suggested that heterotrophic nitrogenase activity in subsurface sediments was more substrate-limited than in surface sediments.

INTRODUCTION

Under nitrogen-depleted conditions diazotrophic bacteria can supplement their nitrogen requirements with ammonium from nitrogen fixation. In many marine environments, including saltmarsh and seagrass ecosystems, nitrogen fixation is not only important as a source of combined nitrogen for bacterial growth (productivity), but also for primary productivity of marine macrophytes and algae with which many bacteria interact (Patriquin and Knowles, 1972; Capone *et al.*, 1979; Capone, 1982; O'Donohue *et al.*, 1991).

The development of the acetylene reduction assay (Stewart *et al.*, 1967; Hardy *et al.*, 1968) as a simple and sensitive method for measuring nitrogenase activity has enabled extensive studies of nitrogen fixation in many aquatic ecosystems. Thus, the role of nitrogen fixation in saltmarsh and seagrass ecosystems has received considerable attention. Both the rates of nitrogen fixation and the importance of fixation to the nitrogen budget of these systems appear to vary considerably (Howarth *et al.*, 1988a). Differences in the ecology of nitrogen fixation in saltmarsh and seagrass ecosystems include spatial and temporal variations, the relative importance of physiological groups involved in nitrogen fixation, and the regulation of rates by physical, chemical, and biological factors.

The purpose of this study was to examine spatial and temporal variations of nitrogen fixation in Langebaan Lagoon, and to assess the links between nitrogen fixation, heterotrophic activity, and primary production described in the previous chapter. The findings are also compared with those of similar systems. Objectives also included an examination of the relative importance of the relevant physiological groups in the lagoon.

METHODS

Sampling Procedure

Two sites were selected for study, Oesterwal (near the mouth of Langebaan Lagoon) and Geelbek (in the upper reaches). Detailed description of physical, chemical, and biological attributes of these sites has been presented previously (Chapter 2). Replicate samples (n=3) of water and sediment were collected from each site for determination of nitrogenase activity. Samples were always collected on the morning of spring tides, at low tide when the sediment flats were exposed, and were transported to the laboratory in an insulated container within 2 hours of collection. Spatial comparisons of nitrogenase activity also included an examination of five sediment microhabitats at each site (see Chapter 2 for a detailed description of microhabitats). In addition to spatial comparisons of nitrogenase activity, seasonality was also examined. A seasonal survey

was initiated during September 1989, and determinations were made in January 1990, April 1990, July 1990, October 1990, and in January 1991.

Nitrogen fixation (acetylene reduction) assays

Water samples (15ml), or sediment samples (25g wet weight), were placed into 25ml glass bottles. Filtered (0.2μm) seawater (2ml), with or without glucose (0.9mg glucose g^{-1} wet weight for sediments, or 15mM final concentration for water), was added, the bottles were sealed, and the headspace replaced with 85% argon and 15% acetylene (Fedgas, SA). Bottles were then incubated in the dark at 20°C on their sides, to facilitate maximum areal exposure between the water or sediment and the headspace. Gas samples (100μl) were withdrawn from the headspace at predetermined time intervals for determination of ethylene content by gas chromatography, as previously described (Chapter 2).

RESULTS

To determine if cyanobacteria have a significant role in nitrogen fixation in Langebaan Lagoon, sediments from five microhabitats (see Chapter 2) and water were examined visually and microscopically for the presence of cyanobacteria during each season. Cyanobacteria were never identified in water or in sediments from burrow openings, burrow linings, or 10cm subsurface. However, *Lyngbya* spp. were occasionally identified as small mats occurring ca. 1mm below the surface of *Zostera* bed sediments and surface sediment between burrows at both sites. Densities (by visual estimation) of these cyanobacteria were apparently higher at Geelbek than at Oesterwal; densities were also apparently higher in *Zostera* beds than in exposed sediment, and were higher in summer than in winter. However, the patchy nature of their distribution, even in *Zostera* beds at Geelbek during summer, is an indication that their numbers were not high overall. These observations reflect the findings of Fielding *et al.* (1988) who found that a high percentage (96%) of the samples they examined from Langebaan Lagoon did not contain evidence of cyanobacteria.

Although *Lyngbya* spp. are non-heterocystous, some species are able to fix nitrogen (Jones, 1990; Paerl *et al.*, 1991). Further examinations of cyanobacteria from Langebaan Lagoon therefore considered whether they could fix nitrogen. Extensive efforts to isolate and purify these strains on a range of media under different conditions (Rippka, 1988) were not successful, and hence DNA could not be extracted from these organisms for probing against *nif* genes. In an alternative approach to this study, rates of acetylene reduction in sediments colonized by cyanobacteria were compared with with those in sediments collected away from the mats. *In vitro* assays were conducted under different conditions of incubation, including different light regimes with or without oxygen (air). In all of these assays, there was no significant difference ($p > 0.05$) in nitrogenase activity in sediments from within mats compared with that in sediments collected away from mats, indicating that these *Lyngbya* spp. were not contributing significantly to nitrogen fixation. Further studies of nitrogen fixation in Langebaan Lagoon subsequently focussed on heterotrophic activity.

Preliminary experiments examined the effect of pC_2H_2 on acetylene reduction. Acetylene reduction activity plateaued between pC_2H_2 of 0.10 and 0.20 atm, and therefore all subsequent assays were conducted with a pC_2H_2 of 0.15 atm. No ethylene production was detected in the absence of added acetylene, nor was ethylene production detected in autoclaved controls. When planning experiments, practicality, economy, minimal disturbance and a design that permitted comparisons of unamended samples with amended samples was sought. Slurries were required to facilitate exposure of the sediment to different amendments. However, amendments were added in the smallest possible volume to minimise disturbance of nutrient concentrations in the sediment (see above). Studies that have employed perfusion technique have indicated that disturbance of ammonium concentrations may affect *in situ* rates of nitrogenase activity (Capone and Carpenter, 1982).

Reduction of acetylene to ethylene (nitrogenase activity) was not detected for water samples. Conditions of incubation of water samples were modified (light/dark, aerobic/anaerobic, and amendment with glucose) in attempts to stimulate activity. However, none of these conditions produced detectable rates of nitrogenase activity in

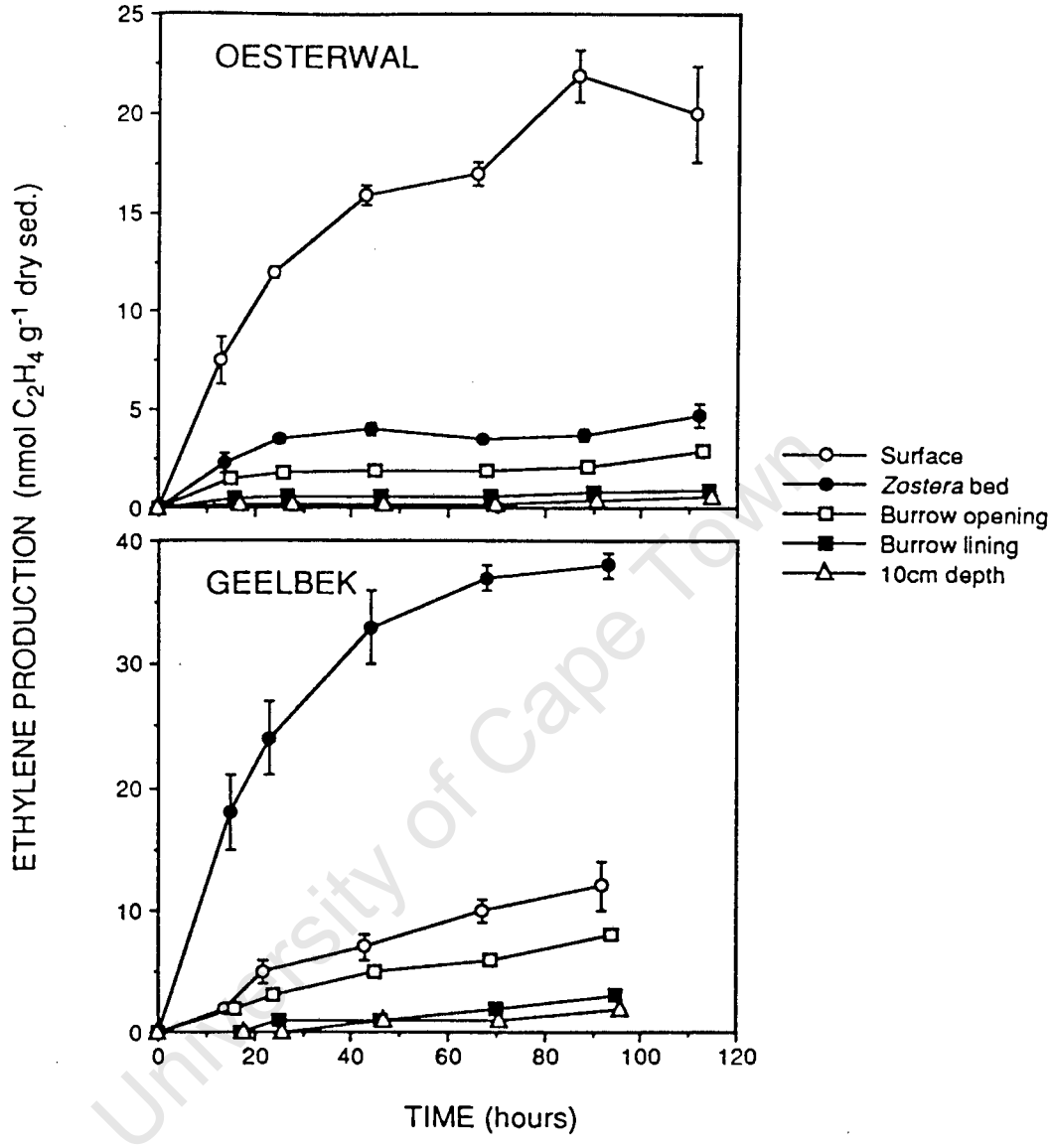

Figure 1. Production of ethylene with time in sediments from Langebaan Lagoon.

water samples. In contrast, rates of ethylene production were usually detectable in sediments. Examples of time-course production of ethylene by unamended sediments are presented in Fig. 1. Rates of ethylene production were linear for at least 12 hours and occasionally for as long as 4-5 days. Rates were generally low (usually < 1 nmol C_2H_4 g^{-1} dry sed. h^{-1}), especially during winter and in subsurface sediments (see below), and therefore ethylene production was often not detectable during incubations

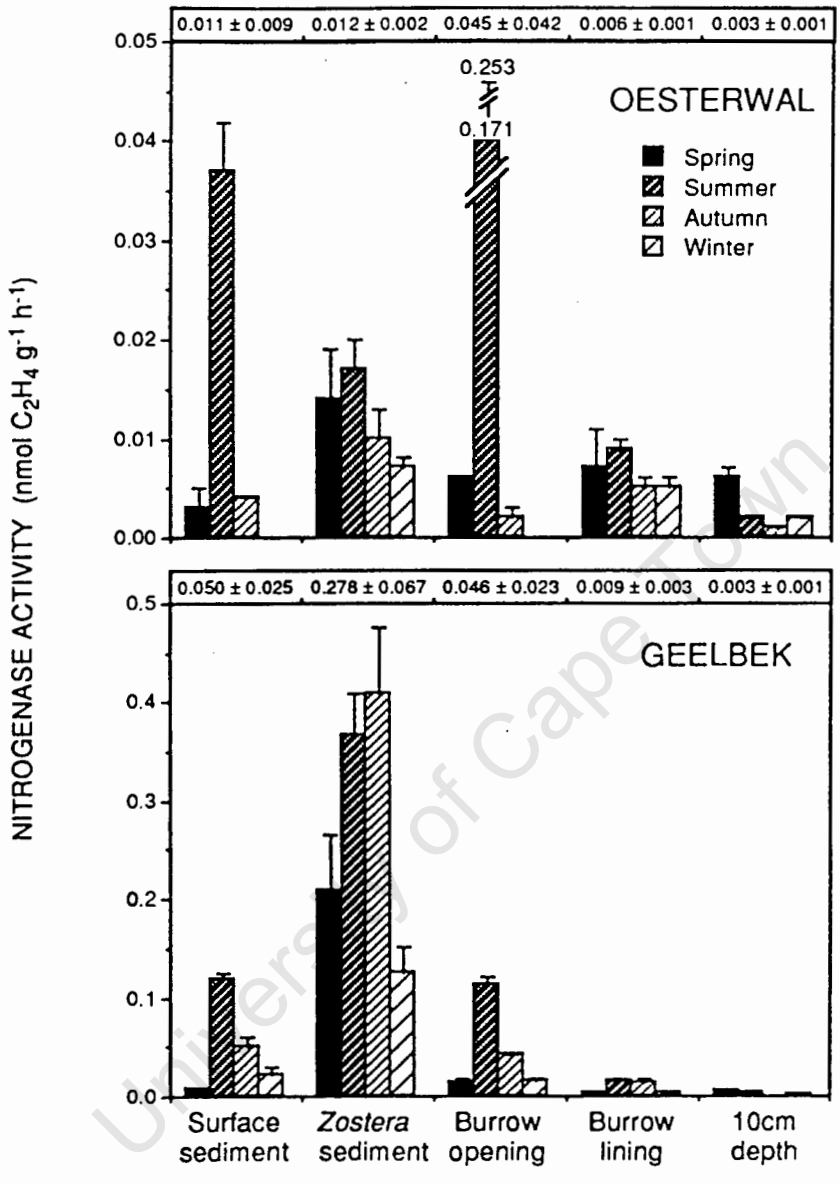

Figure 2. Seasonal variation of nitrogenase activity (means ± SE) in sediments from Langebaan Lagoon. Mean values (± SE) for each habitat are shown above histograms.

of less than 6 hours. However, rates of detectable ethylene production did not indicate a noticeable lag phases (Fig. 1).

Seasonal measurements of nitrogenase activity were made in August - September 1991 (spring), December 1991 - January 1992 (summer), May 1992 (autumn), and July 1992 (winter). Nitrogenase activity in all sediments was generally highest in summer/autumn

and lowest in winter (Fig. 2). Rates in all sediments from Geelbek were higher than those from Oesterwal (significant for surface between burrows, and *Zostera* bed sediment). Nitrogenase activity was 5-fold higher in Geelbek sediments than in Oesterwal sediments when overall means (microhabitats and seasons) were compared. At both sites, rates were highest in surface sediments (surface sediment between burrows, at burrow openings, and from *Zostera* beds), while rates were lower in subsurface sediments (burrow lining and 10cm depth). Rates in sediments from 10cm below the surface were 4- to 17-fold lower than those in surface sediments, and 4- to

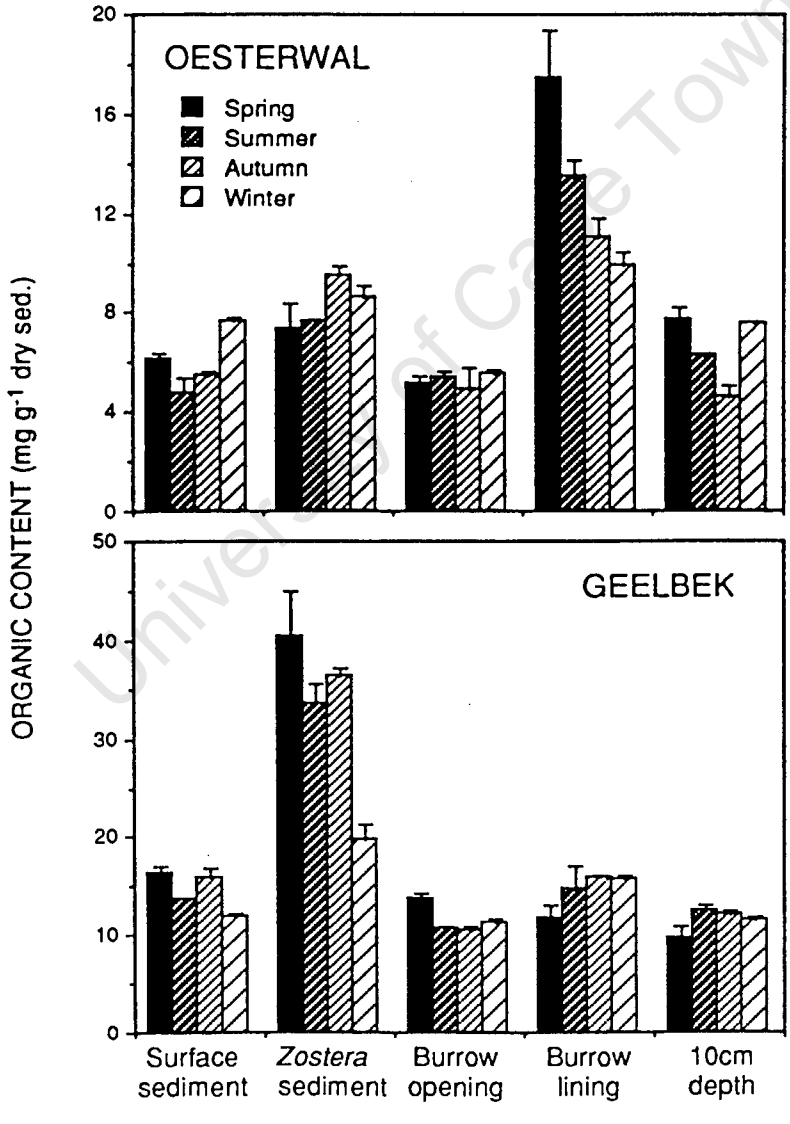

Figure 3. Variation in organic contents of sediments from Langebaan Lagoon during the seasonal survey of nitrogenase activity.

93-fold lower than those in *Zostera* bed sediments. At Oesterwal, rates were highest in burrow opening sediment, although the annual mean activity for this sediment type was strongly biased by relatively high summer values (Fig. 2). Overall, highest rates of nitrogenase activity were measured in *Zostera* bed sediment from Geelbek (annual mean, 0.28 $\pm$ 0.07 nmol C_2H_4 g^{-1} dry sed. h^{-1}) (Fig. 2). Organic contents of sediments were generally higher at Geelbek than at Oesterwal (Fig. 3; see also Chapter 2). Organic contents varied between sediment microhabitats at each site; at Oesterwal, burrow lining had the highest organic content, whereas at Geelbek, *Zostera* bed sediment had the highest organic content (Fig. 3).

Addition of glucose stimulated nitrogenase activity in all sediment types (Table 1). Glucose-stimulated rates were 3- to 1332-fold (average, 220-fold) higher than rates in unamended sediments. There was no clear pattern to the degree of stimulation between seasons or between sites when averages were considered (Table 1). However, glucose-stimulation was significantly ($p < 0.05$) greater in subsurface sediments than in surface sediments (Table 1). Moreover, there was a significant ($p < 0.001$) correlation between

TABLE 1. Ratio of nitrogenase activity in glucose-amended:unamended sediments.

	Surface sediment	*Zostera* sediment	Burrow opening	Burrow lining	10cm depth	Average
OESTERWAL						
Spring	72	259	38	96	148	123
Summer	49	95	7	100	740	198
Autumn	3	335	129	122	1332	384
Winter[a]		211		73	408	231
Average	41	225	58	98	657	216
GEELBEK						
Spring	142	36	20	657	673	306
Summer	16	8	6	129	536	139
Autumn	33	12	7	71	815	188
Winter	48	36	19	249	945	260
Average	60	23	13	277	742	223

[a] Ratios were not available for surface sediment and burrow opening from Oesterwal during winter as nitrogenase activity was not detectable in unamended samples from these microhabitats.

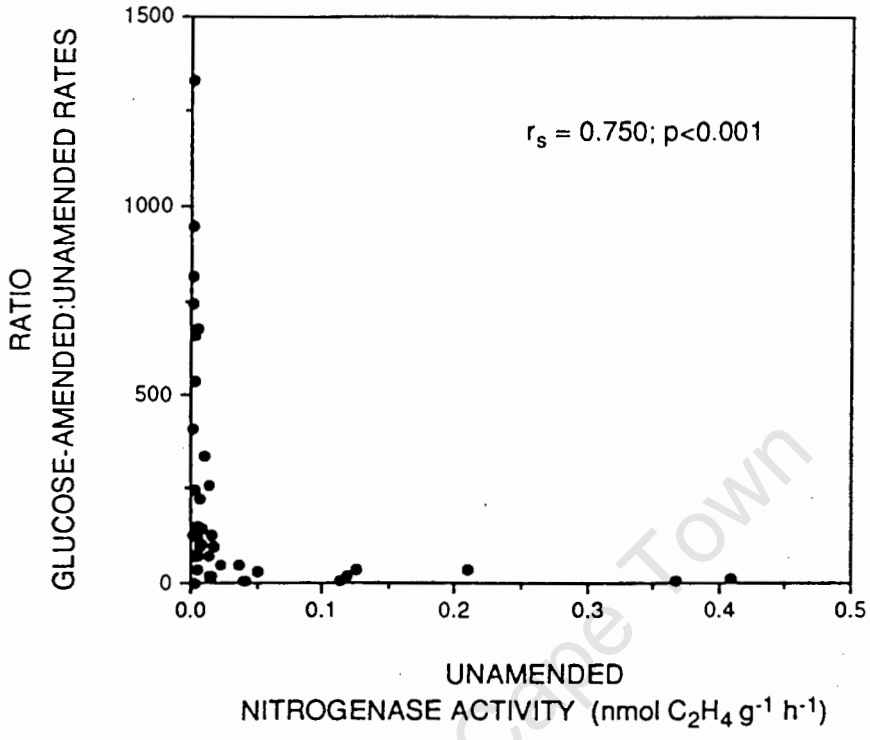

Figure 4. Relationship between the magnitude of glucose-stimulation of nitrogenase activity and activity in unamended sediments. Spearman's non-parametric test by rank for paired data revealed a significant correlation as indicated.

nitrogenase activity in unamended sediments and the magnitude of glucose-stimulation of activity (Fig. 4). The magnitude of glucose-stimulation was higher when activities in the unamended sediments were low.

DISCUSSION

Several workers have reported that cyanobacteria may dominate nitrogen fixation in some saltmarsh environments (Jones, 1974; Carpenter *et al.*, 1978). In other such environments heterotrophic bacteria appear to dominate nitrogen fixation (Whitney *et al.*, 1975; Hanson, 1977a; Teal *et al.*, 1979; Dicker and Smith, 1980c). In the present study, no indication was found that cyanobacteria play a major role in nitrogen fixation in Langebaan Lagoon. Consequently, attention was focussed on heterotrophic nitrogen fixation, for which there was evidence.

Nitrogenase activity was not detected in water from Langebaan Lagoon. This effect was possibly a function of low numbers of nitrogen-fixing bacteria, and relatively oxygen-rich and organic matter-deficient conditions in water, compared with those conditions in sediments (Chapter 2). Factors such as these are known to regulate nitrogen fixation in marine environments (Paerl, 1990). In contrast to the lack of detectable nitrogenase activity in water, activity was detected in sediments. Strong seasonality was noted for nitrogenase activity in all sediment types. Seasonal trends in nitrogenase activity generally indicated low activity during winter with a gradual increase during spring to peak rates during summer/autumn. In subsurface sediments peak rates occurred during spring. This seasonal pattern reflected the higher densities of nitrogen-fixing bacteria in these sediments in summer than in winter (Chapter 2) and with the seasonality of bacterial production (tritiated thymidine incorporation, TTI) in sediments (Chapter 3). Furthermore, the proportion of general heterotrophic isolates from these sediments that were able to reduce acetylene (fix nitrogen), was also higher in summer than in winter (Chapter 2). Seasonality of nitrogenase activity is not unusual; several other workers have reported similar findings for other saltmarsh and seagrass ecosystems (Jones, 1974; Hanson, 1977b; Carpenter *et al.*, 1978; Teal *et al.*, 1979; Capone and Taylor, 1980; Dicker and Smith, 1980b; Capone, 1982; Jones, 1982; Smith and Hayasaka, 1982b; Whiting *et al.*, 1986; O'Donohue *et al.*, 1991).

Spatial patterns of nitrogenase activity reflected patterns of bacterial production (Chapter 3). Overall, nitrogenase activity at Geelbek was 5-fold higher than at Oesterwal. Rates of bacterial production were also higher at Geelbek than at Oesterwal (Chapter 3). Comparisons of microhabitats between sites showed that nitrogenase activity was significantly higher in surface sediment and *Zostera* bed sediment from Geelbek, than in those from Oesterwal ($p < 0.05$, Wilcoxon paired t-test by rank) (Fig. 2). At both sites, nitrogenase activity was highest in surface sediments, while rates were lower in subsurface sediments. This pattern also reflects the pattern of TTI, which was highest in surface sediments and decreased with sediment depth (Chapter 3). Decrease in nitrogenase activity with sediment depth has also been noted for other marine systems (Whitney *et al.*, 1975; Capone and Taylor, 1980; O'Neil and Capone, 1989).

Rates of nitrogen fixation in sediments from Langebaan Lagoon were low when compared with rates for other systems (Table 2). The precise reasons for these relatively low rates are not clear. Besides the limited participation of cyanobacteria in

TABLE 2. Rates of nitrogen fixation in Langebaan Lagoon and similar marine ecosystems.

	Nitrogen fixation[a] (μmol N m^{-2} h^{-1})	Reference
Sediments free of macrophyte cover		
Oesterwal, Langebaan, SA	0.00 - 0.13	This study
Geelbek, Langebaan, SA	0.03 - 0.40	This study
Narragansett Bay, USA	0.4	Seitzinger and Garber, 1987
Moreton Bay, Australia	0.5 - 0.7	O'Donohue *et al.*, 1991
Norton Sound, Alaska	0.8	Haines *et al.*, 1981
Kamishak Bay, Alaska	1.0	Haines *et al.*, 1981
Elson Lagoon, Alaska	2.1	Haines *et al.*, 1981
Great South Bay, USA	3.3 - 8.0	Capone, 1982
Flax Pond (pannes), USA	4.5	Whitney *et al.*, 1975
Flax Pond (mudflat), USA	9.7	Whitney *et al.*, 1975
Sediments with macrophyte cover		
Oesterwal, Langebaan, SA[b]	0.02 - 0.06	This study
Geelbek, Langebaan, SA[b]	0.42 - 1.36	This study
Moreton Bay, Australia[c]	3.0 - 7.3	O'Donohue *et al.*, 1991
Flax Pond, USA[d]	4.6 - 8.3	Whitney *et al.*, 1975
Great South Bay, USA[e]	5.3 - 8.0	Capone, 1982
Sediments with cyanobacterial mat		
Flax Pond, USA	205.0	Whitney *et al.*, 1975

[a] Where necessary, calculations assumed a conversion factor of 3 moles ethylene formed to 1 mole dinitrogen fixed (Stewart *et al.*, 1967)
[b] *Zostera capensis*
[c] *Zostera capricorni*
[d] *Spartina alterniflora*
[e] *Zostera marina*

nitrogen fixation in these sediments (see Table 2 for an example of cyanobacterial nitrogen fixation), and the low densities of heterotrophic nitrogen-fixing bacteria (see Chapter 2), a broad range of environmental and biochemical factors may also influence

nitrogenase activity in benthic environments (Capone, 1988). For instance, detritus derived from vascular plants is a quantitatively significant source of particulate organic matter in saltmarsh and seagrass ecosystems, and thus represents the bulk of particulate organic matter available to heterotrophic bacteria (Hodson *et al.*, 1983; Mann, 1988). In Langebaan Lagoon, saltmarsh and seagrass detritus constituted ca. 90% of the organic carbon content of sediments (Chapter 2). However, detritus derived from vascular marine plants includes significant quantities of complex polysaccharide-based materials, particularly lignocellulose, which are resistant to biodegradation (MacCubbin and Hodson, 1980, Benner *et al.*, 1984). Several workers have indicated that heterotrophic nitrogen fixation in saltmarsh and seagrass ecosystems may be limited by the availability of easily utilizable carbon/energy sources (Hanson, 1977a; Dicker and Smith, 1980b; Capone, 1982; Capone and Budin, 1982). Addition of glucose to sediments from Langebaan Lagoon stimulated nitrogenase activity by 3- to 1332-fold during the seasonal survey. The degree of glucose-stimulation of activity was negatively correlated with the rate of activity in unamended sediments; stimulation tended to be greatest when activity in unamended sediments was low (Fig. 4). These data indicate a significant relationship between nitrogenase activity and the availability of easily utilizable carbon sources in Langebaan sediments. Rates of nitrogenase activity below ca. 0.03 nmol C_2H_4 g^{-1} dry sed. h^{-1} are clearly held at low levels by an inadequate supply of readily utilizable carbon. Above this value, additions of glucose had relatively little effect on nitrogenase activity.

Although the decrease in nitrogenase activity with depth may be linked to a decline in bacterial productivity (TTI), there was also evidence that nitrogenase activity was more subject to organic substrate-limitation in subsurface sediments than in surface sediments. This may be explained if organic matter in subsurface sediments is more refractory, due to microbial utilization of readily available components in the surface layer before or during translocation of some matter to subsurface sediments. The following chapter (5) further explores the way in which nitrogenase activity is modulated by organic substrates commonly found in lagoon sediments.

CHAPTER 5

Nitrogenase Activity in Marine Sediments from a Temperate Saltmarsh Lagoon: Modulation by Organic Substrates, Inorganic Nitrogen, and Physical Factors

Abstract: Nitrogenase activity (acetylene reduction) was examined in marine sediments associated with seagrass beds and thalassinidean prawn burrows, in a temperate saltmarsh lagoon. Nitrogenase activity in unamended sediments from different microhabitats ranged from undetectable values to ca. 0.75 nmol C_2H_4 g^{-1} dry sediment $hour^{-1}$. Higher rates of nitrogen fixation occurred in surface (< 5mm) sediments than in subsurface sediments (10cm below the surface). The effects of additions of fermentable and oxidizable carbon substrates on nitrogenase activity indicated that fermenting bacteria, rather than anaerobes, dominated nitrogen fixation under anaerobic conditions in surface sediments. The bulk of organic material available to heterotrophic bacteria in saltmarsh lagoons is derived from primary production. Amendment of sediments with structural plant polysaccharides showed that xylan and alginate stimulated nitrogenase activity on average by 5- to 18-fold relative to unamended sediments, whereas cellulose and carrageenan were less effective. Amendment of sediments with storage plant polysaccharides produced the greatest stimulation of nitrogenase activity. Addition of laminarin and glycogen (amylopectin) significantly ($p < 0.05$) stimulated nitrogenase activity by 19- to 92-fold. In contrast to these polysaccharides of plant origin, chitin (a polymer of prawn exoskeletons) did not significantly enhance nitrogenase activity in these sediments. Ammonium did not appear to play a major role in the regulation of nitrogenase activity in these sediments, as *in situ* concentrations were ca. 20-fold lower than the concentration (50 μM) which resulted in a slight (0-15%) reduction in nitrogenase activity. Nitrate caused a concentration-dependent inhibition of nitrogenase activity, with ca. 50% inhibition of activity when 100μM nitrate was added to sediments collected near to the mouth of the lagoon. Nitrate (ca. 20μM) is seasonally available to this lagoon due to southern Benguela upwelling during spring and summer. Nitrogenase activity was optimal at 20°C regardless of site, season (spring or summer), or the availability of glucose.

Aerobic conditions stimulated nitrogenase activity by 2- to 20-fold in surface sediments, but not in subsurface sediments, indicating that aerobic or microaerophilic respiration were significant mechanisms of generating energy for nitrogenase activity under aerobic conditions in surface sediments. Oxygen-stimulation of nitrogenase activity was less marked in aerobic sediments around the rim of prawn burrow openings where subsurface sediments had been displaced to the surface by bioturbation. Light stimulated nitrogenase activity in surface sediments from one site under anaerobic, but not under aerobic conditions, suggesting that the observed effect of light on nitrogenase activity may have been due to oxygen from oxygenic photosynthesis.

INTRODUCTION

Rates of nitrogen fixation in the marine environment are often low (Howarth *et al.*, 1988a; Seitzinger, 1988; see Chapter 4). The importance of the control of nitrogen fixation in marine environments has been emphasized (Capone, 1988; Paerl, 1990), since nitrogen fixation can be a major input of nitrogen to saltmarsh and seagrass systems, and other marine environments (Patriquin and Knowles, 1972; Hanson, 1977a; Capone *et al.*, 1979; Capone, 1982; O'Donohue *et al.*, 1991).

The benthic macrofauna of many estuaries in southern Africa is dominated by the thalassinidean prawns, *Callianassa kraussi* and *Upogebia africana*, which reach densities of 350 and 400 individuals m^{-2} sediment respectively (Hanekom, 1980). Thalassinidean prawns exert considerable influence on ecosystem processes through their activities while burrowing and feeding (Bird, 1982; Branch and Pringle, 1987). For instance, the burrowing activities of thalassinids result in profound bioturbation, which has been shown to contribute to sediment transportation (Roberts *et al.*, 1981), increase in oxygenation (Dye, 1978), alteration of the depth-distribution of microalgae (Branch and Pringle, 1987), and has been correlated with enhanced numbers of bacteria (Yingst and Rhoads, 1980; Branch and Pringle, 1987). Bioturbation by thalassinids may also modulate cover by seagrass beds (Suchanek, 1983). Thus, the activities of thalassinids could directly, and potentially indirectly through effects on nitrogen

fixation, affect primary production of diatoms and macrophytes, which provide the food source of these invertebrates as well as many other consumers.

The diversity and variability of benthic microenvironments is likely to be significant in the modulation of nitrogenase activity, since the availability of organic substrates, inorganic nitrogen, and temperature, oxygen, and light have all been shown to be important (Capone, 1988; Paerl, 1990; Bebout *et al.*, 1993). In temperate saltmarsh environments, such as Langebaan Lagoon, variability of physico-chemical factors (Chapter 2) may influence bacterial activity in general (Chapter 3), and nitrogenase activity in particular (Chapter 4). Several workers have reported on the stimulation of nitrogenase activity in marine sediments by mono- and disaccharides (Hanson, 1977b; Dicker and Smith, 1980a; Capone, 1982; Capone and Budin, 1982; O'Neil and Capone, 1989). Studies in Langebaan Lagoon have indicated a significant relationship between nitrogenase activity and the availability of easily utilizable carbon sources (Chapter 4). However, to my knowledge, there have been no reports of similar studies which have examined the potential of polysaccharides to support nitrogen fixation in saltmarsh ecosystems, even though most of the carbon available for bacterial use in saltmarsh ecosystems should be derived primarily from such complex polysaccharide-based plant materials. Bacterial use of complex carbohydrates is quite different from their use of simple ones (Gottschalk, 1986), with possible implications for energy-demanding processes such as nitrogen fixation.

In the present study, acetylene reduction assays were used to measure nitrogenase activity (Hardy *et al.*, 1968) in sediment samples from Langebaan Lagoon. Measurements of nitrogenase activity were compared on a macro-scale (two sites at opposite ends of the lagoon) and on a micro-scale (different microhabitats related to seagrass beds and prawn burrows at each site). Having established that nitrogenase activity is present in sediments but not in the water column (Chapter 4), and that glucose enhanced this activity, this study sought to further explore the modulation of nitrogenase activity by the availability of simple and complex carbohydrates, by the availability of nitrogenous compounds, and by physical factors (temperature, oxygen, and light).

MATERIALS AND METHODS

Sampling Procedure

Two sites, Oesterwal (near the lagoon mouth) and Geelbek (in the southern reaches), were selected for study. Detailed description of physical, chemical, and biological attributes of these sites has been presented previously (see Chapter 2). Spatial comparisons of nitrogenase activity also included an examination of five sediment microhabitats at each site (see Chapter 2 for detailed description of microhabitats). Replicate samples (n=2 or 3, depending on the experiment) of sediment were collected for examination of nitrogenase activity. Samples were always collected on the morning of spring tides, and were transported to the laboratory in an insulated container within 2 hours of collection.

Acetylene Reduction Assays

Sediment samples (25g wet weight) were weighed into glass bottles and amended as described below. The bottles were sealed, and the headspace replaced with 85% argon and 15% acetylene (Fedgas, SA) unless otherwise stated. Bottles were incubated under conditions of light/dark, or different temperatures, as required. Gas samples (100μl) were withdrawn with a gas-tight syringe at predetermined time intervals for determination of ethylene content by gas chromatography, as described previously (Chapter 2).

Assay Amendments

The effects of NH_4^+, organic substrates, and O_2 on acetylene reduction were examined. To determine the effects of O_2 and light, sediment samples were incubated in 100ml glass bottles with 50ml 0.2μm filter-sterilised seawater, either aerobically (ie. 85% air, 15% C_2H_2) or anaerobically (as above), and under light/dark conditions. Bottles were incubated on a shaker to ensure gaseous equilibration. The effects of nitrogenous compounds and different carbohydrates on acetylene reduction were also

examined. Sediments were amended in 25ml wide-neck glass bottles by addition of these substrates in 2ml 0.2μm filter-sterilised seawater (see Chapter 4). The effect of glucose at final concentrations of 0, 0.015, 0.15, 1.5, 15, 150, and 300 mM was examined. The effects of other mono- and disaccharides, including Na-succinate, Na-citrate, Na-lactate, Na-acetate, sucrose, and the sugar alcohol mannitol (all at 15mM final concentration), were also examined. Polysaccharides, including avicel microcrystalline cellulose (FMC Corp.), glycogen (Merck), Na-alginate (BDH), oat spelt xylan, carrageenan, laminarin, starch, and crab shell chitin (all Sigma), were added at 0.9mg g^{-1} wet sediment, which was the mass of substrate approximately equivalent to mono- and disaccharide amendments used by others (Capone and Budin, 1982; Gandy and Yoch, 1988) and approximately equivalent to 15μM glucose (see above). Chitin substrate was prepared according to the method of Reichenbach and Dworkin (1981). Ammonium chloride was added at final concentrations of 0, 50, 100, 500, and 1000 μM. Sodium nitrate was added at final concentrations of 0, 0.1, 1, and 10 mM. Glutamic acid and urea were added at concentrations of 2 and 20 μmoles g^{-1} wet sediment respectively. Yeast extract was added at 0.6mg g^{-1} wet sediment. These concentrations of glutamic acid, urea, and yeast extract were used by Dicker and Smith (1980a).

Statistical Analysis of Data

Statistical analysis of data was carried out by analysis of variance (ANOVA) on untransformed data sets where Bartlett's test for homogeneity of variance indicated homoscedasticity. ANOVA of transformed ($\log_{10}$) data sets was carried out when heteroscedasticity was encountered in untransformed data. Student's t-test was used for statistical comparison of paired means.

RESULTS

Regulation by C-source Availability

Addition of glucose at concentrations from 0.15 to 150 mM stimulated a concentration-dependent increase in nitrogenase activity (acetylene reduction) in sediments from Oesterwal and Geelbek (Fig. 1). No detectable stimulation occurred below 0.15mM glucose, whereas stimulation of rates plateued between 150 and 300 mM glucose. This response was similar between seasons for Geelbek, but summer samples from Oesterwal were more sensitive to stimulation by glucose than samples collected in the spring. A more detailed comparison of the effect of glucose on nitrogenase activity between seasons is presented in Chapter 4. A glucose concentration of 15mM was selected as the standard concentration for further experiments (see below), as this concentration stimulated nitrogenase activity in Langebaan sediments (Fig. 1), and was similar to concentrations used by other workers (Capone and Budin, 1982; Gandy and Yoch, 1988).

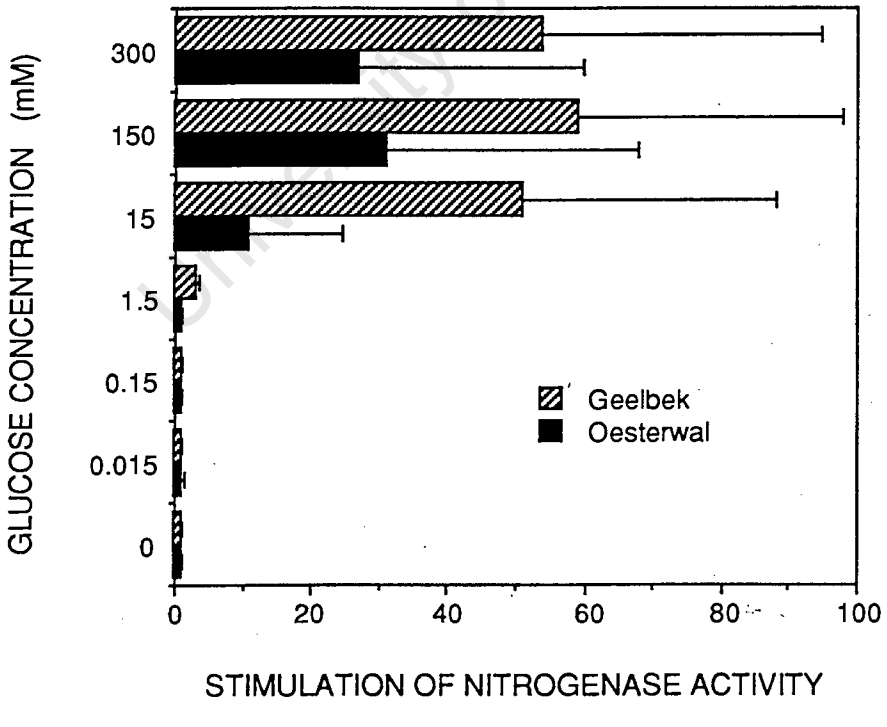

Figure 1. Effect of glucose concentrations on nitrogenase activity. Nitrogenase activity has no units, as values represent ratios of glucose-amended:unamended rates, and thus represent stimulation (by x-fold) of unamended rates. Values are means ± SD of two experiments; one experiment was carried out in September 1991, and the other in February 1992.

The effects of different mono- and disaccharides (fermentable and oxidizable substrates) on nitrogenase activity are presented in Table 1. At both sites (Oesterwal and Geelbek) glucose stimulated nitrogenase activity to the greatest extent. Additions of mannitol and

TABLE 1. Effects of mono- and disaccharides, and mannitol, on nitrogenase activity in surface sediment between burrows.

Substrate	Stimulation[a] Oesterwal	Geelbek
Glucose	3 - 72 (4)	16 - 142 (4)
Mannitol	11 - 63 (2)	11 - 15 (2)
Sucrose	61 (1)	57 (1)
Succinate	1 (1)	6 (1)
Citrate	1 (1)	5 (1)
Lactate	1 (1)	1 (1)
Acetate[b]	1 (3)	1 (3)

[a] Ratios of nitrogenase activity in substrate-amended:unamended sediments. Values represent range of stimulation, with number of experiments in parentheses. Where more than one experiment was carried out, experiments were carried out in different months, usually in different seasons, during 1990-93.

[b] Two experiments with acetate were incubated in the presence of 10% CO_2, 75% N_2, and 15% C_2H_2; the third experiment was incubated without CO_2.

sucrose stimulated nitrogenase activity to a similar extent as glucose. However, relatively poor stimulation of nitrogenase activity was produced by succinate and citrate, while no significant effect was produced by lactate and acetate (Table 1).

The effects of defined polysaccharides on nitrogenase activity are compared with unamended and glucose-stimulated rates in Figs. 2, 3, 4, and Table 2. Rates of nitrogenase activity in unamended (control) sediments from Oesterwal and Geelbek are shown in Fig. 2 (microhabitats at each site) and Table 2 (means $\pm$ SD for each site). Comparison of nitrogenase activity between the two sites (macroscale) showed that rates in unamended samples were greater on average (by 2.4-fold, not significant, $p > 0.05$) in the fine, muddy sediments from Geelbek than in coarse, sandy sediments from Oesterwal during these experiments (Table 2). Spatial comparison on a microscale (different microhabitats at each site) showed that unamended nitrogenase activity was

highest in surface and *Zostera* sediments, and was lowest in subsurface sediments (Fig. 2). *Zostera* bed sediment from Geelbek had the highest organic content (Chapter 2) and also supported the highest rates of nitrogenase activity (Fig. 2).

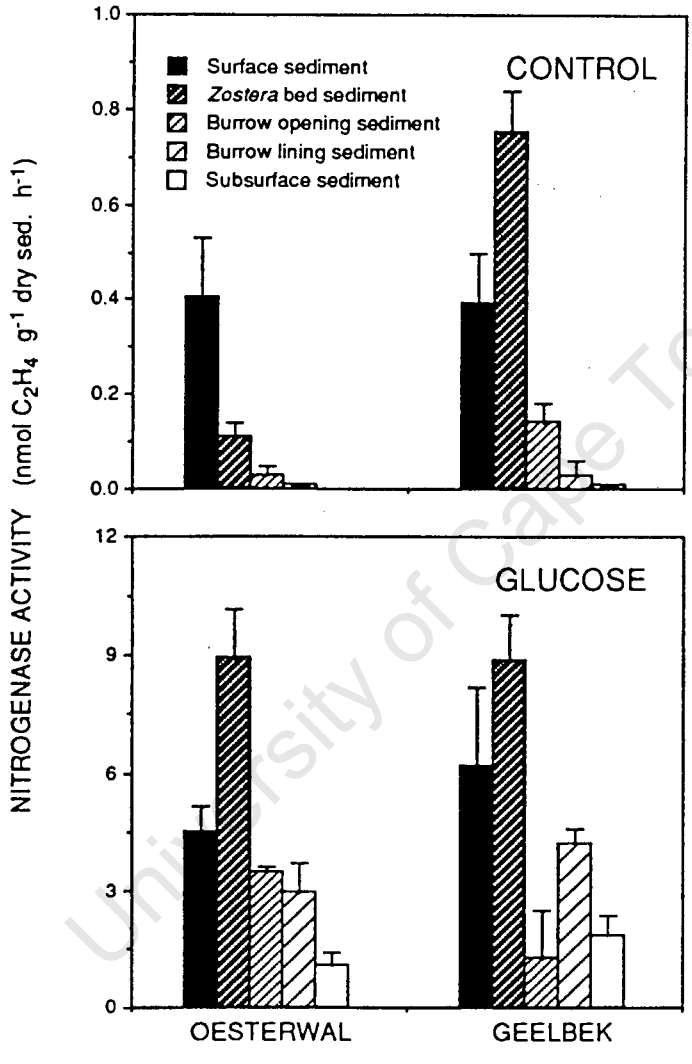

Figure 2. Nitrogenase activity in sediments without amendment (control) or amended with glucose. Note that scales of y-axes are not equal. Sediments were collected from five microhabitats at Oesterwal (12.3.93) and Geelbek (24.3.93). Values represent means ± SD of duplicate samples. Overall means (sites and microhabitats) for each treatment are: Control, 0.19 ± 0.25; Glucose, 4.32 ± 2.87 nmol C_2H_4 g^{-1} dry sed. h^{-1}.

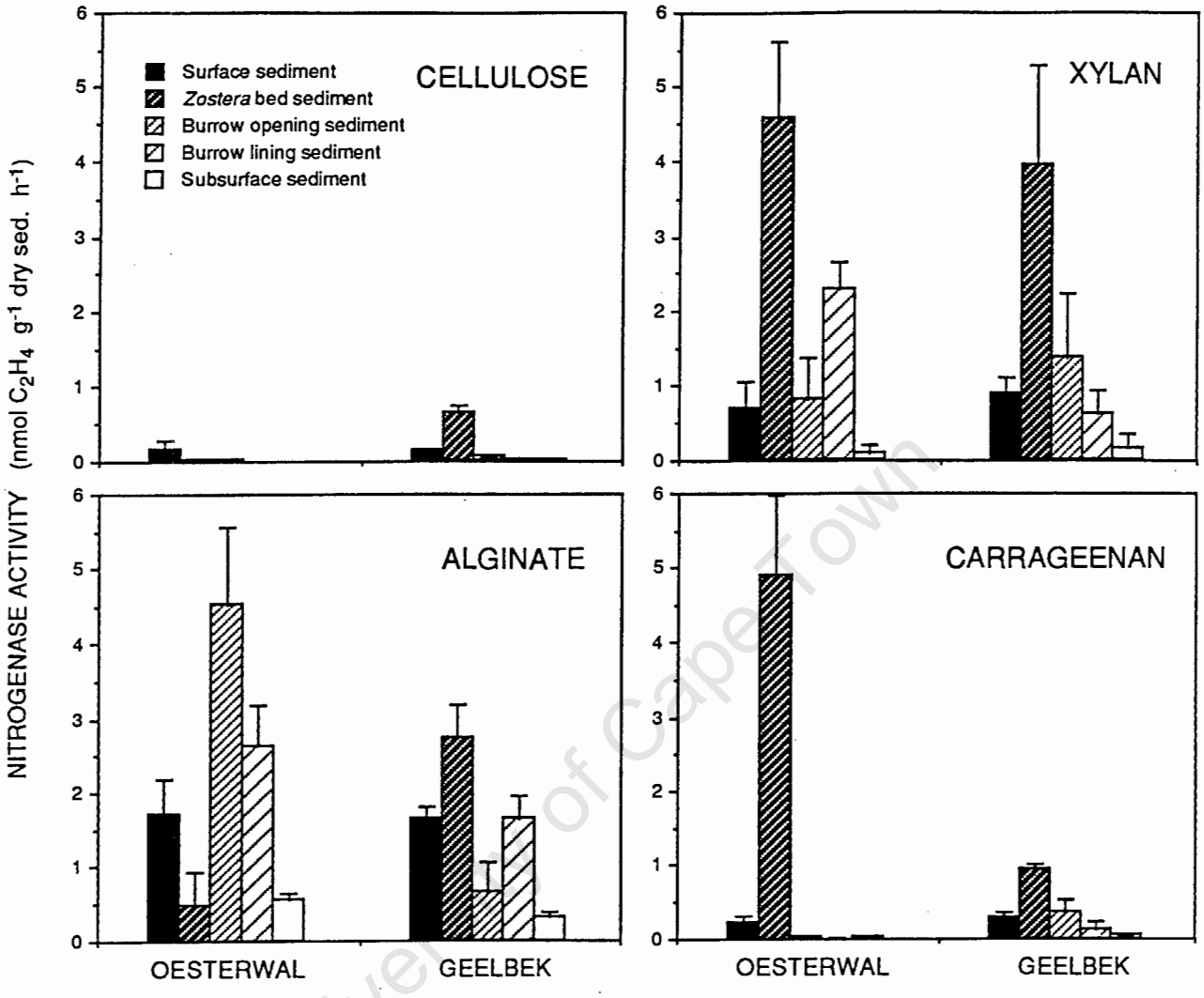

Figure 3. Effects of additions of structural plant polysaccharides on nitrogenase activity in sediments from Oesterwal and Geelbek. Sediments were collected from five microhabitats at Oesterwal (12.3.93) and Geelbek (24.3.93). Values represent means ± SD of duplicate samples. Overall means (sites and microhabitats) for each treatment are: Cellulose, 0.11 ± 0.20; Xylan, 1.55 ± 1.57; Alginate, 1.69 ± 1.32; Carrageenan, 0.70 ± 1.50 nmol C_2H_4 g^{-1} dry sed. h^{-1}.

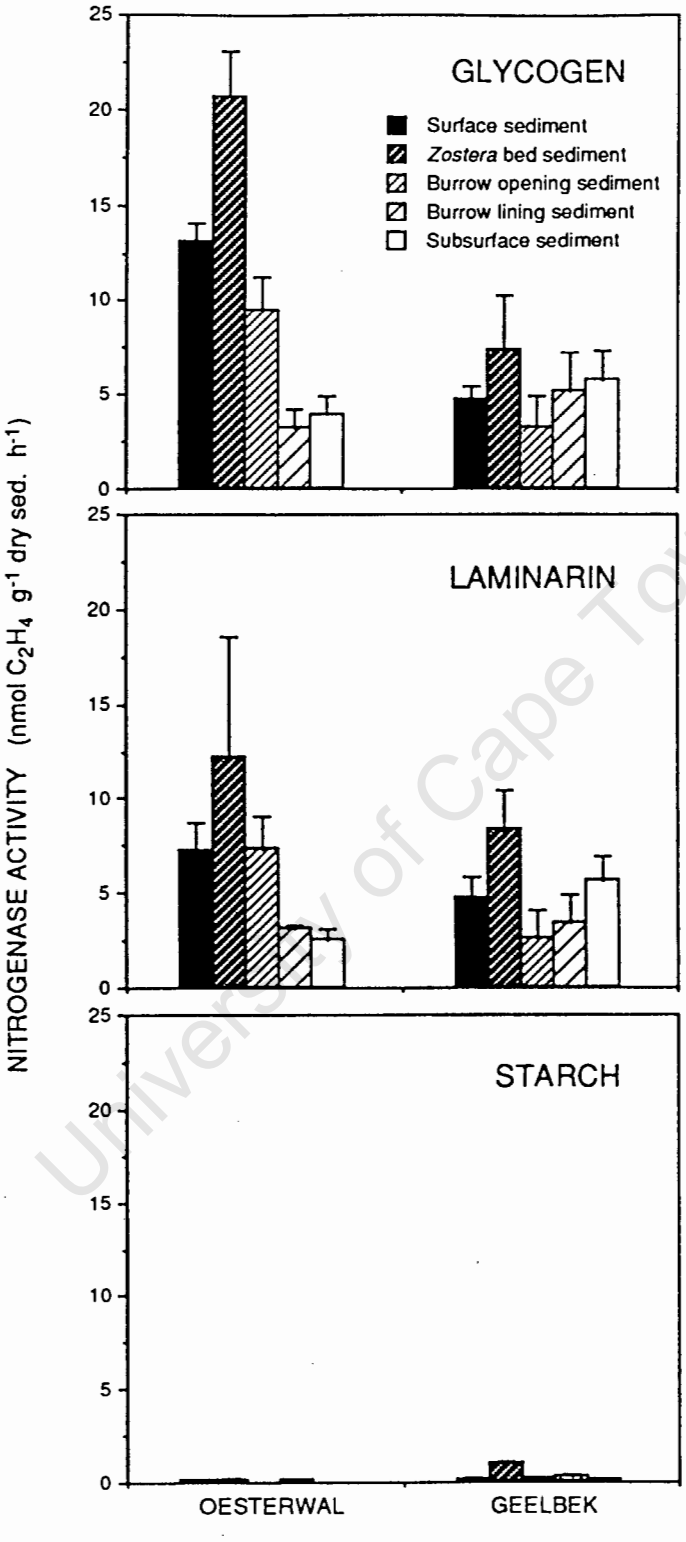

Figure 4. Effects of additions of storage plant polysaccharides on nitrogenase activity in sediments from Oesterwal and Geelbek. Sediments were collected from five microhabitats at Oesterwal (12.3.93) and Geelbek (24.3.93). Values represent means ± SD of duplicate samples. Overall means (sites and microhabitats) for each treatment are: Starch, 0.21 ± 0.31; Glycogen, 7.65 ± 5.52; Laminarin, 5.78 ± 3.14 nmol C_2H_4 g^{-1} dry sed. h^{-1}.

TABLE 2. Effects of different polysaccharides and glucose on nitrogenase activity in sediments from Langebaan Lagoon.

Treatment	Site	Nitrogenase activity[a]	Ratio[b]	
None (control)	Oesterwal	0.11 ± 0.17	1	
	Geelbek	0.26 ± 0.31	1	
Glucose	Oesterwal	4.17 ± 2.93	38	*
	Geelbek	4.47 ± 3.15	17	*
Cellulose	Oesterwal	0.05 ± 0.07	0	
	Geelbek	0.18 ± 0.27	1	
Xylan	Oesterwal	1.69 ± 1.80	15	
	Geelbek	1.40 ± 1.49	5	
Alginate	Oesterwal	1.98 ± 1.67	18	*
	Geelbek	1.40 ± 0.95	5	*
Carrageenan	Oesterwal	1.03 ± 2.15	9	
	Geelbek	0.36 ± 0.35	1	
Glycogen	Oesterwal	10.07 ± 7.19	92	*
	Geelbek	5.27 ± 1.53	20	*
Laminarin	Oesterwal	6.59 ± 3.93	60	*
	Geelbek	4.96 ± 2.24	19	*
Starch	Oesterwal	0.07 ± 0.05	1	
	Geelbek	0.35 ± 0.40	1	
Chitin	Oesterwal	0.16 ± 0.19	1	
	Geelbek	0.38 ± 0.44	1	

[a] Mean ± SD of all microhabitats at each site. Units: nmol C_2H_4 g^{-1} dry sed. h^{-1}.

[b] Activity of treatment/activity of control.

* Denotes treatment activity significantly (ANOVA, $p<0.05$) different to that of the unamended control.

Since nitrogen fixation is an energy-demanding process, the availability of suitable organic substrates may also control rates of heterotrophic nitrogen fixation *in situ*. Since most of the organic material available for bacterial use in saltmarsh ecosystems should be derived from primary production, the effects of complex polysaccharides on nitrogenase activity were examined. Four structural and three storage polysaccharides of plant origin were chosen for comparison. Moreover, because of the high prawn densities in this lagoon, the effect of chitin on nitrogenase activity was also tested.

contrast to polysaccharides of plant origin, additions of chitin were not effective at stimulating nitrogenase activity relative to the control (Table 2).

These data for the effects of plant polysaccharides on nitrogenase activity (Fig. 3 and 4) are supported by results of other experiments. Similar experiments carried out during November, 1991 (Oesterwal), December, 1991 (Oesterwal), January, 1992 (Geelbek), and May, 1992 (Oesterwal and Geelbek) also yielded results showing that glycogen, alginate, and xylan were effective substrates for supporting nitrogenase activity in these sediments (results not shown). Similarly, cellulose and carrageenan were not found to stimulate nitrogenase activity during incubations of < 48h. Laminarin and starch were not tested during these experiments.

Effects of Polysaccharides on Nitrogenase Activity during Extended Incubations

Although cellulose, carrageenan and starch generally did not stimulate nitrogenase activity over short incubations (< 24 hours), these substrates did stimulate ethylene production during extended incubations (4-5 days) (Table 3). Ethylene production by substrate-amended and unamended sediments, was inhibited by 10mM NH_4Cl during extended incubations, indicating that it was most likely due to nitrogenase activity (results not shown). The effect of microcrystalline cellulose (Avicel) on nitrogenase activity over extended incubations was compared to that of carboxymethylcellulose (CMC), a synthetic cellulose polymer. Additions of CMC had no effect on nitrogenase activity, whereas Avicel stimulated nitrogenase activity in certain sediments (Table 3). Although CMC can induce expression of, and be digested by, bacterial cellulases, its methyl-group substitutions render it difficult for bacteria to metabolize further. Interference by methyl groups with bacterial use of these substrates to support nitrogenase activity was also noted for methyl-αD-glucopyranoside, a glucose monomer with a single methyl-group substitution; glucose stimulated nitrogenase activity in burrow lining sediment from Oesterwal by 167-fold, whereas there was no stimulation of nitrogenase activity by glucopyranoside (results not shown). These results indicate that stimulation of nitrogenase activity by cellulose (Avicel) is dependent upon carbon

Benthic nitrogen fixation in saltmarsh and seagrass environments can be stimulated by additions of easily-utilizable carbon sources such as glucose (Dicker and Smith, 1980b; Capone, 1982; Capone and Budin, 1982). Similar effects were measured in Langebaan Lagoon where addition of glucose to sediments stimulated nitrogenase activity by >10-fold relative to unamended controls (Fig. 2). The glucose-effect is included here because it shows that heterotrophic nitrogen fixation in Langebaan Lagoon is limited by the availability of easily utilizable carbon sources, and provides a 'yardstick' against which the effects of polysaccharides can be compared.

Amendment of sediments with defined, complex polysaccharides stimulated nitrogenase activity differently according to the substrate and sediment type (Fig. 3 and 4). Of the four structural plant-polysaccharides tested, xylan and alginate were more effective than cellulose and carrageenan at stimulating nitrogenase activity in most sediment types (Fig. 3, Table 2). Activities (means for sites) stimulated by xylan were 5-15 fold greater (not significant, $p>0.05$) than those of unamended controls, whereas activities stimulated by alginate were 5-18 fold greater (significant, $p<0.05$) than those of controls (Table 2). Amendment of sediments with carrageenan caused sporadic stimulation of nitrogenase activity among microhabitats and sites (Fig. 3), but the mean rates stimulated by carrageenan were not significantly ($p>0.05$) greater than those of the controls (Table 2). The cellulose polymer, microcrystalline cellulose, stimulated nitrogenase activity only after extended lag phases (>60 hours), and only in certain types of sediments (see below).

Of all the polysaccharides examined, the storage polysaccharides, glycogen and laminarin, were the most effective at stimulating nitrogenase activity (Fig. 4, Table 2). Glycogen significantly ($p<0.05$) stimulated nitrogenase activity (means for sites) by 20-92 fold, while laminarin significantly ($p<0.05$) stimulated rates by 19-60 fold, relative to the control values (Table 2). Starch required incubations of > 24 hours for stimulation of nitrogenase activity (see below). In general, rates of nitrogenase activity stimulated by plant polysaccharides (Fig. 3 and 4) reflected the pattern among microhabitats of the unamended samples (Fig. 2); ie. rates were lowest in subsurface sediments, while *Zostera* bed sediment generally supported the greatest activity. In

TABLE 3. Effects of organic substrates on ethylene production by different sediments during extended incubations[a].

Habitat	CMC[b]	Avicel	Carrageenan	Starch
Oesterwal				
Surface sediment	1 ± 1 (2)	1 ± 1 (3)	2 ± 0 (2)	76 (1)
Zostera sediment	1 ± 0 (2)	1 ± 0 (3)	20 ± 26 (2)	123 (1)
Burrow opening	1 ± 1 (2)	42 ± 33 (3)	215 ± 170 (2)	200 (1)
Burrow lining	1 ± 0 (2)	357 ± 162 (3)	264 ± 22 (2)	400 (1)
10cm depth	1 ± 0 (2)	474 ± 230 (3)	62 ± 35 (2)	353 (1)
Geelbek				
Surface sediment	1 (1)	5 ± 5 (2)	17 ± 9 (2)	23 (1)
Zostera sediment	1 (1)	1 ± 0 (2)	1 ± 1 (2)	5 (1)
Burrow opening	1 (1)	9 ± 11 (2)	18 ± 7 (2)	9 (1)
Burrow lining	1 (1)	26 ± 27 (2)	58 ± 60 (2)	104 (1)
10cm depth	1 (1)	29 ± 40 (2)	177 ± 249 (2)	209 (1)

[a] Values are ratios of ethylene production by substrate-amended:unamended sediments during extended incubations (4-5 days). Values represent means ± SD (for n experiments). Where more than one experiment was carried out, experiments were carried out in different months, usually in different seasons, during 1991-93.
[b] Carboxymethylcellulose.

and energy derived from the substrate following hydrolysis of the polymer, and is not a consequence of induction of nitrogenase activity by the presence of cellulose.

Carrageenan and starch stimulated nitrogenase activity in most sediments during extended incubations (Table 3). The effect of cellulose on nitrogenase activity was more variable. Additions of cellulose had no effect on ethylene production in surface sediment and *Zostera* bed sediment from Oesterwal; however, cellulose stimulated ethylene production in burrow-associated and subsurface sediments at Oesterwal during extended incubations (Table 3). This pattern was marked and highly reproducible, occurring in 3 out of 3 experiments, which were carried out in spring, summer and autumn. Additions of cellulose produced a more variable response in ethylene production at Geelbek; during a summer experiment, avicel had no effect on ethylene production in any of the Geelbek sediments, whereas some stimulation was measured in most Geelbek sediments during autumn. Stimulation of ethylene production by cellulose was greater at Oesterwal than at Geelbek.

TABLE 4. Effects of different organic substrates on microfloral characteristics of subsurface sediment from Oesterwal during extended incubation[a].

Substrate	Time (d)	Aerobic		Anaerobic[b]	
		CMC	Xylan	CMC	Xylan
None	0	8 (51)	9 (33)	1 (2)	2 (4)
	2	7 (23)	3 (6)	22 (1)	8 (8)
	5	8 (6)	8 (2)	110 (2)	82 (1)
Glucose	0	8 (51)	9 (33)	1 (2)	2 (4)
	2	42 (2)	43 (4)	38 (13)	40 (3)
	5	130 (2)	110 (4)	850 (1)	750 (4)
Xylan	0	8 (51)	9 (33)	1 (2)	2 (4)
	2	54 (11)	100 (90)	54 (15)	49 (90)
	5	170 (11)	230 (97)	970 (6)	900 (81)
Avicel	0	8 (51)	9 (33)	1 (2)	2 (4)
	2	7 (19)	1 (6)	13 (2)	8 (6)
	5	14 (18)	9 (12)	180 (7)	140 (10)

[a] Values represent numbers of CFU (x 10^5) on CMC or xylan plates, with the proportion (%) of CFU positive for CMC-ase (cellulase) or xylanase respectively in parentheses.

[b] Aerobic incubation includes aerobic and facultative strains; anaerobic incubation includes anaerobic and facultative strains.

The occurrence of a lag phase prior to stimulation of nitrogenase activity in sediments amended with carbon sources, is common (Nedwell and Aziz, 1980; Roszak and Colwell, 1987; Gandy and Yoch, 1988). However, extended bioassays are potentially problematic as the status of *in situ* bacterial populations may change. Proliferation or enrichment of populations during and after the lag phase are problematic for interpretation of the data. The relevance of data from extended incubations to the mechanisms that were controlling nitrogen fixation in the sediments at time of sample collection, is questionable if the *in situ* populations change with respect to nitrogen fixation. However, it is highly unlikely that the *in situ* microfloral populations of seagrass ecosystems are in a perpetual static state; environmental fluctuations such as seasonal growth and die-back of seagrasses and saltmarsh macrophytes, or the effect of storms, results in a dynamic supply of detrital material, rather than a static one.

Changes in numbers of CFU and the proportion of cellulolytic and xylanolytic CFU were monitored during extended incubations (Table 4). For unamended sediments, little change was noted for numbers of aerobic CFU, whereas numbers of CFU on anaerobic plates increased by up to 2 orders of magnitude over time. Additions of glucose and xylan stimulated an increase in numbers of CFU on both aerobic and anaerobic plates. Addition of cellulose stimulated an increase in numbers of anaerobic CFU, but little change was noted for aerobic CFU (Table 4). Changes in the proportion of the population positive for cellulase (P_c) and xylanase (P_x) activity were also monitored. Addition of glucose did not enhance P_c or P_x on either aerobic or anaerobic plates. The effect of avicel on P_c and P_x is difficult to conclude, since P_c and P_x were only slightly higher in the presence of avicel than in unamended sediments or in the presence of glucose. The effect of xylan was most clear; addition of xylan stimulated increase in numbers of CFU and P_x, but not P_c (Table 4). Thus, there is evidence for both proliferation and enrichment during extended incubations in these studies.

TABLE 5. Effects of nalidixic acid, rifampicin, and chloramphenicol on ethylene production in sediments from Langebaan.

Site	Inhibitor[a]	nmol C_2H_4 g^{-1} dry sed.[b] Without glucose	With glucose
Oesterwal	None	3.94 ± 0.05	761.19 ± 213.3
	Nal	2.25 ± 0.27	234.19 ± 8.70
	Rif	2.54 ± 0.35	2.76 ± 0.02
	Cm	2.53 ± 0.05	2.33 ± 0.22
Geelbek	None	6.06 ± 0.51	1132.3 ± 21.24
	Nal	3.60 ± 0.20	1045.4 ± 487.7
	Rif	2.88 ± 0.09	686.65 ± 192.8
	Cm	2.50 ± 0.64	2.56 ± 0.42

[a] Sediments were amended with nalidixic acid (200μg ml^{-1}), rifampicin (200μg ml^{-1}), chloramphenicol (100μg ml^{-1}) in 50ml filtered (0.2μm) seawater, with or without glucose (15mM) as indicated.

[b] Ethylene production was assayed after extended incubation for 5 days. Experiment was carried out during October, 1991. Values represent the means ± SD for replicate samples.

The effects of metabolic inhibitors provided further information about nitrogenase activity during extended incubations (Table 5). Nalidixic acid, an inhibitor of DNA replication and therefore cell division, inhibited between 8 and 69% of ethylene production during incubation for 4 days. The transcriptional inhibitor, rifampicin, inhibited 36-53% of ethylene production in unamended sediments, and 39-99% of ethylene production in glucose-amended sediments. Chloramphenicol, an inhibitor of translation, inhibited 36-59% of ethylene production in unamended sediments, and > 99% of ethylene production in glucose-amended sediments. Unfortunately, no data were available for ethylene production during the first 24 hours incubation, and hence the effects of these inhibitors over short incubations is not clear. However, in similar experiments Gandy and Yoch (1988) concluded that nalidixic acid did not affect rates of nitrogenase activity in saltmarsh sediments during incubations < 30 hours.

Regulation by N-source Availability

Nitrogenase activity in different sediments from Langebaan Lagoon responded differently to additions of NH_4^+ (Fig. 5). Nitrogenase activity in the Geelbek muds showed little response to 0-1mM NH_4^+. In contrast, additions of 0-1mM NH_4^+ to sandy sediments (Oesterwal) caused a concentration-dependant inhibition of nitrogenase activity, with 90% inhibition of activity when 1mM NH_4^+ was added (Fig. 5). To assess the relevance of these effects, these results should be compared with *in situ* NH_4^+ concentrations (Chapter 2). *In situ* concentrations of NH_4^+ were ca. 20-fold lower overall than the lowest addition of NH_4^+ (50 μM), which had no affect on nitrogenase activity in muddy sediments, and caused only a 15% drop in rates in sandy sediments (Fig. 5).

Further investigations of the role of NH_4^+ in the regulation of nitrogenase activity used methionine-DL-sulfoximine (MSX) to inhibit glutamine synthetase, an enzyme involved in the regulation of nitrogenase activity (Chapter 1). Gandy and Yoch (1988) concluded that *in situ* NH_4^+ was responsible for regulation of nitrogenase activity in certain marine sediments, as additions of MSX stimulated nitrogenase activity. In the present

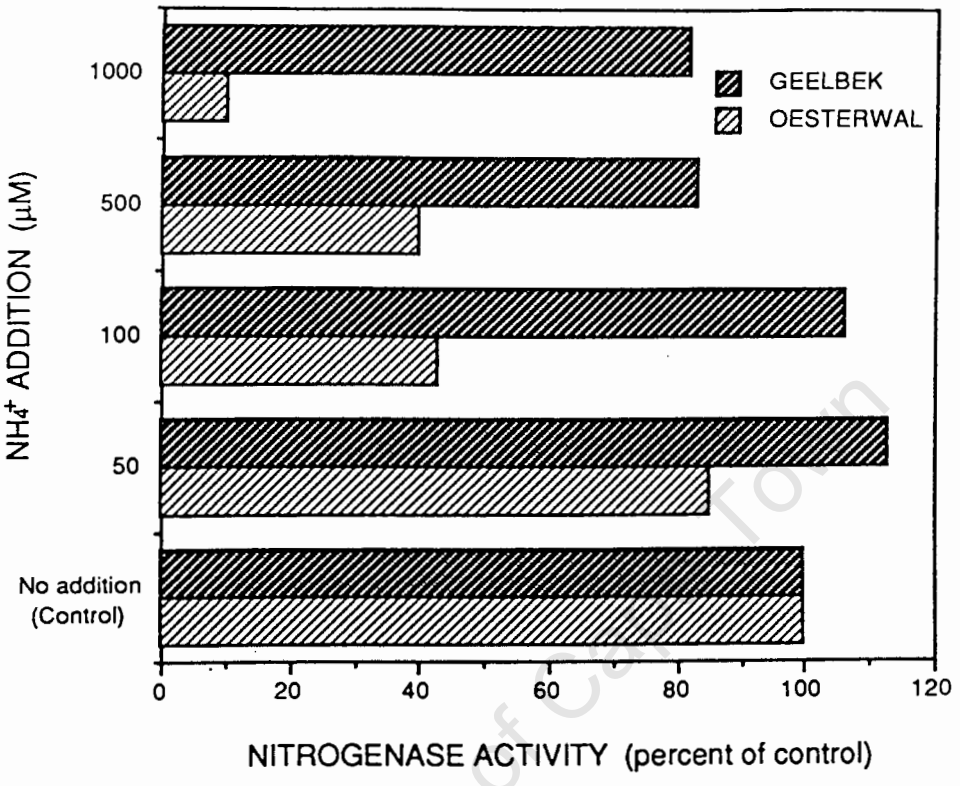

Figure 5. Effect of ammonium chloride on nitrogenase activity in surface sediments. Experiments were carried out on 17.2.92. Values are expressed as percentage of the activity of unamended controls (0.005 ± 0.004 nmol C_2H_4 g^{-1} dry sed. h^{-1}, Oesterwal; 0.24 ± 0.08 nmol C_2H_4 g^{-1} DW h^{-1}, Geelbek), which were made to equal 100%. Values are means of triplicate samples.

study, surface and subsurface sediments were selected for MSX experiments; surface sediment supported relatively high rates of nitrogenase activity (see Chapter 4), whereas subsurface sediment supported low rates of nitrogenase activity and had relatively high concentrations of interstitial ammonium (Chapter 2). Additions of MSX did not stimulate nitrogenase activity in Langebaan sediments (Table 6). There was, however, evidence that MSX was interfering with bacterial metabolism since rates in the presence of MSX were lower than in sediments without MSX. Thus, these experiments do not suggest a significant role for ammonium-control of nitrogen fixation at *in situ* concentrations in Langebaan Lagoon sediments.

TABLE 6. Effect of methionine-DL-sulfoximine on nitrogenase activity in sediments from Langebaan lagoon[a].

Habitat	nmols C_2H_4 g^{-1} dry sed. h^{-1}			
	- Glucose		+ Glucose	
	- MSX	+ MSX	- MSX	+MSX
Oesterwal				
Surface sed.	1.25 ± 0.04	0.27 ± 0.07	142.65 ± 5.96	9.62 ± 1.06
10cm depth	0.01 ± 0.04	0	13.60 ± 3.62	0.08 ± 0.08
Geelbek				
Surface sed.	1.56 ± 0.09	0.45 ± 0.09	14.15 ± 5.03	0.57 ± 0.11
10cm depth	0	0	0.06 ± 0.04	0

[a] Samples were amended with MSX (1mM) and glucose (15mM) as indicated. Ethylene production was assayed after 20h incubation, and values represent means ± SD of replicate samples (n=3).

The effect of nitrate on nitrogenase activity was examined in two experiments. The results of one of these experiments are presented in Fig. 6. Concentrations of nitrate from 0-10mM caused a concentration-dependent inhibition of nitrogenase activity in surface sediment from Oesterwal. Chlorate is a structural analogue of nitrate and is also reduced by nitrate reductase, but contains no nitrogen and therefore cannot be implicated in nitrogen control of nitrogenase activity. Additions of chlorate (0-40mM) also caused a concentration-dependent inhibition of nitrogenase activity in surface sediment from Oesterwal. The effects of nitrate and chlorate on nitrogenase activity were different at Geelbek for this experiment. Concentrations of 100μM nitrate, and 400μM and 4mM chlorate, stimulated nitrogenase activity (although not significantly, $p>0.05$) in Geelbek surface sediment. Nitrogenase activity at Geelbek was completely inhibited by 10mM nitrate and 95% inhibited by 40mM chlorate. This experiment was repeated to further examine the effects at Geelbek. The results of the second experiment showed a concentration-dependent inhibition of nitrogenase activity and did not show the stimulation-effect prevously recorded in the former experiment at Geelbek. Thus, the reasons for the pattern during the first experiment are unclear.

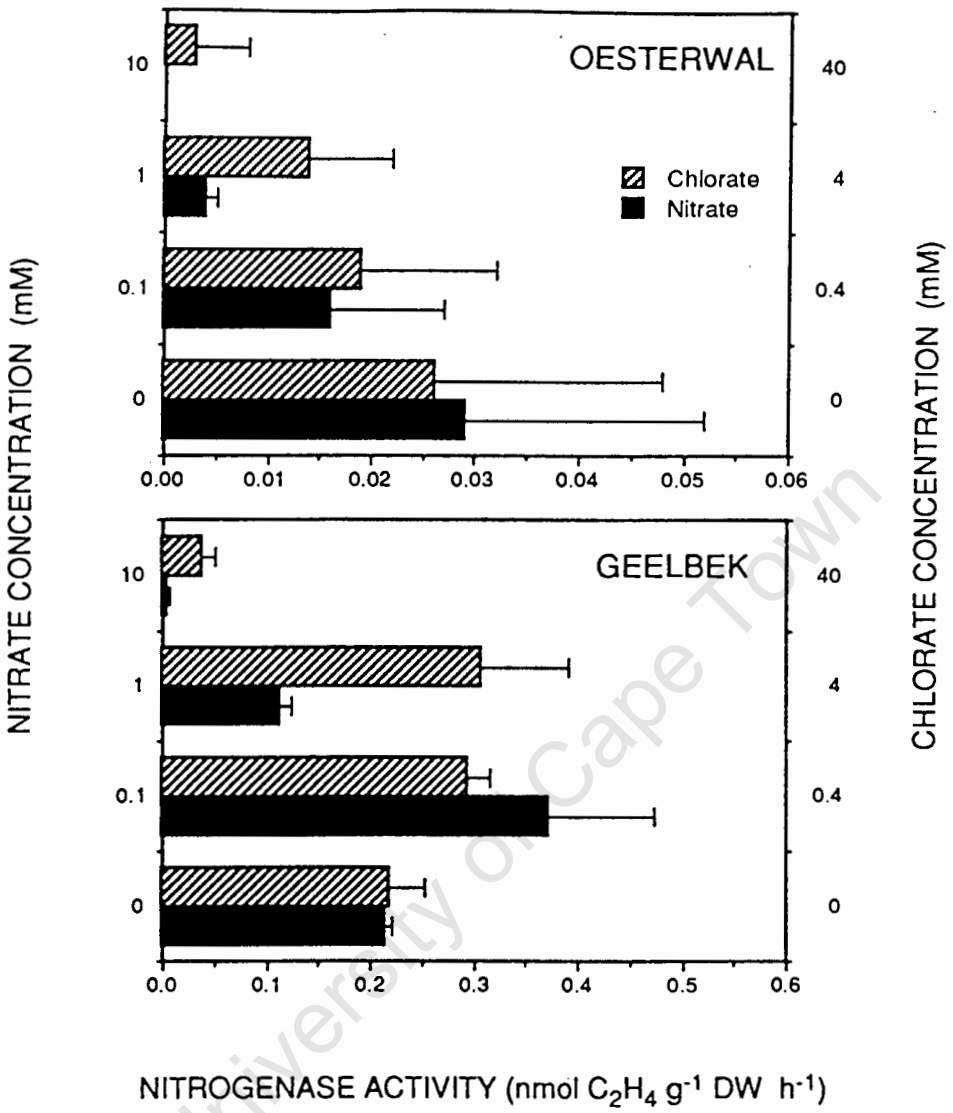

Figure 6. Effect of nitrate and chlorate on nitrogenase activity in surface sediments.

Regulation by Physical Factors

The response of nitrogenase activity in surface sediments to temperature was examined during spring and summer (Fig. 7). Nitrogenase activity was low at 10°C and 37°C, and highest at 20°C for both glucose-amended and unamended sediments. The effect of temperature on nitrogenase activity was similar in spring and summer.

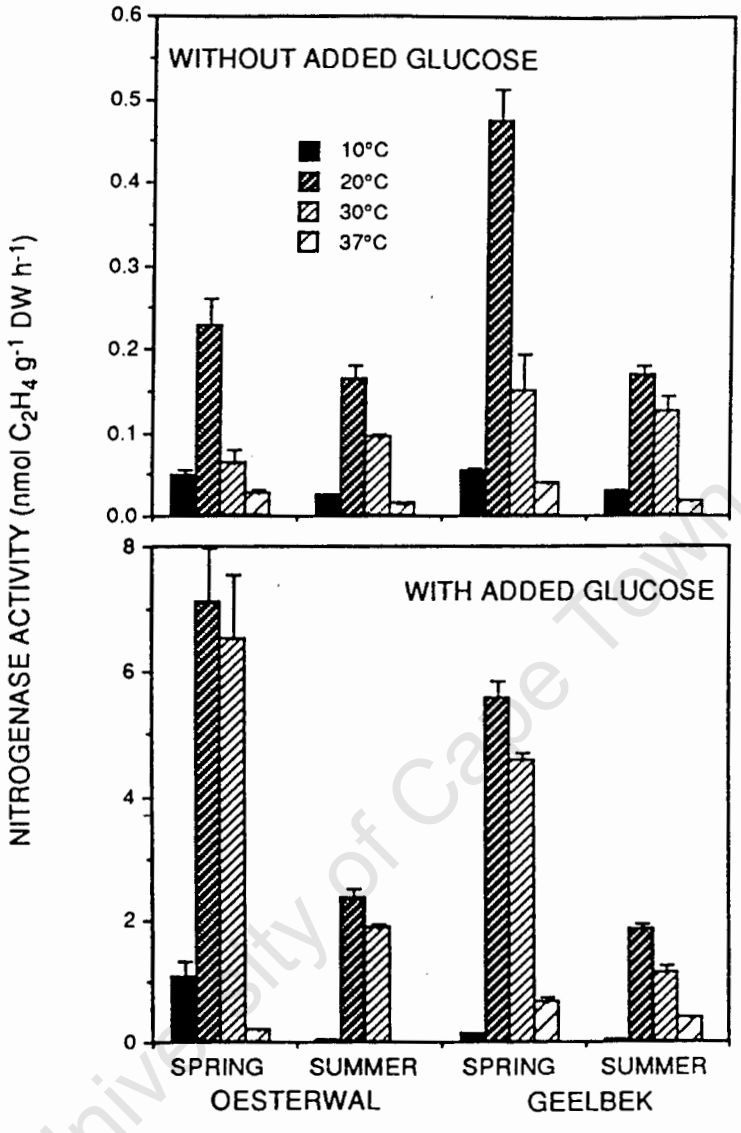

Figure 7. Effect of temperature on nitrogenase activity in surface sediments, with or without added glucose.

The effect of oxygen on nitrogenase activity in Langebaan sediments is shown in Table 7. Nitrogenase activity was significantly ($p < 0.05$) stimulated in the presence of air in surface sediments at Geelbek. This effect was most dramatic in *Zostera* bed sediments where nitrogenase activity was ca. 20-fold greater in the presence of air than in its absence. Although O_2 did stimulate nitrogenase activity in surface sediments at Oesterwal as well, these increases were not significant ($p > 0.05$). Oxygen (air) did not stimulate nitrogenase activity in subsurface sediments at either site. Stimulation of nitrogenase activity by O_2 also occurred in other experiments (see below).

TABLE 7. Effect of O_2 on nitrogenase activity in different sediments.

Sediment	nmol C_2H_4 g^{-1} dry sed. h^{-1} [a]			
	Oesterwal		Geelbek	
	$-O_2$	O_2	$-O_2$	O_2
Surface	0.03 ± 0.00	0.08 ± 0.06	0.07 ± 0.01 *	0.73 ± 0.06
Zostera bed	0.07 ± 0.03	0.29 ± 0.20	0.59 ± 0.13 *	11.29 ± 1.62
Burrow opening	0.02 ± 0.01	0.03 ± 0.03	0.04 ± 0.02 *	0.33 ± 0.11
Burrow lining	0.01 ± 0.01	0.02 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
10cm depth	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.01

[a] values represent mean rates ± SD of replicates (n=3), measured after 15h. Experiments were carried out during December, 1992.

* represents significant ($p < 0.05$) difference between treatments as determined by Student's t-test.

The effects of oxygen and glucose, and possible interactions between these variables were investigated. Independently, both glucose and oxygen significantly ($p < 0.0001$) stimulated nitrogenase activity in surface sediments (Tables 8 and 9). However, there was a significant negative interaction between oxygen- and glucose-stimulated nitrogenase activity; glucose-supported rates were lower in the presence of oxygen than in its absence.

TABLE 8. Effects of oxygen and glucose on nitrogenase activity in surface sediment from Langebaan lagoon[a].

Site	Headspace	nmol C_2H_4 g^{-1} dry sed. h^{-1}	
		- Glucose	+ Glucose
Oesterwal	Aerobic	0.083 ± 0.006	1.917 ± 0.286
	Anaerobic	0.043 ± 0.006	8.260 ± 0.798
Geelbek	Aerobic	0.150 ± 0.010	1.500 ± 0.269
	Anaerobic	0.083 ± 0.006	9.033 ± 0.570

[a] Samples were amended with glucose (5μmol g^{-1} wet sed.) as indicated, and incubated either aerobically (air) or anaerobically (argon). Ethylene production was assayed after incubation for 20h at 25°C. Values represent means ± standard deviations of replicates (n=3).

TABLE 9. ANOVA results showing significant effect of glucose and oxygen (air) on nitrogenase activity in surface sediment from Oesterwal and Geelbek[a].

Treatment	DF	ANOVA SS	Mean square	F value	Pr > F
Oesterwal					
Glucose	1	231.616	231.616	424.69	0.0001
Oxygen	1	90.640	90.640	166.20	0.0001
Glu*O_2	1	92.075	92.075	168.83	0.0001
Geelbek					
Glucose	1	2275.905	2275.905	830.93	0.0001
Oxygen	1	1185.643	1185.643	432.87	0.0001
Glu*O_2	1	1174.536	1174.536	428.82	0.0001

[a] Experiments were carried out during October 1991. Statistical analyses used data for ethylene production (nmol C_2H_4 g^{-1} dry sed. h^{-1}) determined during the first 24 hours' incubation.

The effect of light and oxygen, and possible interactions between these two variables were also explored. Nitrogenase activities in surface sediment and *Zostera* bed sediment under light/dark and aerobic/anaerobic conditions are presented in Table 10, while the statistical analyses of these data are presented in Table 11. Both light and oxygen stimulated nitrogenase activity in sediments from Oesterwal (effect significant ($p<0.05$) for O_2, but not significant ($p>0.05$) for light). Higher rates in Oesterwal sediments in the presence of light may have been partly due to oxygen production by photosynthesis, since light had little effect on nitrogenase activity under aerobic conditions. At Geelbek, oxygen stimulated nitrogenase activity in certain sediment types, whereas light had no significant effect (Tables 10 and 11).

DISCUSSION

Effects of C-Source Availability

Several workers have reported on the regulation of heterotrophic nitrogen fixation in marine sediments by the availability of organic substrates. These reports have generally focussed on the effects of mono- and disaccharides (Hanson, 1977a; Dicker and Smith,

TABLE 10. Effects of aerobic/anaerobic and light/dark incubations on nitrogenase activity (acetylene reduction) in sediments from Langebaan Lagoon.

Habitat	Headspace	nmol C_2H_4 g^{-1} dry sed. h^{-1}[a]	
		Dark	Light
Oesterwal			
Surface sed.	Anaerobic	0.073 ± 0.071	0.446 ± 0.373
	Aerobic	0.345 ± 0.238	0.383 ± 0.249
Zostera sed.	Anaerobic	0.042 ± 0.030	0.184 ± 0.121
	Aerobic	0.580 ± 0.140	0.516 ± 0.076
Geelbek			
Surface sed.	Anaerobic	0.070 ± 0.035	0.098 ± 0.015
	Aerobic	0.497 ± 0.086	0.555 ± 0.123
Zostera sed.	Anaerobic	0.265 ± 0.078	0.362 ± 0.121
	Aerobic	2.567 ± 0.103	2.580 ± 0.310

[a] Rates were calculated from ethylene production after 20h incubation. Experiments were carried out during March 1992.

TABLE 11. ANOVA results showing effects of oxygen (air) and light on nitrogenase activity in sediments from two sites in Langebaan lagoon[a].

Treatment	DF	ANOVA SS	Mean square	F value	Pr > F
Oesterwal					
Sediment	1	0.002	0.002	0.06	0.8130
Oxygen	1	0.440	0.440	11.54	0.0001
Light	1	0.090	0.090	2.36	0.1439
Sed*Light	1	0.041	0.041	1.07	0.3161
O_2*Light	1	0.108	0.108	2.83	0.1118
Geelbek					
Sediment	1	7.786	7.786	408.01	0.0001
Oxygen	1	10.921	10.921	572.31	0.0001
Light	1	0.014	0.014	0.76	0.3962
Sed*Light	1	0.0002	0.0002	0.01	0.9189
O_2*Light	1	0.001	0.001	0.06	0.8049

[a] Experiments were carried out during March 1992. Statistical analyses were performed on data for ethylene production (nmol C_2H_4 g^{-1} dry sed. h^{-1}) determined during 20 hours' incubation.

1980b; Capone, 1982; Jones, 1982). A wide range of fermentable and oxidizable substrates have been found to stimulate nitrogenase activity in a variety of benthic environments (Capone, 1988). Glucose produced the greatest stimulation of nitrogenase activity in sediments from Langebaan Lagoon. Glucose (15mM) stimulated nitrogenase activity in surface sediments from Oesterwal and Geelbek by 50-fold on average, and by up to three orders of magnitude in other sediments (see Chapter 4). Similar effects have been noted for glucose-stimulated nitrogenase activity in other systems (Patriquin and Knowles, 1972; Jones, 1982). The effectiveness of glucose at stimulating nitrogenase activity in sediments is not surprising as it is the 'universal substrate' in bacterial metabolism. The sugar alcohol, mannitol, also stimulated nitrogenase activity in Langebaan sediments. Mannitol is the primary photosynthate of kelp, and comprises 77% of the free sugars and polyols released into the water column during fragmentation of these macrophytes (Newell *et al.*, 1980). Although kelps are not present at Oesterwal and Geelbek, they do occur in the adjacent Saldanha Bay. The response of nitrogenase activity to mannitol indicates that this hexitol is likely to be, at least occasionally, available to heterotrophic diazotrophs in Langebaan Lagoon when uprooted or fragmented kelps are swept into the lagoon. Sucrose, a disaccharide, stimulated nitrogenase activity to a similar extent as the stimulation produced by glucose and mannitol. The biodegradation and mineralisation of saltmarsh organic material includes complex microbial interactions (Capone and Kiene, 1988). Depolymerization of complex polysaccharides yields fermentable substrates, which are fermented to low molecular weight organic acids, alcohols, and H_2. These products are subsequently oxidized to complete mineralization by anaerobically respiring bacteria (Sorensen *et al.*, 1981). In certain marine sediments, sulfate reducing bacteria (SRB) dominate these terminal oxidative processes (Capone and Kiene, 1988). Organic acids, including succinate (4-C), lactate (3-C), and acetate (2-C), were not as effective as glucose, mannitol, and sucrose at stimulating nitrogenase activity in surface sediments, suggesting that either nitrogen-fixing SRB in these sediments were not substrate-limited (which seems an unlikely event in natural systems), or that anaerobic nitrogen fixation in surface sediments was due primarily to fermenters. The latter possibility was explored further by amendment of sediments with 20mM Na-molybdate, a 'specific' inhibitor of SRB (Oremland and Capone, 1988). In the presence of glucose, molybdate

inhibited nitrogenase activity by 15-47%, whereas in unamended sediments activity was inhibited by 67%. These latter data should be interpreted with caution however, as subsequent *in vitro* experiments indicated that high concentrations (20mM) of molybdate inhibited nitrogenase activity in some facultative, nitrogen-fixing isolates (results not shown); hence the inhibitory effect of molybdate on nitrogenase activity may not be specific for SRB.

Although considerable effort has been focussed on the effects of key monomers, few studies have addressed the modulation of nitrogenase activity in marine environments by the availability of polysaccharides. Paerl *et al.* (1987) showed that the amendment of seawater from the coast of North Carolina with combinations of mono- or disaccharides and homogenized *Zostera marina* or *Spartina alterniflora* leaves, enhanced rates of nitrogen fixation. Whereas the sugars may have provided an energy source for bacterial metabolism and nitrogen fixation, Paerl *et al.* attributed the effect of detrital particles to their function as a surface for O_2-depleted microzone formation, and thus as suitable sites for O_2-sensitive nitrogen fixation. Detrital material may also be utilized by bacteria as a substrate for growth.

The results of the present study show different responses of nitrogenase activity to amendments with defined mono- and polysaccharides. These responses varied according to both the substrate and microhabitat type. Additions of xylan to lagoonal sediments stimulated nitrogenase activity in all microhabitats at both sites; rates stimulated by xylan were ca. 7-fold greater than those of unamended controls. Alginate was another structural polysaccharide which was effective at stimulating nitrogenase activity, with the overall magnitude of stimulation similar to that produced by xylan (ca. 8-fold). Alginate is a structural polysaccharide in the Phaeophyta (brown algae) which, although largely absent from the study sites in Langebaan Lagoon, are more abundant in the adjacent Saldanha Bay (see Chapter 2). Acylated alginic acid is also produced in an extracellular mucilage by certain bacteria (Gorin and Spencer, 1966), which have been isolated from saltmarsh sediments (Dicker and Smith, 1981).

Cellulose and hemicellulose (xylan) constitute the major structural polysaccharides of saltmarsh and seagrass macrophytes, and some algae. Hemicellulose (xylan) accounts for 15-30% of plant material and may shield the cellulose polymer from attack by cellulase enzymes (Dekker and Lindner, 1979; Orpin, 1988). In contrast to the effects of xylan and alginate, additions of microcrystalline cellulose did not stimulate nitrogenase activity in incubations of <30 hours. The effective degradation of microcrystalline cellulose is a complex process usually requiring a synergistic attack by three classes of cellulase enzymes. Such enzyme systems are probably not ubiquitous among natural assemblages (Béguin, 1990). Carrageenan did support nitrogenase activity in some sediment types, but the pattern of its effect was not marked nor consistent between sites. Additions of cellulose and carrageenan did stimulate nitrogenase activity in some sediments during extended incubations (>60 hours), indicating that benefit could, eventually, be derived from these substrates by diazotrophic bacteria in saltmarsh ecosystems. In order to obtain this benefit, bacterial enzyme systems may need to be induced, or communities may change with respect to abundance or physiological composition. However, the findings of extended incubations reported here are still significant considering the dynamic supply of organic matter in saltmarsh ecosystems, and the potential value of diazotrophic growth on these materials to primary and secondary producers (see Chapter 7).

Of all the polysacharides tested, glycogen and laminarin were the most effective at stimulating nitrogenase activity. Laminarin is the characteristic glucan of Phaeophyta (eg. *Laminaria* spp.). Instead of starch, some algae include amylopectins or glycogens, which are closely related in chemical structure. Starch stimulated nitrogenase activity only after extended incubations. Starch is a mixture of amylose and amylopectin, and is the storage polysaccharide of many macrophytes. Stimulation of nitrogenase activity by the storage polysaccharides glycogen and laminarin was significantly ($p<0.05$) greater than that produced by structural polysaccharides (xylan, alginate, cellulose, and carrageenan). Furthermore, of those tested, polysaccharides common to many algae (alginate, glycogen, and laminarin) were more effective at stimulating nitrogenase activity than those dominant in saltmarsh and seagrass macrophytes (cellulose, xylan, and starch), even in sediments from beds of *Z. marina*. The dominant macroalga at

Oesterwal and Geelbek is *Gracilaria verrucosa*, although this is not a significant producer relative to the production of saltmarsh and seagrass macrophytes.

Chitin is the major structural polymer in Crustacea, and since saltmarsh lagoons often support large populations of these animals, ecdysis of their exoskeletons may represent a major input of carbon into these environments. In the context of these studies, chitin therefore provided an interesting comparative substrate to polysaccharides of plant origin. Both cellulose and chitin are ß(1-4)-linked polysaccharides, but whereas cellulose consists of monomers of glucose, chitin consists of monomers of *N*-acetyl-D-glucosamine. The anaerobic degradation of chitin in saltmarsh sediments has been previously addressed with respect to two other biogeochemical cycles, namely sulfate reduction and methanogenesis (Boyer, 1986). In those studies, no sulfate-reducing or methanogenic isolates were capable of chitin utilization. However, in mixed cultures of chitin degraders and sulfate reducers or methanogens, additions of chitin stimulated sulfide or methane production respectively (Boyer, 1986). However, the present study showed that chitin was a poor stimulator of nitrogenase activity, indicating that this substrate is not an important regulator of nitrogen fixation in the habitats studied at Langebaan Lagoon.

Effect of N-Source Availability

The regulation of nitrogenase activity by ammonium is well-known (Postgate, 1982). Several workers have reported inverse correlations between *in situ* ammonium concentrations and nitrogenase activity in saltmarsh sediments (Carpenter *et al.*, 1978; Patriquin and Keddy, 1978; Teal *et al.*, 1979). However, other reports have not concluded that it has a major and consistent role in this regard (Hanson, 1977a, 1977b; Dicker and Smith, 1980a). Additions of ammonium (0-1mM) to sediments from Langebaan Lagoon had little effect on nitrogenase activity in muddy sediments, but caused a concentration-dependent inhibition of nitrogenase activity in sandy sediments. Ammonium-control of nitrogen fixation in these sediments may not be significant, however, since *in situ* NH_4^+ concentrations (Chapter 2) were ca. 20-fold lower than additions (50μM) which inhibited nitrogenase activity in sandy sediments by only 15%,

and had no effect on rates in muddy sediments. Furthermore, evidence for control of nitrogenase activity by *in situ* concentrations of ammonium was not found when sediments were amended with MSX, an inhibitor of glutamine synthetase. These findings, especially with respect to the Geelbek muds, contrast with the reports of other workers who have noted lower threshold concentrations of 100-200 μM NH_4^+ for the inhibition of nitrogenase activity in other saltmarsh ecosystems (Carpenter *et al.*, 1978; Teal *et al.*, 1979).

Although nitrate does not generally accumulate to significant levels in sediments (Capone, 1988), upwelling of nutrient-rich South Atlantic Central Water, with a nitrate content of ca. 20μM (Andrews and Hutchings, 1980), occurs along this coastline during spring and summer. Thus, the availability of nitrate to Langebaan Lagoon varies seasonally (Chapter 2). Additions of nitrate at concentrations from 0-10mM caused a concentration-dependent inhibition of nitrogenase activity, with full repression at 10mM. The exact mechanism by which nitrate inhibits nitrogenase activity is not clear. Nitrate can be reduced, through both assimilatory and dissimilatory pathways, to ammonium (Hattori, 1983), which may subsequently inhibit nitrogenase activity. Alternatively, Dicker and Smith (1980a) have argued that nitrate inhibition of nitrogenase activity may be a consequence of a competition for available reducing power between nitrogenase and nitrate reductase. Experiments similar to those of Dicker and Smith were carried out in the present work. Increasing concentrations of chlorate, a non-physiological nitrate analogue, caused a concentration-dependent inhibition of nitrogenase activity in sediments from Langebaan Lagoon, which is consistent with the proposal of Dicker and Smith. However, interpretation of such evidence should take cognizance of the toxicity of chlorite, the reduced product of chlorate. Thus, such data may be ambiguous, since it may not clarify whether lower rates of acetylene reduction are due to physiological inhibition of nitrogenase activity or due to the toxic effects of chlorite.

Effects of Physical Factors

Nitrogenase activity in surface sediment from Langebaan Lagoon was optimal at 20°C. There was no evidence that temperature optima varied between seasons or between sites. This optimum temperature corresponds with the mean annual temperature for both sites, which was ca. 20°C (Chapter 2). Nitrogenase activity in Langebaan sediments was lowest at 10°C and 37°C, indicating that during mid-winter and in high-summer, nitrogenase activity may be inhibited at times. These results reflect the findings of Jones (1982) who reported that nitrogenase activity in estuarine intertidal sediments could not be detected at temperatures below 10°C, but contrast with those of Jones (1982) and Smith and Hayasaka (1982b), who noted temperature optima between 30°C and 35°C for nitrogenase activity in estuarine sediments and seagrass rhizosphere, respectively. Temperature fluctuations on both diel (ca. 4-6°C) and seasonal (ca. 10°C) scales (Chapter 2) are thus likely to influence nitrogen fixation in Langebaan Lagoon.

Despite the extremely oxygen-labile nature of nitrogenase, nitrogen-fixing bacteria have diverse physiologies, ranging from strict anaerobes to strict aerobes. Aerobic diazotrophs (eg. *Azotobacter* spp.) possess physiological mechanisms which shield their nitrogenase from contact with oxygen. The trade-off between the energy-requirement and oxygen-sensitivity of nitrogenase is emphasized by many reports that have described different responses of *in situ* nitrogenase activity to the presence of oxygen. Capone and Budin (1982) found that nitrogenase activity associated with roots and rhizomes of *Z. marina* was greatest under microaerophilic conditions. Smith and Hayasaka (1982a, 1982b) detected O_2-enhanced rates of nitrogenase activity in rhizoshere sediments of seagrass ecosystems, while Dicker and Smith (1980c) found large populations of aerobic diazotrophs (*Azotobacter* spp.) were present in sediments from a Delaware saltmarsh. Oxygen also significantly ($p < 0.05$) stimulated nitrogenase activity in surface sediments from Geelbek. This oxygen-effect was pronounced in sediments from beds of *Z. capensis*, but was also recorded in exposed, surface sediments located away from macrophyte cover.

Bioturbation of sediments by benthic infauna, such as thalassinids, may increase the aeration of subsurface sediments (Dye, 1978). Indeed, Capone (1988) has commented "the effect of O_2, along with those factors controlling O_2 penetration into sediments (temperature, rate of consumption, organic load, bioturbation, etc.) deserve further investigation." Although oxygen had little effect on nitrogenase activity in subsurface sediments from Langebaan Lagoon, it was noteworthy that O_2-stimulation of nitrogenase activity in burrow opening sediment was not as marked as in adjacent surface sediment or *Zostera* bed sediment (Table 7). Turnover of sediments by prawns (bioturbation) may thus limit the extent of O_2-stimulation of nitrogenase activity in surface sediments, as microaerophilic or aerobic diazotrophs are displaced from the surface around the burrow opening by the effects of bioturbation.

Both oxygen and glucose significantly ($p < 0.0001$) stimulated nitrogenase activity in surface sediments. However, the presence of oxygen inhibited full stimulation of nitrogenase activity by glucose, indicating that glucose-supported nitrogenase activity was largely due to nitrogen-fixing fermenters with an O_2-sensitive nitrogenase system, rather than diazotrophs with O_2-tolerant nitrogen fixation, such as *Azotobacter* spp. Light stimulated nitrogenase activity in surface and *Zostera* bed sediments at Oesterwal (although not significantly, $p > 0.05$), but not at Geelbek (Tables 10 and 11). In the presence of O_2, however, light had no effect on nitrogenase activity at Oesterwal. These data suggest that higher nitrogenase activities in the presence of light at Oesterwal may have been due to the effect of oxygen (from photosynthesis) rather than from light itself. Unfortunately, data for the effect of oxygenic photosynthesis inhibitors (eg. 3-(3,4-dichlorophenyl)-1,1-dimethylurea) on the response of nitrogenase activity at Oesterwal to light, were not available. Therefore, a strong role for light in the regulation of nitrogenase activity in Oesterwal sediments cannot be concluded.

Seasonal die-back of macrophyte beds in temperate saltmarsh lagoons, such as Langebaan, results in an increased flux of detrital material for bacterial use (Mazure and Branch, 1979). This material, typically low in N, becomes N-enriched by bacterial colonization. The N-requirements of bacterial growth on plant materials can be subsidized by inorganic nutrients taken from the environment or, as this study shows,

by bacterial dinitrogen fixation. It appears that certain polysaccharide constituents of this detrital material are more available for heterotrophic, diazotrophic growth than others. These findings improve our understanding of the dynamics of energy flow and the carbon and nitrogen cycles of these ecosystems. Aerobic or microaerophilic respiration also appears to be a significant energy source for nitrogenase activity in these ecosystems, despite the O_2-sensitivity of nitrogenase. Bioturbation has a negative effect on O_2-stimulation of nitrogenase activity when surface sediments in these microhabitats are displaced by subsurface sediments. It is tempting to speculate on the consequences of a feedback loop between bioturbation, the control of nitrogen fixation, and the nitrogen requirements of primary production of diatoms and saltmarsh/seagrass macrophytes, in studies of the quantity and nutritional value of food-sources available to benthic invertebrate detritivores, which rely primarily on plant material as an energy source. In the next chapter (6) nitrogen-fixing bacteria were isolated and characterized so that their physiology could be related to the ecology of nitrogen fixation in Langebaan Lagoon, and to determine if any strains possessed unusual characteristics with respect to nitrogen fixation in general.

CHAPTER 6

Characterization of Nitrogen-Fixing Bacteria from a Temperate Saltmarsh Lagoon, including Isolates that Produce Ethane from Acetylene

Abstract. Nitrogen-fixing bacteria were isolated from sediments and water of a saltmarsh lagoon on the west coast of South Africa, and characterized according to factors which regulate nitrogen fixation in the marine environment. The majority of isolates were assigned to the *Photobacterium* or *Vibrio* genera on the basis of physiological and biochemical characteristics. One isolate was further assigned to the species *Vibrio diazotrophicus*. Carbohydrate utilization by each diazotrophic isolate was examined. Abilities of the isolates to utilize a range of mono-, di-, and polysaccharides largely reflected the predicted availability of organic carbon and energy in the lagoon, except that chitin was not utilized. Biochemical tests on the utilization of combined nitrogen showed that one isolate could utilize nitrate, and that this strain was susceptable to full repression of nitrogenase activity by 10mM nitrate. Urease activity was not detected in any of the isolates. In the absence of molybdenum two of the isolates, a *Photobacterium* spp. and *V. diazotrophicus*, reduced acetylene to ethylene and ethane, a property frequently associated with the activity of alternative nitrogenases. Addition of 25μM molybdenum inhibited ethane production by *V. diazotrophicus*, but stimulated ethylene and ethane production by the *Photobacterium* isolate. Addition of 28μM vanadium did not appear to regulate ethane production by either strain. Assays of nitrogenase activity in sediments from which some isolates were obtained indicated that molybdenum was not limiting nitrogenase activity at naturally-occurring concentrations. Southern hybridizations of the chromosomes of these strains with the *anfH* and *vnfH* genes of *Azotobacter vinelandii* and the *nifH* gene of *Klebsiella pneumoniae*, indicated the presence of only one nitrogenase in these isolates.

INTRODUCTION

Unravelling the significance of biological nitrogen fixation in saltmarsh ecosystems is an elusive topic of considerable ecological interest. Notwithstanding a usual deficiency of inorganic nitrogen, saltmarsh ecosystems typically sustain high primary production, support rich biodiversity, and may make significant nutritional contributions to adjacent marine ecosystems (Thayer *et al.*, 1975; Valiela and Teal, 1979). It is possible however, that the potential limitation of primary production imposed by nitrogen impoverishment in saltmarsh and seagrass ecosystems, may frequently be ameliorated by bacterial dinitrogen fixation (Hanson, 1977a; O'Donohue *et al.*, 1991). Assessing the contributions of combined nitrogen to the nitrogen cycle of saltmarshes by nitrogen-fixing bacteria is therefore of ecological importance in understanding the biogeochemical processes that govern nitrogen cycling in these nearshore marine communities.

The ecology of nitrogen fixation in marine environments requires a knowledge of the distribution, enumeration and characterization of nitrogen-fixing bacteria. Several reports have addressed the distribution and enumeration of diazotrophs in marine environments (Maruyama *et al.*, 1970; Dicker and Smith, 1980c; Guerinot and Patriquin, 1981; Guerinot and Colwell, 1985). However, there have been few reports on the biochemical and physiological characterization of nitrogen fixing isolates from different marine habitats (Guerinot and Colwell, 1985; Shieh *et al.*, 1989).

Three genetically distinct nitrogenase systems have been identified in *Azotobacter vinelandii* (Jacobson *et al.*, 1989; Joerger *et al.*, 1989; Joerger *et al.*, 1990). The best characterized nitrogenase is the classical type, nitrogenase-1, which contains molybdenum (Mo) at the probable active site (Shah and Brill, 1977). Two alternative, Mo-independent nitrogenases have also been identified. One of these, nitrogenase-2 contains vanadium (V) (Robson *et al.*, 1986), while the other, nitrogenase-3, apparently does not contain either molybdenum or vanadium (Chisnell *et al.*, 1988). Expression of the alternative nitrogenases (2 and 3) is regulated by the presence or absence of molybdenum and vanadium (Joerger and Bishop, 1988). Whereas

nitrogenase-1 has been found in all known diazotrophs, alternative nitrogenases are not ubiquitous. Recent evidence for the occurrence of alternative nitrogenases in species from different genera has received much interest (Kentemich *et al.*, 1988, Scherer, 1989; Lehman and Roberts, 1991; Kimble and Madigan, 1992), but until now studies have not included marine bacteria.

The ecological and physiological significance of alternative nitrogenases is not clear. One possibility is that alternative nitrogenases may be advantageous to diazotrophs under conditions where molybdenum levels in the environment are too low to support the activity of nitrogenase-1. Although concentrations of molybdenum reported for seawater should be sufficient to support nitrogenase-1 activity (Collier, 1985), it is conceivable that the availability of molybdenum within bacterial cell microenvironments may be more limited. More recently, Dilworth *et al.* (1987) demonstrated that alternative nitrogenase activity reduced acetylene not only to ethylene, but also to ethane. In *A. vinelandii* and *Azotobacter chroococcum* this characteristic distinguishes alternative nitrogenase activity from that of nitrogenase-1, which reduces acetylene only to ethylene. Thus ethane production has been considered to indicate the likely presence of alternative nitrogenases (Dilworth *et al.*, 1987).

The present studies are aimed at elucidating the pathways of carbon and nitrogen fixation and their flows into microbial consumers. One component of these studies includes the characterization of resident diazotrophic populations. The present chapter focusses on the biochemical and physiological characterization of nitrogen-fixing isolates from Langebaan Lagoon, including an investigation of alternative nitrogenases.

METHODS

Study Sites

Two sites in Langebaan Lagoon were selected for study: Oesterwal, near the lagoon mouth, and at Geelbek in the southern reaches. Previous chapters have shown that *in situ* rates of acetylene reduction are greater in the muds at Geelbek than in the sands at

Oesterwal (Chapter 4). Sediment and water samples were taken from both sites (see Chapter 2). Sediment samples were taken to a depth of 5mm from within *Zostera* beds and from exposed sediment surfaces away from *Zostera* beds, while subsurface samples were taken from sediment cores at 10cm below the surface. Samples were always taken on the morning of spring tides, and were transported to the laboratory in an insulated container.

Media

Nitrogen-deficient glucose (NDG) medium consisted of the following components: (i) NaCl, 24.95g; $MgSO_4 \cdot 7H_2O$, 3.0g; $MgCl_2 \cdot 6H_2O$, 2.0g; KCl, 0.75g; $CaCl_2 \cdot 2H_2O$, 0.12g; Tris, 6.0g; disodium EDTA, 0.001g; trace metal solution, 1ml; Milli-Q water (Millipore), 500ml; adjusted to pH 7.8; (ii) glucose, 5g; yeast extract (Difco), 0.01g; Milli-Q water, 400ml; (iii) K_2HPO_4, 0.8g; KH_2PO_4, 0.2g; Milli-Q water, 100ml. Components (i), (ii) and (iii) were combined after each had been autoclaved separately, and $FeSO_4 \cdot 7H_2O$ and $Na_2MoO_4 \cdot 2H_2O$ were added to final concentrations of 0.015g l^{-1} and 0.005g l^{-1} respectively from stock solutions. Trace metal solution contained (g l^{-1} Milli-Q water): H_3BO_3, 2.86; $MnCl_2 \cdot 4H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, 0.079; $Co(NO_3)_2 \cdot 6H_2O$, 0.0494; $NiCl_2 \cdot 6H_2O$, 0.005. Nitrogen-free glucose (NFG) medium was NDG medium without yeast extract. Nitrogen-deficient glucose agar (NDGA) and nitrogen-free glucose agar (NFGA) were NDG and NFG with 1.5% (w/v) agar (Merck). Marine agar (MA) contained 1.5% (w/v) agar (Merck) in marine broth (Difco). Seawater agar (SWA) contained two components: (i) peptone, 2.5g; yeast extract, 1.0g; glucose, 1.0g in 750ml seawater, which had been filtered through Whatman No.1 filter paper; (ii) agar, 15g in 250ml distilled water. The two components were autoclaved separately and then combined. Tryptone yeast extract broth (TY) contained (g l^{-1} distilled water) tryptone, 5.0; yeast extract, 3.0; $CaCl_2 \cdot 2H_2O$, 1.0. Luria broth (LB) was made according to Sambrook *et al.* (1989). Marine Luria broth (MLB) was LB with 75% filtered seawater base, as above. Tryptone yeast extract agar and Luria agar contained 1.5% (w/v) agar in TY and LB respectively.

Isolation of nitrogen-fixing bacteria

Samples of water and sediment were serially diluted in NDG medium, and 100μl of each dilution inoculated in triplicate into 10ml NDG medium in Hungate tubes. Tubes were sealed, the headspace gas replaced with oxygen-free nitrogen, and incubated in the dark at room temperature. After 1 day incubation, 0.05atm. acetylene (Fedgas, SA) was added to each tube. The tubes were then incubated for a further 2 days before headspace gas samples were assayed for ethylene content by gas chromatography (see Chapter 2). Tubes positive for ethylene production were passed twice through 10ml NDG medium, and rechecked for ethylene production during each passage before cultures were spread onto NDGA for colony isolation. Individual colonies were then streaked for purity on NDGA and NFGA to determine growth requirements (Watanabe and Barraquio, 1979). Cultures in the early stages of isolation were always handled in an anaerobic chamber (Forma Scientific) until facultative metabolism for each isolate had been confirmed.

Assays of in situ *nitrogenase activity, and statistical analyses of data*

Triplicate sediment samples (25g wet weight) were weighed into 25ml wide-neck glass bottles, and were amended with ions ($Na_2MoO_4 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, $VOSO_4 \cdot H_2O$, $MgSO_4 \cdot 7H_2O$) or carbon sources (glucose, xylan, microcrystalline cellulose) as solutions of substrate in 2ml SM buffer (0.37M NaCl, 9mM KCl, 25mM $MgSO_4$, 23mM $MgCl_2$, 10mM Tris, pH 7.8). The bottles were sealed, and the headspace replaced with 85% argon and 15% acetylene (Fedgas). Bottles were incubated in the dark at 20°C, and the headspace gas was assayed for ethylene and ethane after 43 and 84 hours, as below. No ethylene or ethane production was detected in the absence of added acetylene, nor was ethylene production detected in autoclaved controls. Statistical analysis of data was carried out using analysis of variance (ANOVA) on untransformed data sets; Bartlett's test for homogeneity of variance did not indicate heteroscedasticity.

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All marine isolates were maintained on NFGA and SWA, except strain H24C and *V. diazotrophicus* ATCC33466 which were maintained on NFGA and MA. *A. vinelandii* was maintained on modified Burk's medium (Strandberg and Wilson, 1968). All strains were grown under aerobic conditions at 30°C, or 37°C in the case of *Escherichia coli*. For DNA isolations all marine strains were grown in MLB, and *A. vinelandii* was grown in LB.

Characterization of strains

Diazotrophic isolates were characterized biochemically and genetically, and compared with the characteristics of type strains, as follows: Standard biochemical tests, described in detail by Smibert and Krieg (1981) were used for biochemical and physiological characterization of the isolates. Each test was replicated (n=5) for each isolate. The methods of Sambrook *et al.* (1989), Murray and Thompson (1980), and Wilson (1989) were used for chromosomal DNA isolation. Restriction digests (*Pst*I, *Cla*I) of genomic DNA samples were electrophoretically separated on 0.7% agarose gels in Tris-borate-EDTA buffer (Sambrook *et al.*, 1989) prior to Southern transfer. Southern transfers of electrophoretically separated DNA samples were performed using Hybond N^+ membranes (Amersham, UK), according to the protocol recommended by the manufacturer. Plasmids pSJVH and pLWH3 containing *anfH* and *vnfH* respectively (Table 1) were kindly supplied by Dr P. Bishop (North Carolina State University). The origin of DNA used as probes is depicted in Fig.1. DNA inserts were recovered from the plasmids as follows: *nifH* was recovered by digesting pSA30 with *Nco*I, *vnfH* was recovered by digesting pSJVH with *Pst*I, and *anfH* recovered by digesting pLWH3 with *Hin*dIII and *Eco*RI. Probes were purified by gel electroelution as described by Sambrook *et al.* (1989). Probes were separated on low melting point agarose prior to a second separation on 1% (w/v) agarose before electroelution. Purified probe DNA was radiolabelled with ^{32}P-ATP (Amersham) for detection according to the method of

TABLE 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Reference or origin
Isolates		
OZRW5	nif^+	This study, OZS
O25	nif^+	This study, OZS
H1B	nif^+	This study, OSS
H24C	nif^+	This study, O10
WI1	nif^+	This study, GW
G21	nif^+	This study, GW
G25	nif^+	This study, GZS
OCT2	nif^+	This study, GZS
OCT3	nif^+	This study, GZS
Type strains		
Azotobacter vinelandii ATCC 478	nif^+, vnf^+, anf^+	Bush and Wilson (1959)
Vibrio diazotrophicus ATCC 33466	nif^+	Guerinot *et al.* (1985)
Plasmids		
pSA30	pBR322 containing *K. pneumoniae nifHDK*	Cannon *et al.* (1979)
pSJVH	pGEM3 containing *A. vinelandii vnfHFd*	Joerger *et al.* (1988)
pLWH3	pUC19 containing *A. vinelandii anfH*	Joerger *et al.* (1989)

OZS, Oesterwal *Zostera* bed sediment; OSS, Oesterwal exposed surface sediment; O10, Oesterwal sediment from 10cm depth; GW, Geelbek water; GZS, Geelbek *Zostera* bed sediment.

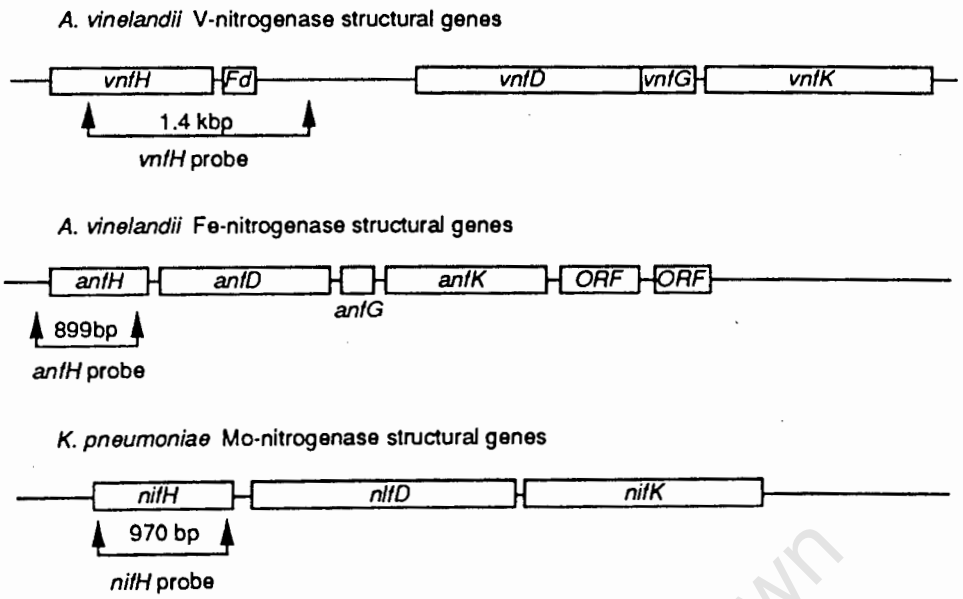

Figure 1. Regions of *vnfH*, *anfH*, and *nifH* used as probes for Southern hybridizations.

Sambrook *et al.* (1989). Hybridization (using 7% (w/v) SDS, 0.5% (w/v) non-fat dry milk powder, 1mM EDTA, 0.5M phosphate buffer, pH 7.2) and washing procedures (twice with 5% SDS, 1mM EDTA, 40mM phosphate buffer, pH 7.2) according to the protocol recommended by Church and Gilbert (1984), were carried out at 65°C.

RESULTS

Isolation of nitrogen-fixing bacteria

Bacterial strains capable of reducing acetylene to ethylene were isolated from a selected range of intertidal microhabitats in Langebaan Lagoon during an annual study (Table 1). Benthic microhabitats which were sampled included regions where primary production was dominated by seagrass or diatoms, the principle food-sources of some intertidal invertebrates (Harris, 1993). The sampling regime also extended to subsurface sediments and overlying waters, since these may also contribute combined nitrogen to the intertidal communities. Few isolates of acetylene-reducing bacteria were obtained from lagoonal seawater samples using these methods; samples which did yield isolates were from the organically-rich waters of Geelbek, typically after sediments had

been disturbed. These results correlate with previous studies which showed rates of acetylene reduction in these water samples to be undetectable (Chapter 4). In contrast, sediments from within *Zostera* beds yielded most of the nitrogen-fixing bacteria isolated during this study (Table 1). Most isolates in Table 1 represent groups of strains which showed identical biochemical and physiological characteristics. All isolates were able to grow in NFG medium under N_2, indicating that they could fix N_2 without requirement for complex organic growth factors.

Biochemical characterization and identification of isolates

All strains isolated during this study were motile, gram-negative rods with one or two polar flagellae. They fermented glucose and required NaCl for growth (Table 2). These characteristics indicate that these strains belong to the family Vibrionaceae. On further characterization, and in particular the ability to produce gas from glucose (Table 2), strains OZRW5, O25, G21, G25, OCT2 and OCT3 have been assigned to the genus *Photobacterium*, whereas strains H1B and H24C have been assigned to the genus *Vibrio*. Strain WI2, which is catalase and oxidase negative, could not be assigned to any genus in the Vibrionaceae. Other workers have also noted the occurrence of marine bacteria which are catalase and oxidase negative (Guerinot and Colwell, 1985; Shieh *et al.*, 1989). In addition to NaCl, strain WI2 also required high levels of Mg^{2+} for growth; it is unclear what the physiological basis for this requirement might be. Furthermore, it was interesting that strain H24C, which was able to reduce nitrate to nitrite (Table 2), was also susceptible to total repression of nitrogenase activity when 10mM $NaNO_3$ was added to the assay medium.

Within the group assigned to the *Photobacterium* genus, strains were differentiated mainly according to the following tests: oxidase, indole, methyl red, CMC-ase, and colony morphology. Biochemically similar strains (eg. G21 and G25) were further separated on the presence or absence of plasmids; strain G25 was found to contain a plasmid whereas plasmids were not detected in strain G21 (results not shown). Strain OCT3 is considered to be a biochemically atypical member of the *Photobacterium*

TABLE 2. Biochemical and physiological characteristics of nitrogen-fixing bacteria, isolates from this study and *V. diazotrophicus*[a].

Characteristic	Result for strain[b]									
	OZRW5	O25	H1B	H24C	WI2	G21	G25	OCT2	OCT3	*V.diazo.*
Catalase	+	+	+	+	-	+	+	+	+	+
Oxidase	-	-	+	+	-	+	+	+	-	+
Gas from glucose	+	+	-	-	+	+	+	+	-	-
Indole	+	-	+	-	-	+	+	+	-	+
Methyl red	v	v	v	+	+	v	+	-	+	+
Voges-Proskauer	+	+	-	-	v	+	+	+	-	-
Growth in:										
0% NaCl	-	-	-	-	-	-	-	-	-	-
3% NaCl	+	+	+	+	+	+	+	+	+	+
7% NaCl	+	+	+	+	+	+	+	+	+	+
Acid production from:										
Arabinose	+	+	v	+	-	+	+	+	v	+
Cellobiose	+	+	+	+	+	+	+	+	+	+
Galactose	-	-	-	v	v	-	-	-	+	v
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	+	-	-	-	-	-	+	-
Maltose	+	+	+	+	+	+	+	v	+	v
Mannitol	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	v	v	+	+	+	+	v	-
Salicin	v	v	+	-	+	v	v	v	-	v
Sucrose	+	+	+	+	+	+	+	+	-	+
Xylose	v	+	-	+	-	v	v	+	v	+
Sensitivity to O/129[c]	+	+	+	+	+	+	+	+	v	+
Growth on TCBS	-	-	-	+	-	-	-	-	-	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+
Lysine decarboxylase	-	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-
Agarase	-	-	-	-	-	-	-	-	-	-
Alginase	+	+	-	-	+	+	+	+	-	-
CMC[d]-ase	-	+	+	+	+	+	-	+	-	-
Xylanase	+	+	+	-	+	+	+	+	-	+
Starch hydrolysis	+	+	v	+	+	+	+	+	+	-
Chitinase	-	-	-	-	-	-	-	-	-	-
Protease[f]	-	-	v	-	+	+	-	+	-	-
Lipase[g]	-	-	v	+	-	-	-	-	-	-
DNase	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	v
Nitrate reductase	-	-	-	+	-	v	-	-	-	+
Luminescence	-	-	-	-	-	-	-	-	-	-
Pigmentation	-	-	-	-	-	-	-	-	o	-

[a] *Vibrio diazotrophicus* ATCC33466
[b] All strains were Gram negative, motile rods with fermentative metabolism
[c] 2,4-diamino-6,7-diisopropylpteridine (150μg.ml^{-1}); [d] thiosulfate citrate bile sucrose agar; [e] carboxymethylcellulose; [f] milk powder; [g] Tween 20
v, variable; o, orange

group; OCT3 was V-P negative, fermented galactose and lactose, but not sucrose, produced neither CMC-ase nor xylanase, and had bright orange pigmentation (Table 2). Strain OCT3 also produced ethane in acetylene reduction tests (Table 4), which further differentiated it from the rest of the *Photobacterium* group.

Two strains were assigned to the genus *Vibrio*. Strain H24C was assigned to the species *V. diazotrophicus* on the basis of colony morphology and biochemical tests, including growth on TCBS agar (Table 2). The high degree of similarity in biochemical characteristics, as well as the similarity in ethane production (Table 4), supports this classification. Strain H24C was isolated from subsurface sediment at Oesterwal.

Studies on the utilization of carbon sources showed that 7 of the 9 isolates produced a xylanase, 6 isolates were able to hydrolyse CMC, 5 isolates produced an alginase, and all isolates utilized starch. All strains were able to utilize cellobiose and glucose. Arabinose was utilized by most strains, while xylose was utilized to a lesser extent among the group (Table 2). These findings reflect the predominance of plant-derived materials as substrates for bacterial growth in saltmarsh and seagrass environments. None of the isolates were able to utilize microcrystalline cellulose (Avicel). However, the complex enzyme system required to hydrolyse this highly crystalline polymer is probably not ubiquitous among bacterial populations (Béguin, 1990). Four strains produced a protease, only 2 strains produced a lipase, and none of the isolates produced a chitinase.

Ethane production

Ethane production has occasionally been detected in acetylene reduction assays of nitrogen fixation in Langebaan Lagoon sediments. Rates of acetylene reduction from unamended sediment samples were generally low and ethane peaks were not detected. However, when sediments were amended with different carbohydrate sources, which stimulated acetylene reduction rates, small amounts of ethane were occasionally measured. During experiments carried out in January 1992, sediments from different

TABLE 3. Assays of acetylene reduction *in situ* for which both ethane and ethylene production were detected.

Sediment type[a]	Amendment[b]	Ratio[c]
Oesterwal, BO	Glucose	0.02
Geelbek, ZB	Xylan	0.01
Geelbek, ZB	Xylan	0.07
Geelbek, BL	Xylan	0.01

[a] Sediment samples were collected in triplicate from 5 microhabitats (exposed surface sediment, *Zostera* bed (ZB), prawn burrow opening (BO), prawn burrow lining (BL), and 10cm subsurface) at each site.
[b] Sediments were amended with 0.9 mg substrate g^{-1} wet sediment.
[c] Percent molar ratio of ethane to ethylene production. Results of assays for which ratio > 0 are shown.

benthic microhabitats at Oesterwal (sand) and Geelbek (mud) were amended with glucose, xylan and cellulose. Ethane production was detected for four out of a total of 90 samples, and formed 0.01 to 0.07% of ethylene production (Table 3). As ethane production is frequently considered to be a property of alternative nitrogenases, and the expression of alternative nitrogenases in *A. vinelandii* is regulated by Mo and V availability, further studies examined whether ethane production reflected a possible limitation of nitrogenase-1 activity in these sediments by low concentrations of Mo. Enrichment of sediment, in which ethane production had been previously detected, with Mo, Fe, and V, did not significantly ($p > 0.05$) enhance acetylene reduction relative to unamended controls (Fig. 2a). Elevated rates of nitrogenase activity *in situ* should exert greater demands on naturally-occurring supplies of Mo, Fe, and possibly V. However, nitrogenase activity stimulated by glucose was not further enhanced by additions of Mo, Fe, and V (Fig. 2b). Howarth and Cole (1985) proposed that the availability of Mo to bacteria in seawater might be restricted by SO_4^{2-}. Sulfate is a structural analogue of molybdate, and they argued that the relatively high concentrations of SO_4^{2-} in seawater could restrict Mo uptake by bacteria. Paulsen *et al.* (1991) examined the possible limitation of nitrogen fixation by Mo in seawater by using additions of sulfate to interfere with molybdate uptake by bacteria. The same approach was used to study lagoonal sediments in the present work, and conlusions similar to

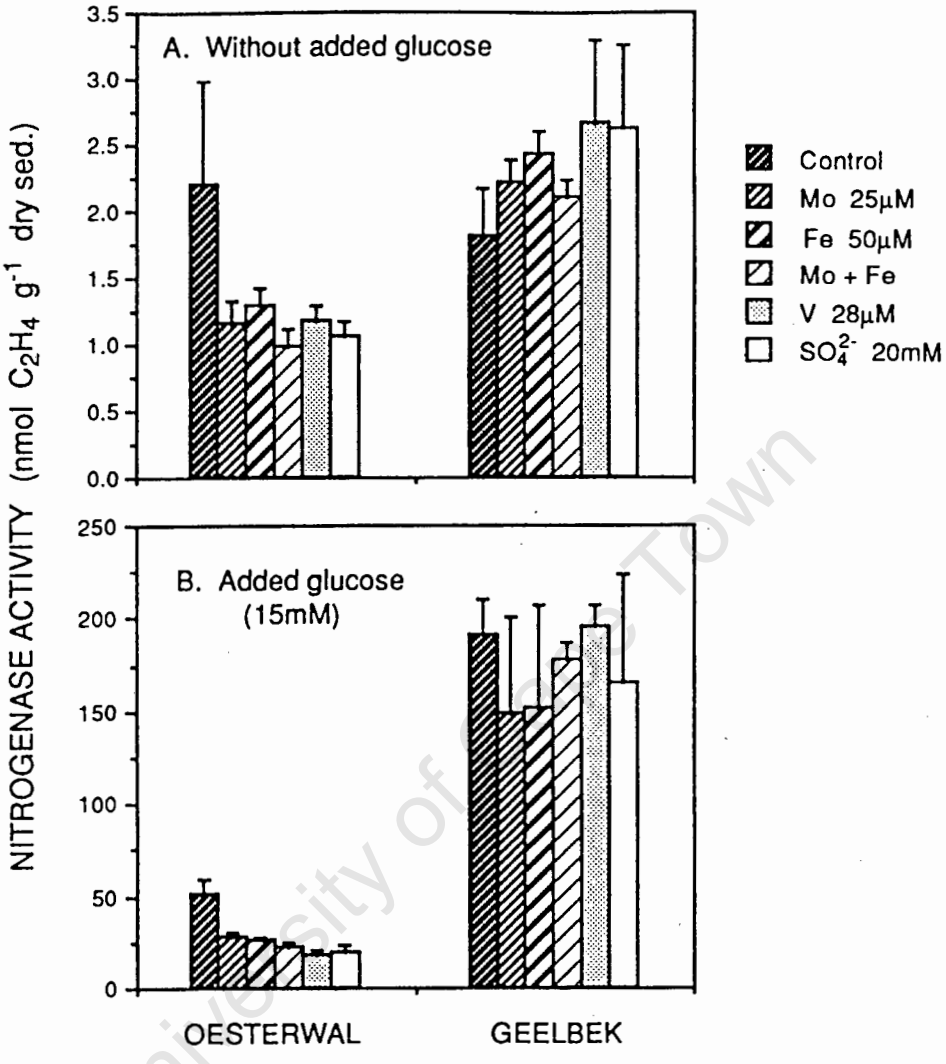

Figure 2. Effect of additions of different ions on nitrogenase activity in surface sediments with or without added glucose. Values represent means ± SD of replicate samples (n=3); ethylene determinations after incubation for 43 hours.

those of Paulsen *et al.* (1991) were reached. Additions of SO_4^{2-} did not significantly ($p > 0.05$) lower rates of acetylene reduction relative to samples without added SO_4^{2-} (Fig. 2), and so it seemed that Mo was not limiting nitrogenase activity in these samples. Further studies of ethane production focussed on pure cultures of nitrogen-fixing bacteria isolated from Langebaan Lagoon.

Acetylene reduction to ethylene and ethane was detected for two isolates, strains H24C and OCT3, and *V. diazotrophicus* ATCC33466, when Mo was omitted from the assay medium. Strain OCT3 was isolated from *Zostera* sediment at Geelbek, which had supported ethane production during previous *in situ* acetylene reduction assays (Table 3). Addition of Mo to cultures of nitrogen-fixing isolates starved of Mo stimulated rates of acetylene reduction for all isolates, suggesting that the nitrogenases of these strains used Mo. Results for three strains, OCT3, *V. diazotrophicus*, and H24C, are presented in Table 4. In contrast to Mo, addition of V did not enhance rates of acetylene reduction in these strains.

TABLE 4. Reduction of acetylene to ethylene and ethane by cultures of ethane-producing strains under different ionic conditions[a].

Strain	+Fe +Mo C_2H_4[b]	Ratio[c]	+Fe C_2H_4	Ratio	+Fe +V C_2H_4	Ratio
OCT3	100	0.17	38	0.17	42	0.18
H24C	100	0	51	0.15	53	0.14
V. diazotrophicus	100	0	60	0.22	64	0.17

[a] Fe, 50μM; Mo, 25μM; V, 28μM
[b] percentage of ethylene production by cultures with added Fe and Mo
[c] percent molar ratio of ethane/ethylene production

Ethane production by strain H24C and *V. diazotrophicus* ceased when 25μM Mo was added to the medium (Table 4). Ethane production is unusual and is frequently associated with the activity of alternative nitrogenases. Therefore, Southern hybridization of the chromosomal DNA of ethane-producing strains with probes for the alternative nitrogenase *anfH* and *vnfH* genes of *A. vinelandii*, and the *nifH* gene of *K. pneumoniae* was used to determine if these strains contained more than one nitrogenase. Hybridization of *Pst*I-digested *A. vinelandii* chromosome, as the control, with each of the 3 different nitrogenase gene probes showed three distinct bands, which varied in intensity when hybridizations with different probes were carried out (Fig. 3). Multiple banding hybridization patterns similar to the control would be expected if, in

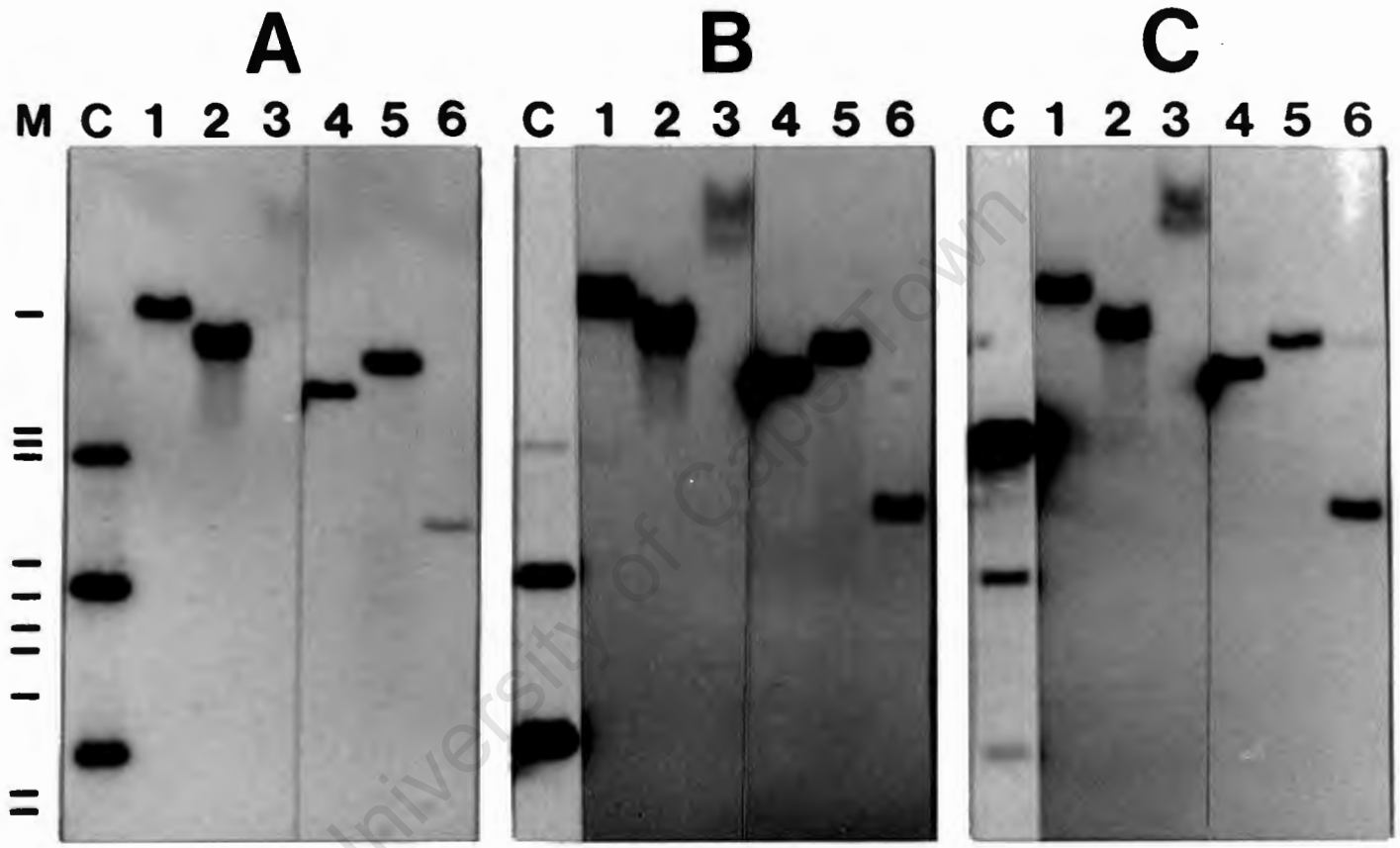

Figure 3. Hybridization of *nifH*, *vnfH*, and *anfH* probes to the chromosomes of strains that produced ethane from acetylene. Lanes 1, 2, 3: DNA of strain OCT3, *V. diazotrophicus*, and strain H24C respectively, digested with *Pst*I; lanes 4, 5, 6: DNA of strain OCT3, *V. diazotrophicus*, and strain H24C respectively, digested with *Cla*I; lane C: control, *A. vinelandii* DNA digested with *Pst*I. Panels A, B, and C: hybridization patterns with *nifH*, *vnfH*, and *anfH* probes respectively. Autoradiographs were performed for 92h, except for the control lanes in panels B and C (homologous probes), which were for 48h. The relevant lanes from 48h and 92h autoradiographs were combined for this figure. Molecular weight markers (M) are fragments of *Pst*I-digested lambda DNA. Digestion of strain H24C DNA was better with *Cla*I than with *Pst*I.

addition to the conventional nitrogenase, alternative nitrogenases were also present in the marine strains. Hybridizations with *Pst*I and *Cla*I digests of chromosomal DNA, however, clearly show only one band in each lane (Fig. 3), indicating that not more than one nitrogenase was present in each of the marine strains.

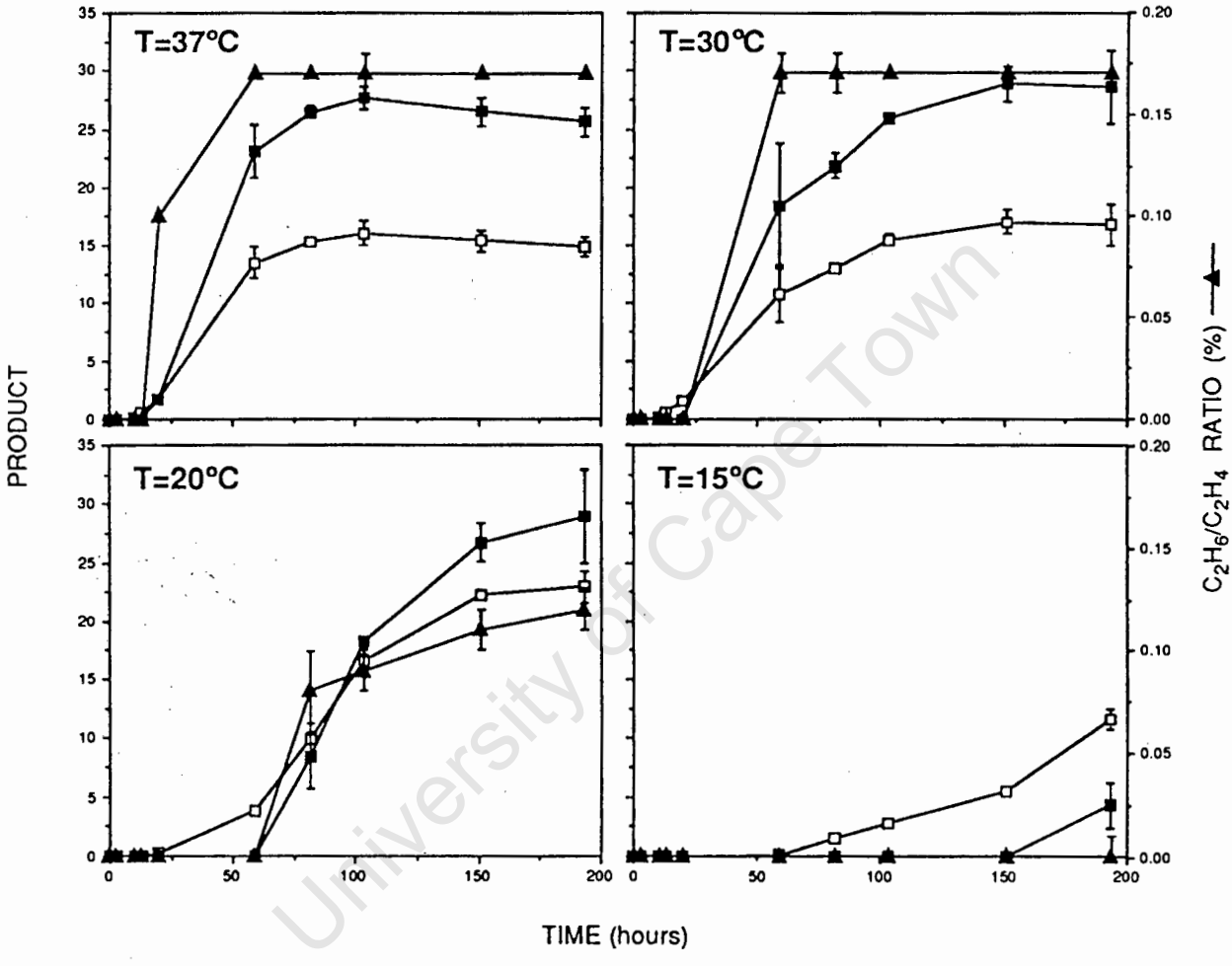

Figure 4. Effect of temperature on ethylene and ethane production by strain OCT3. Production rates are given as μmol C_2H_4 mg^{-1} protein (—□—) and nmol C_2H_6 mg^{-1} protein (—■—).

Addition of 25μM Mo did not alter the ratio of ethane:ethylene production by strain OCT3. Addition of Mo to diazotrophic cultures of OCT3 stimulated both ethane and ethylene production and maintained the ratio of these two products (Table 4). Furthermore, addition of 28μM vanadium did not appear to regulate ethane production in either OCT3 or *V. diazotrophicus* (Table 4). Ethane production by strain OCT3 may

be considered unusual as it was not affected by Mo or V, and did not appear to be the product of alternative nitrogenase activity. Under elevated temperatures nitrogenase-1 of *A. vinelandii* may produce small amounts of ethane (R. Eady, R. Pau, pers. comm.). The effect of temperature on ethane production by strain OCT3 was examined (Fig. 4). No change in the ethane:ethylene ratio was observed when the incubation temperature was raised from 30°C to 37°C. However, at lower temperatures (20°C and 15°C) rates of acetylene reduction were slower, and the ratio of ethane:ethylene production decreased. Compared to ethylene production, so little ethane was produced by OCT3 at 15°C, even after extended incubations (ca. 200h), that the mean ratio did not rise above 0.00% (Fig. 4).

DISCUSSION

Physiological characterization of the diazotrophic isolates obtained in this study indicated that their utilization of polysaccharides reflected the organic material available to bacteria in saltmarsh ecosystems. Although plant material probably represents the majority of organic material available to heterotrophic bacteria in Langebaan Lagoon, saltmarsh ecosystems also support large populations of crustaceans. Thus, chitin may represent a further source of organic material which could be utilized by bacteria in these environments. However, none of the isolates were able to produce chitinase, indicating that these strains depended on organic carbon and energy from plant materials rather than from chitin. It is possible that consortional interactions between diazotrophs and chitinolytic bacteria may be energetically beneficial to nitrogen fixation. However, the trend in polysaccharide utilization among these isolates correlates with the results of previous work, which showed that additions of xylan and cellulose, but not chitin, to sediment samples from Langebaan Lagoon, stimulated *in situ* rates of acetylene reduction (Chapter 5).

Investigations into the physiology associated with repression of nitrogenase activity by sources of combined nitrogen showed that strain H24C was able to reduce nitrate to nitrite (Table 2). In contrast to the other isolates, strain H24C was also subject to repression of nitrogenase activity by nitrate. The exact mechanism by which nitrate

represses nitrogenase activity is not clear. Reduction of nitrate by assimilatory pathways may produce ammonium, which inhibits nitrogenase activity (Hattori, 1983). However, Dicker and Smith (1980c) have proposed that nitrate induces the expression of nitrate reductase, which competes with nitrogenase for available reducing power. Nitrate and other nutrients are seasonally available to the south west coast of South Africa, including Langebaan Lagoon, when upwelling introduces nutrient-rich South Atlantic Central Water to this region during spring and summer (Andrews and Hutchings, 1980). The availability of nitrate might therefore regulate nitrogen fixation in Langebaan Lagoon on a seasonal basis, although strong evidence for this is not indicated among the nitrogen fixers isolated in this study where only one strain was able to utilize nitrate.

Another source of combined nitrogen for bacteria in coastal lagoons is urea, an excretion product of some animals (eg. zooplankton) that inhabit these ecosystems. However, none of the isolates in the present study were able to produce urease (Table 2). Although previous work indicated that only ca.10% of general heterotrophic isolates from this lagoon were able to utilize urea (Chapter 2), the absence of urease activity among the diazotrophic isolates was surprising, since the energetic cost of obtaining combined nitrogen from urea should be less than that of nitrogen fixation.

The presence of *nif* genes on large plasmids is a characteristic of plant-associated bacteria such as *Rhizobium* spp. and the lignin degrading strain, *Lignobacter* K17 (Casse *et al.*, 1979; Darylo *et al.*, 1981). In contrast, the *nif* genes of plasmid-bearing *Azospirillum* strains, which are also plant-associated, are chromosomally located (Plazinski *et al.*, 1983). As two of the isolates were found to contain megaplasmids of 125 to 200 megadaltons, I was interested in whether the *nif* genes of these isolates were plasmid-borne. Southern hybridization studies using a probe specific for the *nifHDK* genes of *K. pneumoniae* showed that *nif* genes were not located on the plasmids of these strains (results not shown).

Members of the family Vibrionaceae are common to marine habitats. In particular, it has been shown that the genus *Vibrio*, previously considered to lack the ability to fix

nitrogen, may be important to the ecology of nitrogen fixation in some marine environments (Guerinot and Patriquin, 1981; Guerinot and Colwell, 1985; Shieh *et al.*, 1989). The first species of *Vibrio* shown to fix nitrogen was *V. diazotrophicus*; strains of *V. diazotrophicus* have been isolated from the gastrointestinal tract of sea-urchins, from cockles, oysters and whelks, and from seawater and marshwater from England and the United States (Guerinot *et al.*, 1982). Isolates of *V. diazotrophicus* have also been obtained from saline rivers and lakes in Spain (Urdaci, 1987).

Two isolates, *V. diazotrophicus* and *Photobacterium* strain OCT3, reduced acetylene to ethylene and ethane when molybdenum levels in the medium were low. In addition, the type strain *V. diazotrophicus* ATCC33466, also produced ethane. Ethane production by *V. diazotrophicus* ceased when molybdenum (25μM) was added to the medium (Table 4). Addition of 25μM Mo stimulated acetylene reduction by the ethane-producers, which indicated that these strains contained at least an Mo-type nitrogenase. This effect may be considered indicative of alternative nitrogenase activity, since Mo and V are known to regulate the expression of alterative nitrogenases in some organisms (Dilworth *et al.*, 1987; Joerger and Bishop, 1988). The reduction of acetylene to ethylene and ethane has not been reported for any species of *Vibrio* (Guerinot *et al.*, 1982; Urdaci *et al.*, 1988), but most studies of acetylene reduction in the vibrios have included Mo in the assay medium.

Kimble and Madigan (1992) have recently reported evidence for alternative nitrogenases in the phototrophic bacterium *Heliobacterium gestii*. Their work was based on nitrogenase reduction of acetylene under different ionic conditions, but without genetic evidence. Lehman and Roberts (1991) have identified an alternative nitrogenase system in *Rhodospirillum rubrum* by Southern hybridization of *nifH*, *anfH*, *vnfH*, and *anfD* of *A. vinelandii* to the *R. rubrum* chromosome. Their results suggest that the alternative nitrogenase of *R. rubrum* is similar to the enzyme encoded by *A. vinelandii anfHDK* genes. A similar approach was used to investigate the possibility of alternative nitrogenase genes in ethane-producing marine strains, using DNA probes specific to the *anfH*, *vnfH* genes of *A. vinelandii*, and the *nifH* gene of *K. pneumoniae*. Results of these experiments indicated that the ethane-producing marine bacteria examined here

contained only one nitrogenase, and that acetylene reduction was reliant on Mo. Addition of molybdenum, even at quite high concentrations, failed to inhibit ethane production in *Photobacterium* strain OCT3; increased molybdenum concentrations stimulated both ethane and ethylene production by strain OCT3, and the molar ratio of ethane/ethylene production was maintained (Table 4). This represents an uncharacteristic feature of Mo-nitrogenase reduction of acetylene.

Addition of molybdenum to cultures of an *A. vinelandii* strain with deletions in the genes for both Mo- and V-type nitrogenases, stimulated ethane production (Pau *et al.*, 1989). It was proposed that this transient change in reactivity was due to the incorporation of the FeMo cofactor into nitrogenase-3 to produce a hybrid enzyme. Further, they proposed that addition of Mo and subsequent synthesis of the FeMo cofactor would be accompanied by repression of the synthesis of nitrogenase-3 (Pau *et al.*, 1989). It is unlikely that a similar explanation could be applied to the results for strain OCT3 in our studies, since nitrogenase derepression was performed in medium containing molybdenum. However, the work of Pau *et al.* indicates that the different nitrogenase polypeptides can accept different nitrogenase cofactors, to produce changes in reactivity during acetylene reduction. Since Southern hybridization studies could not confirm the presence of *anf* or *vnf* genes in strain OCT3, it may be possible that OCT3 has a Mo-type nitrogenase with the ethane-producing characteristic of the alternative nitrogenases of *A. vinelandii*. However, it cannot be concluded from these data precisely which nitrogenase (1, 2, or 3) is present in OCT3, since some cross-hybridization occurs between the different probes used here (see lanes of *A. vinelandii* chromosomal DNA in Fig. 3). It may also be possible therefore, that OCT3 contains only an alternative nitrogenase, which could accept MoFeco, as shown by Pau *et al.* (1989). If so, this would indeed be a unique diazotrophic strain.

The response of ethane production by strain OCT3 to a range of temperatures representative of those at Langebaan was examined. Nitrogenase-1 of *A. vinelandii* may produce small amounts of ethane under elevated temperatures (R. Eady, R. Pau, pers. comm.). Ethane production by OCT3 decreased at lower incubation temperatures, and formed a negligible fraction of ethylene production at the lowest temperature, 15°C.

Ethane production in OCT3 appears to be a normal function of this organism's nitrogenase. The reduction in ethane production at 15°C simply reflects enzyme inactivity, since the rate of acetylene reduction at 15°C was about 10-fold lower than at 37°C.

This work shows that the physiological characteristics of diazotrophs related to control of nitrogenase activity generally reflect the environment from which these isolates were obtained. We have occasionally detected small ethane peaks in acetylene reduction assays carried out on sediment samples from Langebaan Lagoon. Ethane production has been considered to be indicative of alternative nitrogenase activity, which would implicate molybdenum and/or vanadium availability in the ecological control of nitrogen fixation in this lagoon. However, the results obtained from this study of isolates from Langebaan Lagoon places significant doubt on such interpretations, and questions the validity of ethane production, or the regulation of ethane production by Mo or V availability, as tests for the presence of alternative nitrogenases in different genera.

CHAPTER 7

Synthesis: Ecology and Physiology of Nitrogen Fixation in Langebaan Lagoon.

Heterotrophic bacterial activity and nitrogen fixation are two processes that are central to nutrient regeneration and nitrogen cycling in saltmarsh ecosystems. Rates of nitrogen fixation in many marine environments tend to be low (Howarth *et al.*, 1988a). The mechanisms that control rates of nitrogen fixation in these environments are important since primary and secondary producers are often reliant on this process to supplement their nitrogen requirements.

Bacterial activity and nitrogen fixation are influenced by environmental characteristics, and saltmarsh ecosystems can be considered as a 'mosaic' of habitats. In Langebaan Lagoon, tidally-controlled water circulation and limited wave action are two physical processes that influence the distribution of different types of sediments. Geelbek, in the sheltered, southern reaches of this lagoon, has fine, muddy sediments which are relatively anaerobic, whereas Oesterwal, near the lagoon mouth is exposed to tidal currents and limited wave action, and has sandy sediments which are relatively aerobic. Biotic components of the ecosystem have also influenced the characteristics of microhabitats within the 'framework environment' shaped by physical factors. For instance, sediments associated with seagrass beds have a higher organic content and have higher densities of bacteria than exposed surface sediments. Furthermore, burrowing prawns, which occur in high numbers and dominate the benthic macrofauna at Oesterwal and Geelbek, modify the environment by lining their burrows with organically-rich mucus, and turn over significant quantities of sediment. Their activities thus increase oxygenation of subsurface sediments (Dye, 1978), and alter the depth-distribution of microbiota (Branch and Pringle, 1987). These observations prompted consideration of how (to what extent) macrocommunity structure (including macrophytes and macrofauna) influenced bacterial production and nitrogen fixation in Langebaan Lagoon, and what factors (physical, chemical, and biological) were primarily responsible for regulating nitrogenase activity.

These questions were addressed by using ecological and physiological methods. An ecological background study assessed the status of environmental parameters important to bacterial activity in general, and nitrogen fixation in particular. Since there has been some debate in the literature about the validity of assumptions underlying methods (tritiated thymidine incorporation, TTI) used to estimate bacterial production in natural systems, the methodology of estimating bacterial production in Langebaan Lagoon was examined. Established and modified methods were subsequently used to measure rates of bacterial productivity and nitrogen fixation on a seasonal basis in sediments and water from the lagoon. Since rates of nitrogenase activity were generally lower than those reported for similar ecosystems, detailed examinations were carried out to determine which factors were primarily responsible for regulating nitrogenase activity. During the course of these studies, different strains of diazotrophic bacteria were isolated from the lagoon, and the physiologies of these strains were examined for further information about the genetic and physiological processes regulating nitrogenase activity.

Although diazotrophic bacteria were present in lagoon water, nitrogenase activity was not detected in water column samples. Diazotrophic bacteria isolated from water were facultatively anaerobic heterotrophs, with O_2-sensitive nitrogenase activity. Paerl *et al.* (1987) concluded that heterotrophic nitrogen fixation in seawater was largely limited by the availability of organic substrates. Paerl *et al.* showed that free sugars provide a source of energy for bacterial metabolism, whereas detrital particles provide surfaces upon which bacterial aggregates in the water column can establish O_2-depleted microzones, thus enabling O_2-sensitive nitrogen fixation. However, additions of different organic substrates to Langebaan Lagoon water under aerobic or anaerobic conditions failed to stimulate nitrogenase activity to detectable levels. The failure to detect nitrogenase activity in lagoon water was most likely a function of low numbers of diazotrophic bacteria in this environment.

Sediments supported higher rates of bacterial production and nitrogen fixation than water on a volume basis. Spatial comparisons showed that fine, muddy sediments at Geelbek supported greater bacterial productivity and nitrogenase activity than those at

Oesterwal. This spatial pattern reflected the patterns of bacterial abundance and the organic contents of these sediments. It is likely that the greater quantity and quality (C:N ratio) of organic matter in Geelbek sediments probably supported the greater bacterial abundance in those sediments compared with Oesterwal sediments; thus, the distribution of sediments by physical factors (including tidal currents and wave action) has contributed to spatial patterns of bacterial activity.

Sediments within *Zostera* beds at Geelbek supported the highest rates of nitrogen fixation overall. *Zostera* bed sediment from Geelbek also had the highest organic content, but highest densities of bacteria were found in subsurface sediments at Geelbek. The regulation of nitrogenase activity in subsurface sediments is discussed below.

One consideration of these studies concerned the nature of bacterial production and nitrogenase activity in sediments associated with the burrows of *C. kraussi* and *U. africana*. Previous studies have reported greater bacterial densities in burrow-associated sediments than in adjacent subsurface sediments (Branch and Pringle, 1987), suggesting that organically-rich burrow lining and relatively oxygen-rich burrow water provide an improved subsurface environment for bacterial activity. The present work shows that bacterial productivity and nitrogenase activity were higher in burrow lining than in subsurface sediment away from burrows. However, bacterial abundance was not significantly higher in burrow lining relative to subsurface sediments; at Geelbek, numbers in subsurface sediment away from burrows exceeded those in burrow lining (Harris, 1993). Thus, bacterial abundance does not explain enhanced bacterial activity in burrow lining, suggesting that an improved subsurface environment for bacterial activity is indeed present in burrow lining, while perhaps increased grazing pressure limits bacterial populations.

While the enhanced organic content of burrow lining may provide substrates for bacterial metabolism to facilitate energy-demanding nitrogenase activity, nitrogen fixation at the burrow sediment/water interface should be affected by oxygen from the burrow water. Nitrogen fixation by aerobes such as *Azotobacter* spp. may be stimulated

in the presence of oxygen from burrow water, whereas nitrogen fixation by facultative or anaerobic strains is likely to be inhibited by oxygen. Further studies therefore examined the effect of oxygen on nitrogen fixation in sediments. Nitrogenase activity in surface sediments (particularly surface sediment between burrows and *Zostera* bed sediment) was stimulated by aerobic conditions by up to 20-fold, relative to rates under anaerobic conditions. These data indicate that populations of aerobic diazotrophs are active in these sediments, and prompted comparison with the effect of oxygen on rates in burrow lining (a subsurface sediment/water interface). Oxygen did not stimulate nitrogenase activity in burrow lining or subsurface sediment away from burrows. It is not clear why evidence for aerobic diazotrophy was found in surface sediments, but not in burrow lining; no significant interaction was noted between light and oxygen on nitrogenase activity in surface sediments. The oxygen-effect was also examined in sediments at burrow openings. The degree of oxygen-stimulation of nitrogenase activity was less in burrow opening sediment than in adjacent surface sediment away from burrows. Burrow opening sediment includes subsurface sediment displaced to the surface due to bioturbation. Surface sediment with aerobic diazotrophs would thus be displaced from the surface, and bioturbation would have a negative effect on oxygen-dependent microbial metabolism in surface sediments. While motile bacteria, displaced by bioturbation, may gradually move back to aerobic zones within burrow opening sediment, the substrates (saltmarsh detritus) available to heterotrophic bacteria in subsurface sediments (and therefore also in burrow opening sediments) are likely to be more refractory than newer detrital material present in surface sediments away from burrows and within *Zostera* beds.

Comparisons of bacterial activity in sediments on a temporal scale showed a seasonal pattern in rates. The usual pattern showed that bacterial production and nitrogenase activity were lowest in winter, and increased during spring to reach peak rates during summer. Whereas, seasonality of bacterial production (TTI) was coincident variations in the supply of organic matter from primary production, seasonality of nitrogenase activity (examined during seasons different to TTI) did not reflect temporal fluctuations in organic contents of sediments or in bacterial abundance. However, seasonality of nitrogenase activity in sediments did reflect changes in numbers of nitrogen-fixing

bacteria, which were also highest in summer and lowest in winter. Furthermore, the proportion that diazotrophs contributed to total heterotrophic population cultured on agar plates also changed between seasons; diazotrophs were relatively more abundant during summer than during winter. The reasons why diazotrophs should form a greater proportion of the total population during summer than during winter are not clear. It is possible that conditions (e.g. increased organic matter availability while nitrogen replete conditions persist) may be more favourable for diazotrophy during summer than during winter. In temperate saltmarsh ecosystems, primary production, the dominant source of organic matter in these ecosystems, increases during spring and summer, with die-back of the macrophytes occurring during late summer to autumn. Mazure and Branch (1979) and I (Chapter 3) have noted significant seasonality of organic matter available for bacterial use in Langebaan sediments, although not all the seasonal studies I undertook revealed seasonality in either quantity or quality of organic contents (Chapters 2 and 4).

Rates of nitrogen fixation in sediments from Langebaan Lagoon were generally low when compared with rates reported for similar ecosystems. Additions of glucose (an easily-utilizable carbon source, and a component of the organic matter available to bacteria in Langebaan Lagoon) stimulated nitrogenase activity by ca. 1 to 3 orders of magnitude. The degree of glucose-stimulation of nitrogenase activity was negatively correlated with the rate of activity in unamended sediments; stimulation of activity was greatest when rates in unamended sediments were low, indicating that nitrogenase activity was dependent upon the availability of easily utilizable carbon sources. There was no distinct seasonal pattern in the magnitude of glucose-stimulation of nitrogenase activity, and hence seasonality of nitrogenase activity could not be linked to the availability of glucose. However, on a spatial scale, the degree of glucose-stimulation suggested that a limited availability of easily-utilizable carbon sources was partly responsible for lower rates of nitrogen fixation in subsurface sediments than in surface sediments. Detritus released from senescent or damaged plants passes initially to surface sediments, where bacterial colonization occurs and readily available materials are utilized. Organic material in subsurface sediments is likely to contain relatively more recalcitrant materials than that in surface sediments.

In surface sediments, where the highest rates of nitrogen fixation were measured, fermentation appeared to be the dominant metabolism supporting nitrogenase activity. Glucose and mannitol are components of the organic material available to bacteria in saltmarsh environments, and were better substrates at supporting *in situ* nitrogenase activity in surface sediment than oxidizable substrates, such as lactate or acetate. Most organic matter that becomes available to bacteria in these environments is, however, complex, polysaccharide-based plant material. Bacteria in Langebaan Lagoon sediments showed different abilities to utilize different polysaccharides to support their requirements for nitrogenase activity. In terms of supporting nitrogen fixation, storage polysaccharides (glycogen and laminarin) were better substrates than structural polysaccharides (cellulose, carrageenan, xylan and alginate). Of the structural polysaccharides, xylan and alginate were more easily utilized than cellulose and carrageenan. Sediment bacteria required extended incubations (2-3 days) with cellulose before detectable stimulation of nitrogenase activity could be measured. This result may be significant, however, since cellulose is a major component of many marine plant materials. Moreover, the supply of detrital material in such systems is dynamic; release of plant materials for bacterial use occurs during seasonal die-back of macrophytes and the physical action of storms. In contrast to the polysaccharides of plant origin, a polymer of prawn exoskeletons, chitin, did not stimulate nitrogenase activity, indicating that the availability of chitin cannot make a significant contribution to the control of nitrogenase activity in Langebaan Lagoon.

The effects of organic substrates on *in situ* nitrogenase activity reflected the physiologies of diazotrophic bacteria isolated from Langebaan Lagoon. Most isolates produced cellulases and xylanases, but none could utilize chitin, indicating that nitrogen fixation in Langebaan Lagoon relies primarily on plant materials as substrates for growth of diazotrophic bacteria. A further consequence of diazotrophic growth on plant materials concerns the role of nitrogen fixation in the nutritional demands of secondary producers. Specifically, the nutritional value of detritus to detritivores in the marine community is largely dependent on the nitrogen content of the food-source (Mann, 1988). The nitrogen content of detrital material may be enhanced when such material is

colonized by bacteria, thus improving its nutritional value to consumers (Newell, 1965; Pomeroy, 1980). The nitrogen demands of microbial growth on detritus may be supplemented with inorganic nitrogen obtained from the environment, or from nitrogen fixation. The present work shows that diazotrophic growth on complex plant polysaccharides may be significant. Since little attention has been paid to the degree to which nitrogen fixation in microbially-mediated nitrogen-enrichment of saltmarsh and seagrass detritus can benefit consumers, future research on this topic is encouraged.

Ammonium and nitrate inhibited nitrogenase activity in Langebaan sediments in a concentration-dependent manner. However, ammonium did not appear to have a significant role in the regulation of nitrogenase activity in Langebaan Lagoon as concentrations of ammonium in interstitial waters were well below those required to inhibit *in situ* activity. The availability of nitrate to Langebaan Lagoon increases during summer when upwelling occurs. One isolate was found to have nitrate reductase activity, indicating that nitrate may modulate nitrogenase activity in some bacteria in Langebaan Lagoon. However, the extent to which nitrate controls nitrogenase activity is uncertain, since *in situ* nitrate concentrations were low compared to the concentrations required to inhibit nitrogenase activity in sediments.

Temperature probably has a dominant role in the regulation of bacterial activity in general, and nitrogen fixation in particular, in temperate saltmarsh ecosystems. In Langebaan Lagoon, highest rates of nitrogenase activity were measured at 20°C, with relatively little activity occurring at temperature extremes of 10°C or 37°C. Seasonal variations of sediment temperature in Langebaan Lagoon ranged from ca. 11°C on winter mornings to more than 28°C on summer afternoons, and thus approach the extremes of the range examined here. Furthermore, diel temperature variations of 4-6°C are also likely to influence nitrogenase activity, as nitrogenase activity of nitrogen-fixing isolates from Langebaan Lagoon was found to be sensitive to a temperature difference between 15°C and 20°C.

The production of ethane during acetylene reduction assays was occasionally noted when sediments were amended with carbohydrates to stimulate nitrogenase activity.

Ethane production is frequently considered to be a property of alternative nitrogenase reduction of acetylene, suggesting that *in situ* nitrogenase activity may be limited by molybdenum or vanadium availability. However, additions of Mo and V to sediments failed to stimulate nitrogenase activity, indicating that Mo and V were not limiting nitrogenase activity at *in situ* concentrations. Further investigations of alternative nitrogenase activity focussed on diazotrophic isolates from the lagoon. Two isolates of nitrogen-fixing bacteria from Langebaan Lagoon reduced acetylene to both ethylene and ethane. Ethane production by both strains was apparently not regulated by vanadium. Molybdenum inhibited nitrogenase activity in an isolate of *Vibrio diazotrophicus*, but not in a *Photobacterium* isolate. Southern hybridizations of genomic DNA from these isolates with *anfH*, *vnfH*, and *nifH* genes indicated the presence of only one nitrogenase in each strain. Ethane production by a single nitrogenase in the *Photobacterium* isolate in the presence of Mo and V presents a particularly interesting question: either ethane production is not necessarily restricted to alternative nitrogenases (contrary to the generally held view that it is), or this *Photobacterium* isolate contains an alterative nitrogenase, without a conventional Mo-nitrogenase. If the latter is true, then the molecular biology and physiology of this strain could provide important information about the regulation of alternative nitrogenases. These questions are particularly deserving of further attention.

From a physiological perspective, this study has demonstrated that diazotrophy in Langebaan Lagoon is very closely coupled to and modulated by the biochemical nature of plant material and the influence of the abiotic physical environment. Evolutionary physiological control mechanisms within the bacteria seemed to be finely tuned to these processes.

The original ecological hypothesis was that nitrogen fixation might significantly contribute to N-supply in what is traditionally regarded as a N-impoverished environment. However, in this particular environment, the seasonal summer upwelling cycle introduces significant concentrations of NO_3^- into the system which is incorporated into primary producers (diatoms, seagrass, and saltmarsh macrophytes) through photosynthesis. In the pelagic marine environment the recycling and

conservation of reduced and regenerated nitrogen is relatively well understood, and dominated by the role of microzooplankton where the physical environment in the upper water column is not particularly important. However, in this lagoon system, this study has demonstrated a complex interaction between primary producers fixing "new" nitrogen (NO_3^-), bioturbation by macrofauna, heterotrophic bacterial activity, diazotrophy and the physical environment (notably sediment particle size and temperature). Conservation of nitrogen would intuitively appear to be high because of the relatively aerobic nature of the sediments (due to bioturbation), which would minimize denitrification (although this was not measured). Quantifying the linkages in mass blance terms remains elusive, but it is clear that there does exist a complex interaction between primary producers and consumers where both heterotrophic bacterial activity and diazotrophy play an important intermediary role.

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