EXTRACELLULAR DIGESTION IN TWO INTERTIDAL MUSSELS AND THE ROLE PLAYED BY THEIR GUT BACTERIA

THESIS

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"Life is not only a merriment;
Life is desire and determination.
Wisdom is not in words;
Wisdom is meaning within words."
The sayings of the brook
Gibran

ABSTRACT

The intertidal mussels, *Perna perna* and *Choromytilus meridionalis* co-occur on the southern coast of South Africa. Mussels ingest mixtures of bacteria, phytoplankton, zooplankton and detritus, with proportions varying according to availability. These bivalves filter similar-sized particles, which might result in interspecific competition. Carbohydrate-digesting enzymes of the mussels and their enteric bacteria, and bacteriolytic enzymes of the mussels were therefore examined to elucidate feeding ecology in these animals, at an enzymatic level.

Style enzymes of both species digested the storage carbohydrates amylose, glycogen and laminarin, and the structural carbohydrate carboxymethyl cellulose (CMC). Differential rates of digestion of these carbohydrates suggests that *Perna perna* relies more on plankton (and possibly bacteria), than on detritus for food, while *Choromytilus meridionalis* relies equally on all components of the seston. There may therefore be some degree of resource partitioning of the seston. The styles of *P.perna* had a lower specific enzyme activity, but higher protein content than those of *C.meridionalis*. *P.perna* could therefore release more glucose from a given concentration of substrate than *C.meridionalis*.

The gut contents and tissue were examined microscopically to determine where the bacterial colonisation sites were. Bacteria were associated primarily with the gut contents, but not the gut walls, of both species. The styles housed spirochaete bacteria (*Cristispira* sp). *Perna perna* housed large numbers of one species, while *Choromytilus meridionalis* had lower numbers of two species. Levels of infection differed between species and localities.

Enteric (but not style) bacteria of *Perna perna* and *Choromytilus meridionalis* always digested the same carbohydrates as the mussels, as well as the structural carbohydrates, mannan and fucoidan. Activity was erratic on the structural compounds, carrageenin and xylan, and absent on alginic acid or inulin. Activity on the storage carbohydrates by enteric bacteria from *C.meridionalis* was higher than by those from *P.perna*. This is probably related to the larger bacterial populations housed by *C.meridionalis* than by *P.perna*.

Bacteriolytic activity by the digestive enzymes of *Perna perna* was higher than for *Choromytilus meridionalis*. In *P.perna* it was due to a combination of different enzymes, one of which is a true lysozyme. *C.meridionalis* did not produce a true lysozyme.

Enzymes produced by the mussels and their enteric bacteria allow the mussels to

utilise all components of the seston. Low endogenous enzyme activity by *Choromytilus meridionalis*, coupled with the high activity by its enteric bacteria, suggests that they rely more on bacterial activity to meet their dietary requirements than does *Perna perna*. The ability of enteric bacteria to digest carbohydrates which the mussels cannot indicates that the bacteria are endosymbiotic, although the sporadic nature of activity of some of the enzymes, and the fact that bacteria are associated with the gut contents, indicates that the relationship is only incidental.

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CHAPTER 1

INTRODUCTION

"The world is the mollusc of your choice"

Terry Pratchet

Filter feeders play an important role in intertidal ecosystem processes by controlling phytoplankton biomass and by acting as biodepositors and nutrient exchangers through the production of faeces and pseudofaeces (Peterson and Black, 1987; Bayne and Hawkins, 1992; Asmus and Asmus, 1993; MacDonald and Ward, 1994). Furthermore, mussel influences can cause changes in the composition of bacterial assemblages within the water column, and they have the potential to increase bacterial productivity and the potential rates of the flow of bacterial carbon to microherbivores (Newell and Krambeck, 1995). Filter feeders form a major component of the benthic biomass in the intertidal zone, with mean standing stocks (including shells) of mussels alone equalling 69.8 tons km^{-1} on the southern coast of South Africa (van Erkom Schurink and Griffiths, 1990).

Filter feeders are opportunistic feeders, ingesting mixtures of bacteria, phyto- and microzooplankton and detritus (Crosby and Reid, 1971; Mayasich and Smucker, 1986; Teo and Sabapathy, 1990; Bayne and Hawkins, 1992), with the proportions of these components varying seasonally (Schleyer, 1981; Cliff, 1982). In specialised environments, such as marshes, bacteria may be utilised more extensively by bivalves than in areas where bacterial populations are limited, such as in open coastal waters (Langdon and Newell, 1990). Many authors have demonstrated that phytoplankton forms the major component of the diet of filter feeders (see Asmus and Asmus, 1993; Newell and Shumway, 1993, for reviews), even though phytoplankton is the most variable component of the particulate organic matter (POM) (Griffiths, 1980; Schleyer, 1981; Cliff, 1982; Talbot and Bate, 1988). Other studies have, however, shown that phytoplankton and detritus together form the major part of the diet of suspension-feeding bivalves (Seiderer and Newell, 1985; Fielding and Davis, 1989). In spite of this, some researchers still feel that phytoplankton cells are not present in large enough numbers to contribute significantly to filter feeders' diets, and that detritus forms the major food source of these animals (Widdows *et al.*, 1979; Griffiths, 1980).

Detritus also shows seasonal variation (Cliff, 1982), but its biomass always exceeds that of bacteria and phytoplankton. Seaweed fragments form the major component of detritus, and one can assume that their carbohydrate constituents will be found in the water column (Crosby and Reid, 1971). Their major storage and structural carbohydrates, as well as those found in phyto- and microzooplankton and bacteria, are listed in Table 1.1. The macroalgae are listed in order of decreasing abundance on the southeast coast of South Africa (Seagrief, 1988). The carbohydrates are listed in order of decreasing concentration in each category.

Table 1.1. Carbohydrate composition of potential food for filter feeders.

	Structural	Storage	References
Red Algae	Carrageenin, xylan, mannan, cellulose	Floridean starch	Me; Pe; Pr
Green algae	Cellulose, mannan, xylan	Starch, inulin	Me; Pe; Pr
Brown algae	Alginic acid, fucoidan, cellulose	Laminarin	Me; Pe; Pr
Phytoplankton			
Flagellates, cyanobacteria		Starch-, glycogen- and amylopectin-like glucans	Me; Pr
Diatoms		Chrysolaminarin	Be; Me; Pe; Pr
Microzooplankton		Glycogen	SW; TS
Bacteria		Glycogen	Bo

References:- Be: Beattie *et al.*, 1961; Me: Meeuse, 1962; Pe: Percival, 1968; SW: Stark and Walker, 1983; Bo: Bohinski, 1987; Pr: Price, 1990; TS: Teo and Sabapathy, 1990.

Various studies have shown that different marine invertebrates are able to digest all the carbohydrates listed in Table 1.1, but that the digestion of alginic acid, carrageenin, xylan, mannan, fucoidan and inulin is not very widespread (eg. Huang and Giese, 1958; Eppley and Lasker, 1959; Sova *et al.*, 1970; Crosby and Reid, 1971; Favarov and Vaskovsky, 1971; Elyakova, 1972; Kristensen, 1972a; Gianfreda *et al.*, 1979; Elyakova *et al.*, 1981; Payne and Thorpe, 1993). A large proportion of detrital matter consists of refractory carbon, which means that a great portion of the carbon in the water column is in fact indigestible. This means that either filter feeders can acquire enough carbon from the fraction that they can digest, or they rely on bacterial mediation to increase their carbon intake (Hawkins and Bayne, 1992).

Microscopic investigations of bacteria within the alimentary canal have shown that invertebrates, from a wide range of phylogenetic groups and habitats, house bacteria associated with various parts of the gut tissue (Popham and Dickson, 1973; Zachary and Colwell, 1979; Deming and Colwell, 1982; Cavanaugh, 1983; Breznak, 1984; Juilfs and Wägele, 1987; Reid and Brand, 1987; Harris *et al.*, 1991; Harris, 1993b; Johnson and Le Pennec, 1995), or the gut contents (Atlas *et al.*, 1982; Garland *et al.*, 1982; Charrier, 1990; Sweijd, 1990).

Many studies have shown the nature of the relationships between bacteria and their invertebrate hosts to be very complex, and includes commensalism, mutualism (obligate or facultative), parasitism and predation (Atlas, 1984). The distribution of bacteria within the gut is also indicative of the function that they may perform. Bacteria attached to the gut contents produce supplementary digestive enzymes, while there is a direct nutrient flux between bacteria attached to the gut epithelium and its host (Harris, 1993a).

As commensals and mutualists, bacteria can perform various functions, but most are directly related to diet. Several studies have shown that bacteria significantly increase activity on some carbohydrates (Dempsey and Kitting, 1987; Musgrove, 1988; Muthu, 1990; Erasmus, 1996), while other bacteria produce enzymes which their hosts do not (Huang and Giese, 1958; Sweijd, 1990; Erasmus, 1996). The dependence of the invertebrate host on bacteria is, however, often questioned - many researchers have shown that bacteria do not contribute significantly to digestion (Galli and Giese, 1959; Horiuchi and Lane, 1966; Payne

et al., 1972; Newell and Langdon, 1986; Harris *et al.*, 1991), while others suggest that bacteria in the gut are only important during periods of food paucity (Lasker and Giese, 1954; Prim and Lawrence, 1975; Moriarty, 1990). Some researchers have, however, shown that treating invertebrates with antibiotics either reduces the animal's growth rate (Vitalis *et al.*, 1988), or leads to the depletion of enzymes, such as cellulases (Wainwright and Mann, 1982). There are, however, cases where the host is dependant on bacterial enzymes, notably Teredinids, wood-boring bivalves (Morton, 1978; Crosby and Reid, 1971; Griffin *et al.*, 1987). These animals are dependant on bacteria found in a special organ, the gland of Deshayes (Popham and Dickson, 1973), for the digestion of cellulose and the fixation of nitrogen (Waterbury *et al.*, 1983).

Some bacteria fix environmental nitrogen or produce essential amino acids, to supplement the hosts' protein deficient diets (Fong and Mann, 1980; Guerinot and Patriquin, 1981; Waterbury *et al.*, 1983). Bacteria may also provide their hosts with growth factors (vitamins), and soluble nutrients (Phillips, 1984; Bonar *et al.*, 1986). In return, the bacteria are provided with a protected environment with a concentrated food supply, although the bacteria may derive non-nutritive benefits (Bonar *et al.*, 1986; Plante *et al.*, 1990).

A benign form of parasitism can occur between transient bacteria and invertebrates: The bacteria digest food within the gut for their own benefit only, but the impact on the host is not great as the bacteria do not remain in the gut for long periods (Plante *et al.*, 1990). Intracellular parasites have been found in the gills and gut epithelia of many bivalves (Harshbarger *et al.*, 1977; Fries *et al.*, 1991; Fries and Grant, 1991; 1992), although mass mortalities of clams in their natural settings have not been attributed to these bacteria (Fries and Grant, 1991). Several other bacteria do, however, cause disease in bivalves (see Austin, 1988, for a comprehensive list).

Finally, bacteria may serve as a food source; as the sole source of nutrition (eg Newell, 1965; Reiswig, 1975; Rieper, 1978; Reid and Brand, 1987; Boetius and Felbeck, 1995), or as an additional food source (Adams and Angelovic, 1970; Birkbeck and McHenery, 1982; Wright *et al.*, 1982; Harvey and Luoma, 1984; Seiderer *et al.*, 1984; Dye and Lasiak, 1987). To utilise enteric bacteria, invertebrates have to produce enzymes and other chemicals which kill and degrade bacteria (eg. Burkholder and Sharma, 1969; Wardlaw and Unkles, 1978; McHenery *et al.*, 1979; Jamieson and Wardlaw, 1989; Plante and Mayer, 1994), and these enzymes have been found in a wide range of invertebrates.

An obligate association occurs between invertebrates found in reducing sediments or at hydrothermal vents and the chemoautotrophic bacteria resident in their gill tissue (eg. Cavanaugh *et al.*, 1981; Cavanaugh, 1983; Southward, 1986; Cavanaugh, 1994; Johnson and Le Pennec, 1995). These bacteria oxidise sulphides, nitrites, ammonia and hydrogen as an energy source, and metabolise organic carbon (eg. Felbeck, 1983; Brock and Madigan, 1991; Robinson and Cavanaugh, 1995). The host can acquire the carbon in several ways: 1) by phagocytosing live bacteria (Johnson and Le Pennec, 1995); 2) by ingesting the dead cells or excretory products (Brock and Madigan, 1991); and 3) through the translocation of carbon from the bacteria to bacteria-free tissue (Herry *et al.*, 1989).

There are four species of mussels which are common on South African shores: *Perna perna* (brown mussel), *Choromytilus meridionalis* (black mussel), *Mytilus galloprovincialis* (European mussel) and *Aulacoyma ater* (ribbed mussel) (van Erkom Schurink and Griffiths, 1990). *P.perna* and *C.meridionalis* are the most common, naturally occurring species on the

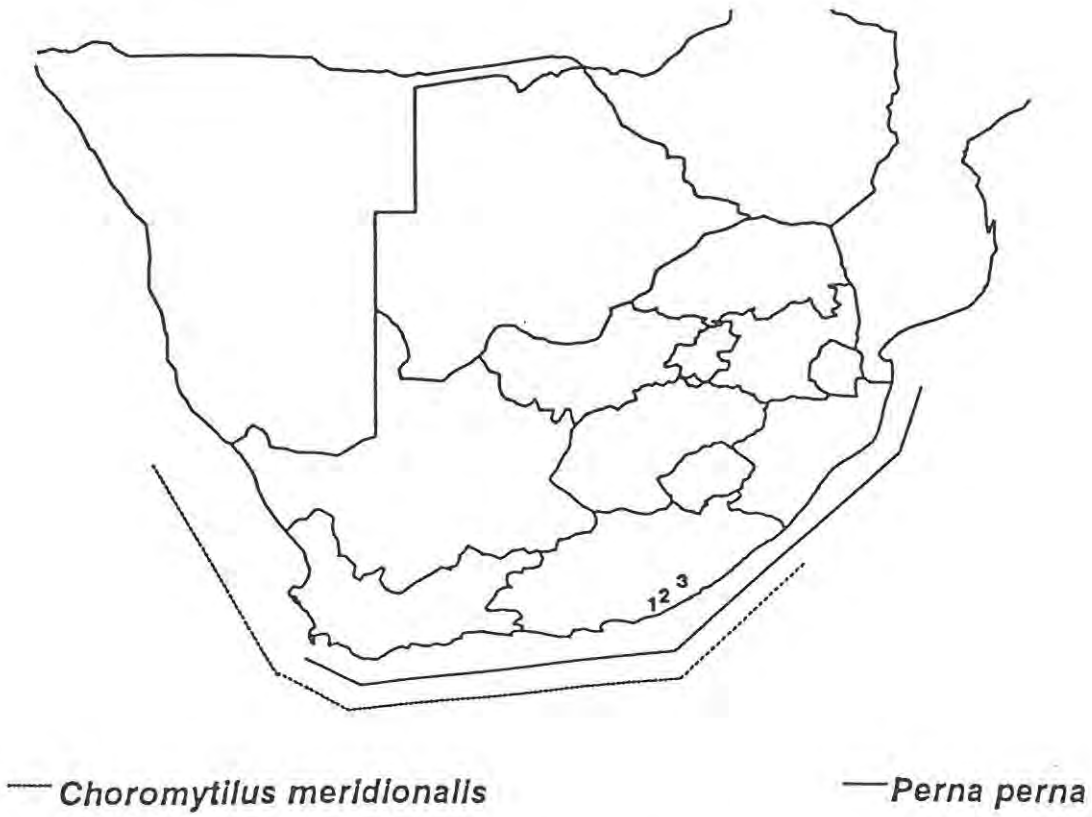


Fig. 1.1. Distribution of *Perna perna* and *Choromytilus meridionalis* (Study sites at: 1 - Port Alfred, 2 - Rufane's River Mouth, 3 - Old Woman's River)

South African coast, and were the only species considered for this study. *P. perna* decreases in abundance and size from east to west, while *C. meridionalis* decreases in abundance and size from west to east (Fig 1.1) (van Erkom Schurink and Griffiths, 1990).

Perna perna is found in dense beds from mid-intertidal to a few meters depth, while *Choromytilus meridionalis* is more common on the low shore on flat reefs, particularly in areas subject to sand cover (van Erkom Schurink and Griffiths, 1990; Marshall and McQuaid, 1993). Mixed species beds do occur, but generally where these mussels co-occur they are subject to some habitat separation. Marshall and McQuaid (1993) concluded that this separation may be a response to differences in anaerobic processes, but it might be further mediated by differences in feeding regimes.

Several studies of the diet of *Choromytilus meridionalis* have been conducted on the west coast of South Africa (Griffiths, 1980; Seiderer *et al.*, 1982; Seiderer *et al.*, 1984; Seiderer and Newell, 1985; Muir *et al.*, 1986; Fielding and Davis, 1989). Constant reference will be made to these studies, and to the fact that these animals are regularly exposed to strong up- and downwelling conditions, and that they inhabit dense kelp-beds (see Branch and Griffiths, 1988, for review). Upwelling occurs in summer, and is caused by strong south-easterly winds, while downwelling is caused by north-westerly winds during winter (Andrews and Hutchings, 1980; Wulff and Field, 1983). During upwelling conditions, cold, nutrient-rich water is brought to the surface, while there is a drop in the particulate load (Andrews and Hutchings, 1980). During this time, the relative proportions of phytoplankton and detritus in the seston decrease, while that of bacteria increases (Seiderer and Newell, 1985). During downwelling, the total particulate load increases (Wulff and Field, 1983). Weak up- and downwelling occur on the east coast, but are not as predictable as on the west coast (Beckley, 1983). The implications of these different conditions will become clear during the course of the thesis.

To date, one study has been done on the enzymes produced by *Perna perna* and *Choromytilus meridionalis* (Seiderer *et al.*, 1982). In this study, activity on only four carbohydrates was examined. In addition, the sample sites of the 2 species were quite far apart, with very different environmental conditions - *C.meridionalis* was collected from a kelp bed, while *P.perna* was collected from plankton-rich water (Seiderer *et al.*, 1982). It is

therefore difficult to ascertain whether the differences noted were species specific or diet induced. *C.meridionalis* has also been demonstrated to digest bacteria as a food source during periods of upwelling (Seiderer *et al.*, 1984; Seiderer and Newell, 1985; Muir *et al.*, 1986). Studies analogous to these have not been performed on *P.perna*, although Berry and Schleyer (1983) have demonstrated that *P.perna* is capable of filtering bacteria from the water column, and proposed that *P.perna* probably relies on bacteria as an additional food source.

The present study therefore aims to answer the following questions:

1) Are *Perna perna* and *Choromytilus meridionalis* able to digest the wide range of carbohydrates available within the water column, and do they have different enzyme suites which allow them to utilize different food sources?
2) Are the specific enzyme activities of the 2 species different?
3) Do the enteric bacteria of these species have the potential to supplement the mussels' enzymes by producing additional enzymes?
4) Do *P.perna* and *C.meridionalis* produce bacteriolytic enzymes which could allow them to exploit bacteria as an additional food source? and finally,
5) Are the enteric bacteria associated primarily with the gut contents, or do they colonise specific sites along the alimentary canal?

CHAPTER 2

ENZYME ACTIVITY OF *PERNA PERNA* AND *CHOROMYTILUS MERIDIONALIS*

INTRODUCTION

The digestion of a wide range of carbohydrates, derived from most algae, has been studied in many marine invertebrates (eg. Huang and Giese, 1958; Kristensen, 1972a; Elyakova *et al.*, 1981). Some studies have concentrated on the digestion of particular substrates by one or several species, such as laminarin (Sova *et al.*, 1970), cellulose (Nair, 1955; Crosby and Reid, 1971; Elyakova, 1972; Gianfreda *et al.*, 1979) and alginic acid (Eppley and Lasker, 1959; Favarov and Vaskovsky, 1971). Others concentrated more on the enzymes produced by one or two particular species, for example the mussels *Perna perna* and *Choromytilus meridionalis* (Seiderer *et al.*, 1982), *P.viridis* (Teo and Sabapathy, 1990), the scallop, *Pectin maximus* (Stark and Walker, 1983) and the oyster, *Ostrea edulis* (Mathers, 1973). Further studies looked at the potential contribution of various food sources, including bacteria, phytoplankton and detritus, to carbon and nitrogen uptake by bivalves (Lucas and Newell, 1984; Seiderer and Newell, 1985; Langdon and Newell, 1990).

In general, the "across the board" comparative studies by Kristensen (1972a), Elyakova *et al.* (1981) and, to a lesser extent, Huang and Giese (1958), showed that molluscs possess an extensive suite of carbohydrases, and that these enzymes were also far more potent than the enzymes produced by crustaceans, echinoderms and annelids. Collectively, molluscs have been shown to digest cellobiose, amylose, glycogen, laminarin, carboxymethyl cellulose (CMC), cellulose, chitin, xylan (Huang and Giese, 1958; Kristensen, 1972a), agar, alginic acid (Favarov and Vaskovsky, 1971; Gómez-Pinchetti and Garcia-Reina, 1993), inulin (Van Weel, 1961), carrageenin (Horiuchi and Lane, 1966) and fucoidan (Huang and Giese, 1958; Alexander *et al.*, 1979).

The only strong correlation between enzyme and diet has been found with proteases, with carnivorous molluscs having more active proteases than herbivores (Morton, 1983). The presence of different carbohydrases is not, however, indicative of diet (Van Weel, 1961; Alexander *et al.*, 1979; Gianfreda *et al.*, 1979). The relative importance of the different enzymes may differ between species, in response to their food (Crosby and Reid, 1971; Kristensen, 1972a). The presence of less common enzymes that digest polysaccharides such as alginic acid, carrageenin, xylan and fucoidan is likely to be a response to continuous exposure to food high in these carbohydrates (cf. Newell and Langdon, 1986). One can therefore make tentative predictions of the importance of different parts of the seston to the

mussels, based on the relative importance of the enzymes which are active on these carbohydrates.

Earlier authors attributed various functions to the style, a hyaline rod found in most bivalves (Owen, 1974; Morton, 1983). The most common functions were thought to be the grinding of food against the gastric shield, and as a food reserve, to be resorbed during starvation (Haselhof, 1888, in Morton, 1952). It is now accepted that the style acts primarily in the release of digestive enzymes during extracellular digestion (eg. Yonge, 1930; Morton, 1952; Owen, 1974; Morton, 1983). Other functions may include the sorting of food particles (Kristensen, 1972b) and the detachment of microorganisms from sediment (Lopez, 1980).

In the Protobranchia, a protostyle, made of compressed faecal matter, is found within the intestine, and does not produce amylolytic enzymes (Morton, 1952; Kristensen, 1972b; Owen, 1974; Morton, 1983). A true crystalline style is found in lamellibranchs and some gastropods (Morton, 1952; Owen, 1974; Morton, 1983). The style is found in a style sac, which may be either separate from the intestine, or conjoined to the midgut (Fig. 2.1). Biochemically, the style contains mainly carbohydrates and proteins, the proportions of which

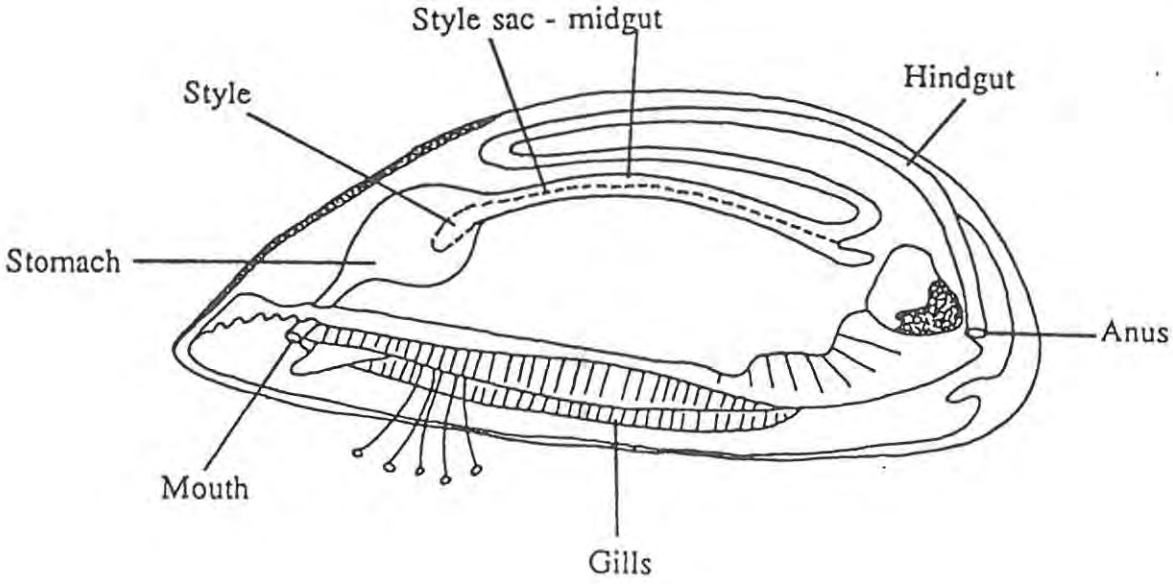


Fig. 2.1 Schematic illustration of the alimentary canal of the mussel.

vary between species (Bailey and Worboys, 1960; Doyle, 1966; Alexander *et al.*, 1979). The enzyme fraction was negligible in terms of weight in the pen shell, *Pinna nobilis* (Bailey and Worboys, 1960).

The anterior end of the style protrudes into the stomach where it rotates against a gastric shield, releasing enzymes into the stomach lumen (Bailey and Worboys, 1960; Kristensen, 1972b; Owen, 1974; Morton, 1983). Extracellular digestion may be supplemented by enzymes secreted by the stomach wall and/or the digestive shield (Payne and Thorpe, 1993). The products of extracellular digestion are absorbed by the digestive cells of the digestive gland (or hepatopancreas), where intracellular digestion occurs (Owen, 1974; Morton, 1983), degrading food particles to their monomeric units. Extracellular digestion of CM-Cellulose, amylose and laminarin is often higher than intracellular digestion (Sova *et al.*, 1970; Elyakova, 1972; Wojtowicz, 1972; Owen, 1974; Elyakova *et al.*, 1981).

The Nelson-Somogyi assay for reducing sugars is a standard biochemical assay that measures the concentration of reducing sugars (Plummer, 1978) and has been used by several researchers to measure enzyme activity (Huang and Giese, 1958; Elyakova *et al.*, 1981; Seiderer *et al.*, 1982; Fielding *et al.*, 1986; Sweijd, 1990; Erasmus, 1996). When polysaccharides are hydrolysed, reducing sugars are released, and the subsequent increase in reducing sugars is indicative of enzyme activity. A calibration curve for the reducing sugar must be determined over a range of concentrations, and the concentration of reducing sugar in the assay sample (ie, containing the substrate and enzyme) is then calculated from this curve. Fielding *et al.* (1986) found that the slopes of the curves of different reducing sugars differ, so that a separate curve has to be determined for each reducing sugar produced from the different polysaccharides. A shortcoming of this assay is that it detects the presence of all reducing sugars, including di-, tri- and oligomers, and not only monomers (Mayasich and Smucker, 1986). This implies that the importance of the enzyme, and subsequently the importance of the substrate that it digests, may be overestimated, as the substrate can only be utilised in metabolic pathways once it has been reduced to its monomeric form (Mayasich and Smucker, 1986; Boetius and Felbeck, 1995). In spite of this, it has been found to be the best technique for determining enzyme activity (Fielding *et al.*, 1986).

This experiment posed the following questions:

1) Do *Perna perna* and *Choromytilus meridionalis* digest the major storage and structural carbohydrates found in the macroalgae, phytoplankton and microzooplankton present in the surrounding waters?
2) Do these species have different enzyme suites, allowing them to utilise different food sources?
3) Are the specific enzyme activities of these two species different?

METHODS AND MATERIALS

Collection of specimens:

Samples of *Perna perna* (60-80mm) and *Choromytilus meridionalis* (35-55mm) were collected from Port Alfred (33°36'S/26°54'E) and Rufane's River Mouth (33°34'S/26°56'E) respectively, on the South East coast of South Africa (Fig. 1.1). *P.perna* were collected in February and March, and *C.meridionalis* in April and May 1995. The animals were transported to the laboratory, and processed within 1.5-2.5 hours.

Preparation of styles:

The mussels were dissected, and their styles removed. The styles were homogenised over ice in a glass tissue grinder at 20 styles per 12ml 20mM phosphate buffer (pH 7.0), containing 150mM NaCl (Seiderer *et al.*, 1982; Fielding *et al.*, 1986). The homogenate was centrifuged at 15,000rpm at 4°C for 5 minutes. The supernatant was decanted and frozen at -20°C and used within a month. The pellet was discarded.

The protein content of the style extracts (ie. the supernatant) was determined by the Folin-Lowry protein assay (Plummer, 1978), using Bovine Serum Albumin (BSA) as a standard.

Calibration curves:

Calibration (standard) curves were determined for the monomers of each polysaccharide (Table 2.1). A series of dilutions, with concentrations of 0.2, 0.1, 0.05. 0.025, 0.0125, 0.0063 $mg.ml^{-1}$, in 20 mM phosphate buffer (pH 6.9) containing 150mM NaCl, was prepared for each monomer. The absorbance readings of the different sugar concentrations were determined using the Nelson-Somogyi reducing sugar assay (Plummer, 1978). Each standard curve was repeated thrice, and a regression curve was generated from the pooled data. These curves were used throughout the experiment.

Enzyme assay:

a) Preparation of substrates:

Polysaccharide solutions (0.5% w/v) were prepared in 20mM phosphate buffer (pH 6.9) containing 150mM NaCl (Seiderer *et al.*, 1982; Fielding *et al.*, 1986). The

polysaccharides used are listed in Table 2.1. A few drops of 1M NaOH had to be added to alginic acid, while amylose and xylan had to be heated slightly to dissolve these carbohydrates. Fresh stock solutions were prepared for each test, as the substrates may hydrolyse spontaneously over time (C. Boyd, Rhodes University, pers. comm.).

Table 2.1. The polysaccharides and their corresponding monomers used to determine their respective calibration curves.

Polysaccharide	Monomer[1]
STORAGE COMPOUNDS	
Amylose	Glucose
Glycogen	Glucose
Inulin	Fructose
Laminarin	Glucose
STRUCTURAL COMPOUNDS	
Alginic acid[2]	Glucose
Carrageenin	Galactose
CM-Cellulose	Glucose
Fucoidan	Fucose
Mannan	Mannose
Xylan	Xylose

[1] = From Percival, 1968.

[2] = The monomers of alginic acid are guluronic and mannuronic acid, but due to the unavailability of these chemicals, glucose was used instead (Galli and Giese, 1959; Sweijd, 1990). This would not affect the detection of activity, but would give a qualitative rather than a quantitative measure of activity.

b) Assay:

Equal volumes (1.9ml) of enzyme extract and substrate solution were mixed and kept in a waterbath at 20°C. Aliquots of 0.4ml were removed at 0, 2, 4, 6, 8, 10, 15, 20 and 25 minutes, and the reaction stopped by adding it to 0.6ml Nelson's Reagent (Nelson's Reagent A:B, 25:1; Plummer, 1978), and boiling for 10 minutes. All test tubes were stoppered with marbles, to prevent the sample solutions from evaporating while boiling. After cooling, 1.2ml arsenomolybdate (Appendix 1) was added, and once the fizzing had stopped, 3.6ml distilled water was added.

The solutions were mixed thoroughly using a vortex-mixer, and filtered through Whatman filter paper (No. 1), to remove the precipitate formed by the denatured protein. The absorbance of each sample was read at 660nm on a Shiamadzu UV-150-02 Double-beam spectrophotometer. Each assay was repeated thrice.

Three controls were prepared, each containing all the reagents, and either 1) 0.4ml phosphate buffer (pH 6.9); 2) 0.2ml enzyme extract and 0.2ml phosphate buffer (pH 7.0); or 3) 0.2ml substrate and 0.2ml phosphate buffer (pH 6.9). The first control was used to blank the machine between readings, while the sum of the absorbance readings of the other 2 were subtracted from the assay readings to account for basal reducing sugars.

Calculation of enzyme activity:

Table 2.2 lists the regression equations from which the reducing sugar concentrations were calculated. These equations were adapted to calculate specific enzyme activity as follows:

1) reducing sugar ($mg.ml^{-1}$) [x] = (absorbance [y] - constant)/m,
where m = the slope of the regression curve.

2) Specific enzyme activity (mg reducing $sugar.ml^{-1}$/mg protein) =
mg $reducing\ sugar.ml^{-1}$/ [(mg $total\ protein.ml^{-1}$)/5],
where total protein = protein concentration of the enzyme extract. The protein concentration was divided by five because only 0.2ml protein extract was used in the assays.

To calculate relative digestion, the maximum reading for each assay was taken to be 100%, and all the other readings from that specific assay were calculated as a percentage of this value.

Table 2.2. Regression equations of the calibration curves of reducing sugar concentrations against absorbance ($P<0.05$ in all cases).

Monomer	Equation: y=mx+c (r^2) (n=3)
Glucose	y= 4.570x+0.032 (0.95)
Fucose	y= 1.795x-0.048 (0.91)
Xylose	y= 4.219x+0.005 (0.94)
Mannose	y= 3.560x-0.052 (0.98)
Galactose	y= 3.078x-0.019 (0.98)
Fructose	y= 4.464x+0.047 (0.94)

Statistics:

All statistical analyses were done using the Statgraphics computer package. Differences in intra- and interspecific rates of digestion were compared using the two-way ANOVA test. Where rates of digestion were found to be significantly different, a multiple range test was performed. Statistical analysis of relative activity was done using the same tests as mentioned above, but the data were transformed by calculating the arcsine of relative digestion before analysis. The sample sizes were too small to test for normality (M. Villet, Rhodes University, Pers. Comm.).

RESULTS

An increase in reducing sugars released with time indicated that *Perna perna* and *Choromytilus meridionalis* digested three of the four storage carbohydrates tested (amylose, glycogen and laminarin), and only one of the structural carbohydrates (Carboxymethyl cellulose, Table 2.3).

Table 2.3. A summary of digestion of storage and structural carbohydrates by the style enzymes of *P. perna* and *C. meridionalis*; CMC = carboxymethyl cellulose, + = positive reaction, - = no reaction.

Carbohydrate	*P. perna*	*C. meridionalis*
STORAGE		
Amylose	+	+
Glycogen	+	+
Laminarin	+	+
Inulin	-	-
STRUCTURAL		
CMC	+	+
Alginic acid	-	-
Carrageenin	-	-
Fucoidan	-	-
Mannan	-	-
Xylan	-	-

In all cases, simple regression analysis showed that the slopes of the change in reducing sugar concentration released through the hydrolysis of alginic acid, carrageenin, fucoidan, inulin, mannan, and xylan were not significantly different from 0 ($P>0.05$). This

indicated that there was no relationship between reducing sugar concentration and time, and that these polysaccharides were not digested.

Digestion by *P.perna*:

Amylose

Activity on amylose was generally low (Fig. 2.2a). Specific enzyme activity exhibited a general levelling off after 4 minutes. The highest recorded activity was 0.30 mg glucose.ml^{-1}.mg protein^{-1}, and the highest mean activity was 0.17 mg glucose.ml^{-1}.mg protein^{-1}.

Carboxymethyl cellulose (CMC)

Specific enzyme activity on CMC was low (Fig. 2.2b). The mean maximum specific enzyme activity was 0.046 mg glucose.ml^{-1}.mg protein^{-1}, with a maximum activity of 0.15 mg glucose.ml^{-1}.mg protein^{-1}. Even though specific enzyme activity fluctuated throughout the assay, there was a general increase in reducing sugar concentration with time.

Glycogen

Specific enzyme activity was low and levelled off after 8 minutes (Fig. 2.2c). The maximum activity was 0.35 mg glucose.ml^{-1}.mg protein^{-1}, and the maximum mean activity was 0.29 mg glucose.ml^{-1}.mg protein^{-1}.

Laminarin

Laminarin showed a stepped increase in activity, with activity levelling off between 6 and 10 minutes, and then again between 15 and 20 minutes (Fig. 2.2d). For the first 4 minutes, there was a low degree of variability, but the variability increased with time. The maximum mean activity was 0.57 mg glucose.ml^{-1}.mg protein^{-1}, and the maximum activity was 0.85 mg glucose.ml^{-1}.mg protein^{-1}.

Intraspecific differences in carbohydrase activity:

The effects of the two factors, time and substrate, on rates of activity on the substrates showing digestion (amylose, glycogen, CMC and laminarin) were compared using a two-way ANOVA. Both factors had significant effects ($P<0.05$), but there was no significant interaction. The multiple range test showed that activities on all the substrates were

significantly different from each other (Fig. 2.3a), and were, in order of decreasing activity, laminarin, glycogen, amylose and CMC.

Relative digestion is an indication of the speed at which 100% digestion is reached. There was no significant difference between activity on glycogen and laminarin, but activity on these carbohydrates was significantly higher than on amylose and CMC ($P<0.05$) (Fig. 2.3b).

Digestion by *Choromytilus meridionalis*:

Amylose

Specific enzyme activity on amylose was variable, and variability increased with time (Fig. 2.2a). Maximum activity was 3.15 mg glucose.ml^{-1}.mg protein^{-1}, with a mean maximum of 2.32 mg glucose.ml^{-1}.mg protein^{-1}.

CM-Cellulose

Digestion of CMC was slight, with specific enzyme activity fluctuating, but with a general upward trend with time (Fig. 2.2b). The maximum activity was 0.50 mg glucose.ml^{-1}. mg protein^{-1}, and the highest mean was 0.36 mg glucose.ml^{-1}.mg protein^{-1}.

Glycogen

Specific enzyme activity increased steadily throughout the assay until it tapered off slightly after 20 minutes (Fig. 2.2c). The maximum activity was 5.75 mg glucose.ml^{-1}.mg protein^{-1}, and the maximum mean was 3.55 mg glucose.ml^{-1}.mg protein^{-1}. Initially, activity by the individual extracts were similar, but the standard deviations around the mean increased with time.

Laminarin

There was a steady increase in specific enzyme activity throughout the assay (Fig. 2.2d). As with several other substrates, standard deviations around the mean were initially small, but increased with time. The mean maximum activity was 2.59 mg glucose.ml^{-1}.mg protein^{-1}, and the maximum value was 3.15 mg glucose.ml^{-1}.mg protein^{-1}.

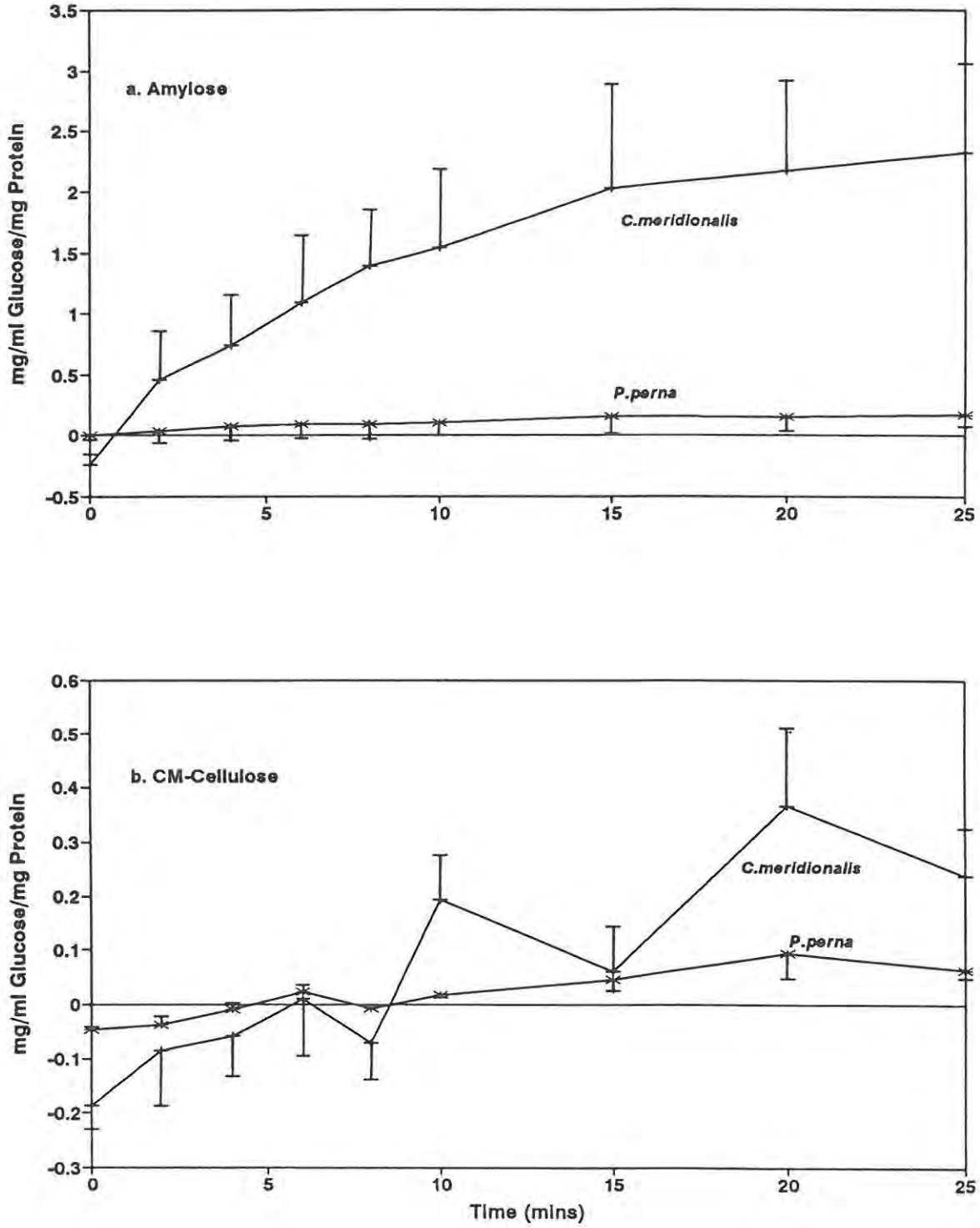


Fig. 2.2 Specific activity by *P.perna* and *C.meridionalis;* a) Amylose, b) CMC.

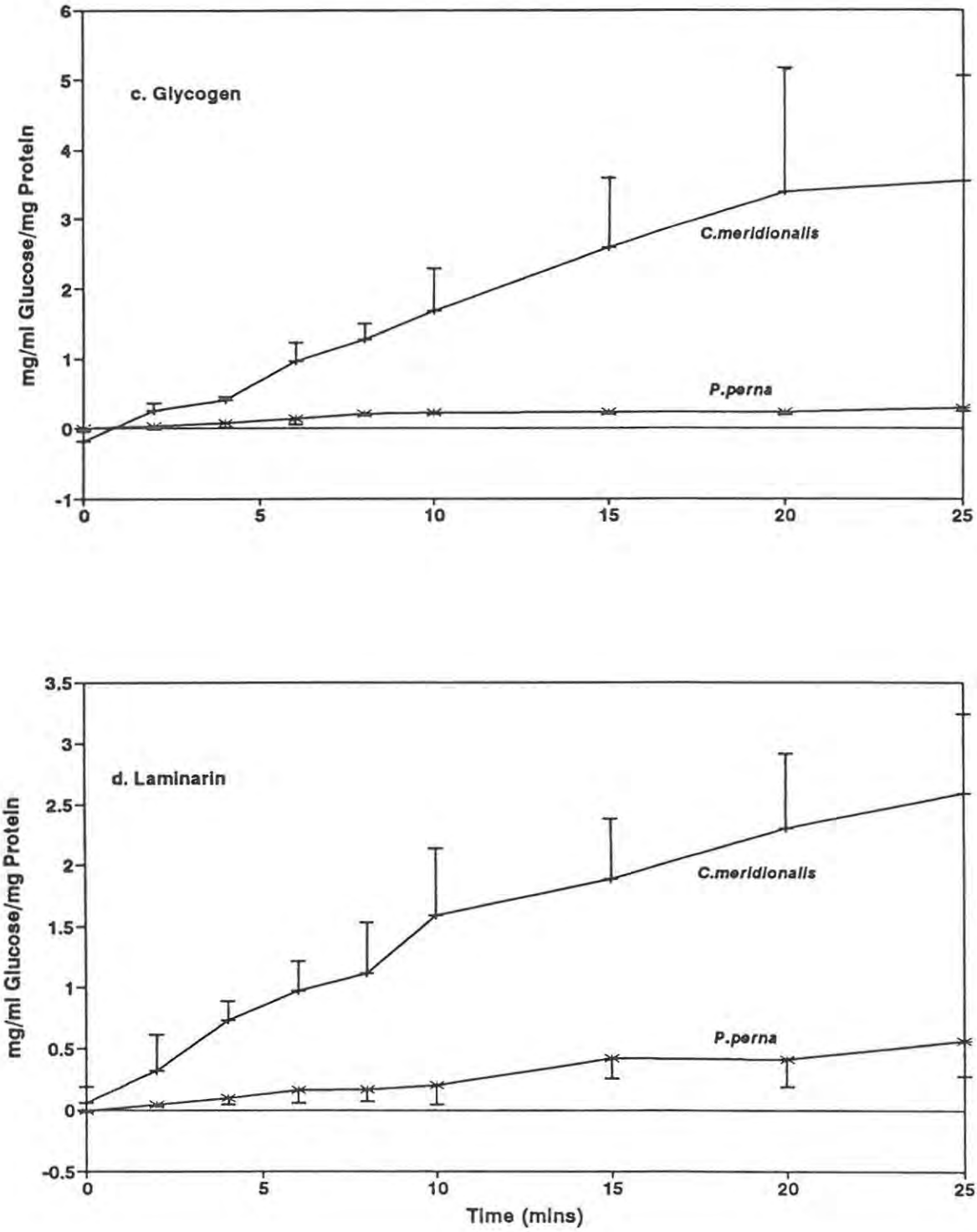


Fig. 2.2 Specific activity by *P.perna* and *C.meridionalis*; c) Glycogen, d) Laminarin.

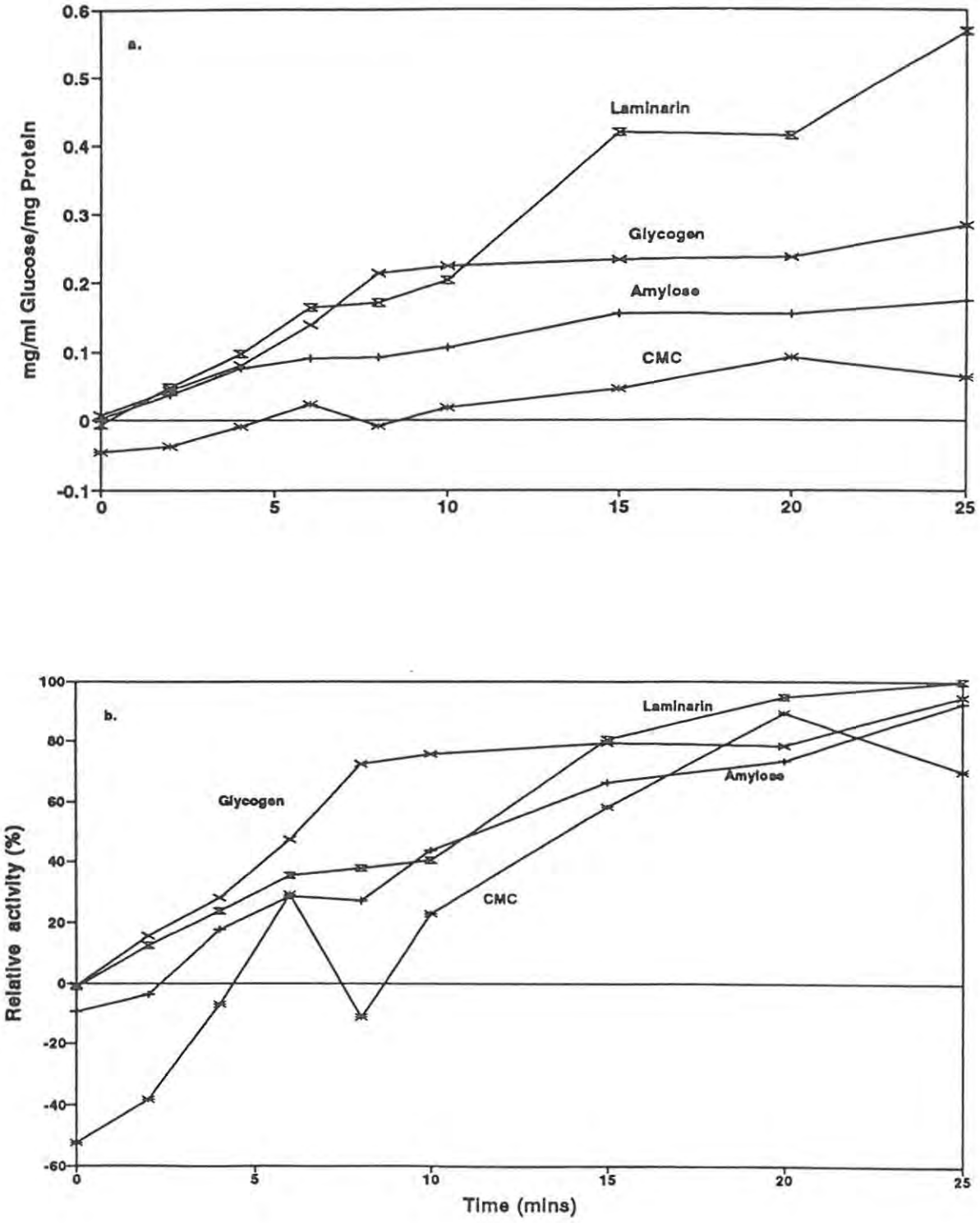


Fig. 2.3 Mean activity by *P.perna*: a) Specific activity, b) Relative activity. Standard deviations omitted for clarity.

Intraspecific differences in rates of digestion:

A two-way ANOVA showed that substrate had a significant effect, with a significant differences ($P<0.05$) in specific enzyme activity on amylose, CMC, glycogen and laminarin (Fig. 2.4a). The multiple range test showed that there was no significant difference in the activity on amylose, glycogen and laminarin, but that activity on these 3 polysaccharides was significantly higher than on CMC.

The same relationship was found for relative activity (Fig. 2.4b). Relative digestion of amylose, glycogen and laminarin increased until it reached 100% after 25 minutes, without reaching a plateau.

Interspecific differences in digestion:

Activities on the different carbohydrates by *Perna perna* and *Choromytilus meridionalis*, were tested separately, using time and species as factors.

Specific enzyme activity by *P. perna* and *C. meridionalis*

Specific enzyme activity by *C. meridionalis* was generally an order of magnitude higher than by *P. perna* (Figs. 2.2a, b & d) and was significantly different ($P<0.05$), for all substrates except for CMC. There was no significant difference ($P>0.05$) between specific enzyme activity on CMC by the two species (Fig. 2.2c).

Relative digestion by *P. perna* and *C. meridionalis*

Relative digestion of amylose was significantly higher for *C. meridionalis* than *P. perna* ($P<0.05$). There was no significant difference between relative rates of digestion of the other three substrates by the two mussels ($P>0.05$ in all cases).

Reducing sugars produced by *P. perna* and *C. meridionalis*

Figures 2.5a-d show the concentrations of glucose ($mg.ml^{-1}$) produced by fixed volumes of enzyme extract of the two species. In the case of amylose, glycogen and laminarin, *P. perna* produced significantly more reducing sugar from a fixed concentration of polysaccharides than *C. meridionalis* ($P<0.05$ in each case). The difference between the individual reducing sugars released from CMC was not statistically significant ($P>0.05$).

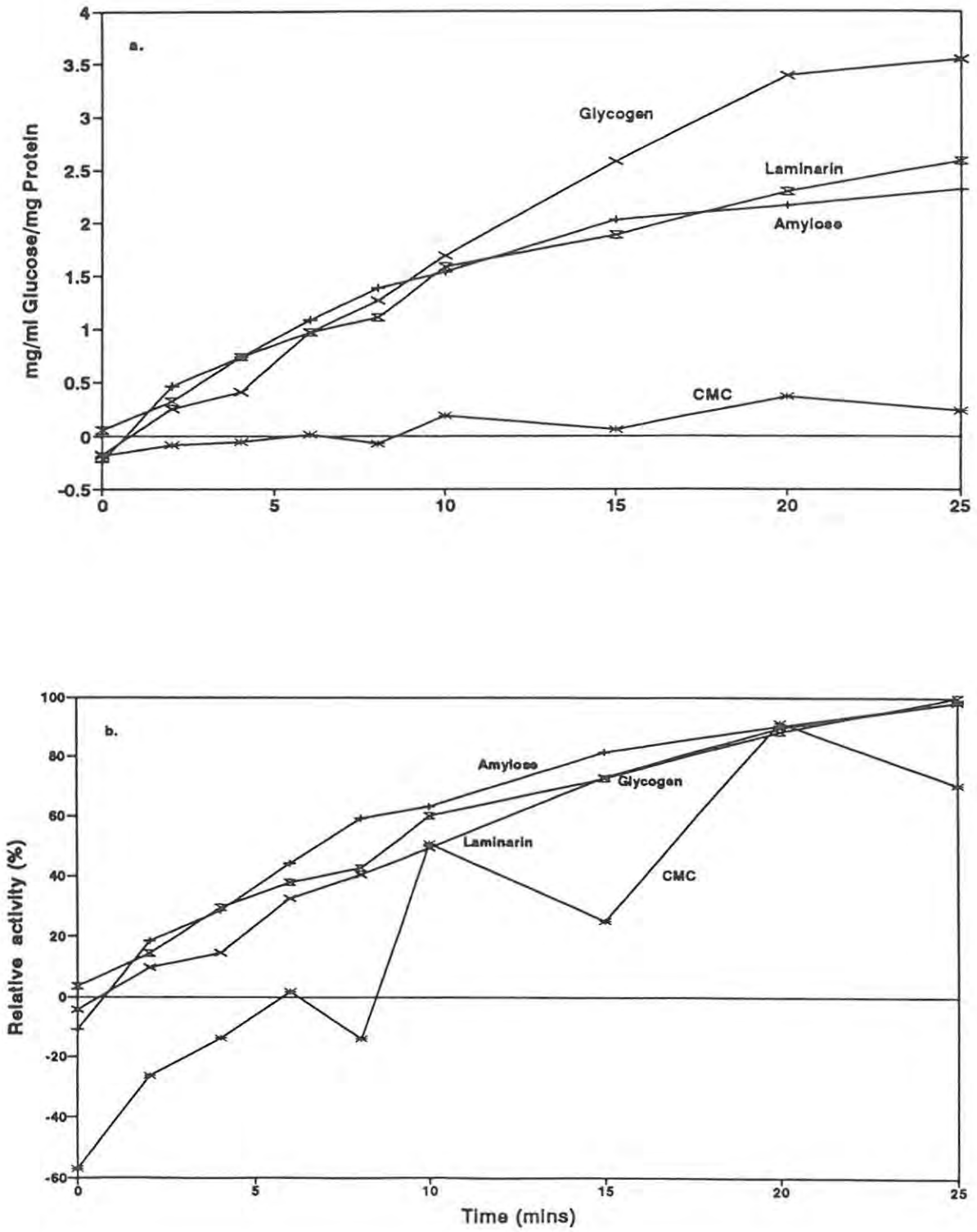


Fig. 2.4 Mean activity by *C.meridionalis*: a) Specific activity, b) Relative activity. Standard deviations omitted for clarity.

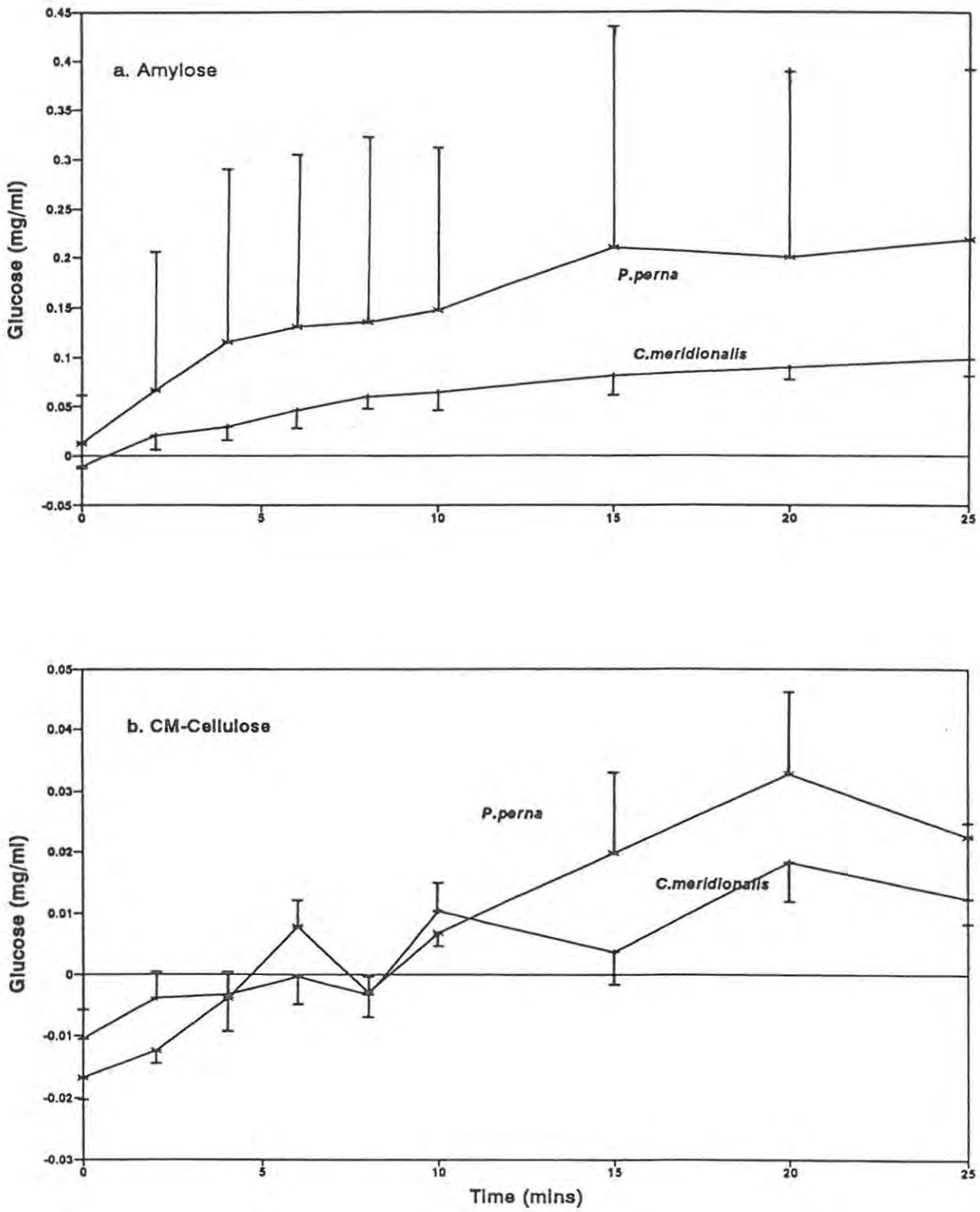


Fig. 2.5 Absolute activity by *P.perna* and *C.meridionalis*: a) Amylose, b) CMC

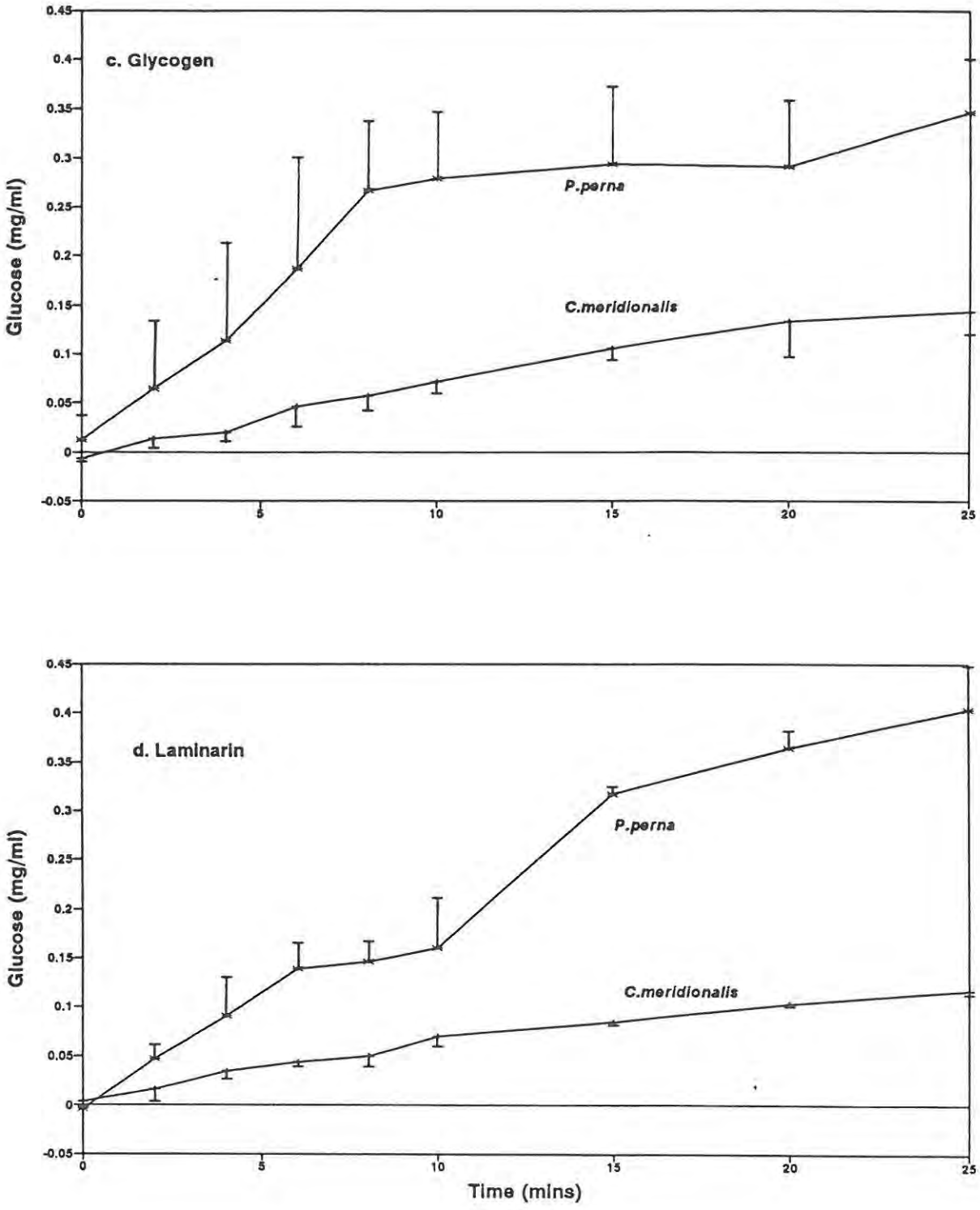


Fig. 2.5 Absolute activity by *P.perna* and *C.meridionalis*: c) Glycogen, d) Laminarin

Characterisation of α-amylase activity:

The results from the initial enzyme assays suggested that enzyme activity was related to protein concentration. To test if this relationship was significant, five additional assays were run for each species. The reaction was stopped after 10 minutes, and the data pooled with the results from the initial assays.

Simple regression analysis showed that, for *Perna perna*, there was a weak positive correlation between protein concentration and specific enzyme activity (Table 2.4). In *Choromytilus meridionalis*, there was a strong negative correlation (Table 2.4).

Table 2.4. Relationship between protein concentration and enzyme activity.

	Regression	r^2	P
P.perna	y= 0.063x - 0.18	50,77	0.047
C.meridionalis	y= -3.733x + 2.28	75.55	0.005

DISCUSSION

Considerable evidence exists in the literature for the digestion of storage carbohydrates (amylose, glycogen, starch and laminarin) by invertebrates (eg. Horiuchi and Lane, 1966; Kristensen, 1972a; Wojtowicz, 1972; Alexander *et al.*, 1979; Elyakova *et al.*, 1981; Stark and Walker, 1983). In addition, these studies also showed that CM-cellulose (CMC) is the most widely digested structural carbohydrate, while digestion of other structural carbohydrates is less common.

Perna perna and *Choromytilus meridionalis* both digested amylose, CMC, glycogen and laminarin, and neither showed any activity on alginic acid, carrageenin, fucoidan, inulin, mannan and xylan (Table 2.3). All the digested sugars were polymers of glucose, indicating that the polysaccharases were all glucanases. This suite of enzymes was almost identical to those found in other bivalves, such as the oyster, *Ostrea edulis* (Mathers, 1973) and the scallop, *Pectin maximus* (Stark and Walker 1983). Evidence for the digestion of each of the carbohydrates will be discussed individually, starting with the sugars which were not digested in the present study.

Carbohydrates not digested:

Digestion of alginic acid has previously been demonstrated in molluscs (Horiuchi and Lane, 1966; Favorov and Vaskovsky, 1971; Kristensen, 1972a; Liu *et al.*, 1984; Gómez-Pinchetti and Garcia-Reina, 1993; Erasmus, 1996; G.G. Foster, Rhodes University, pers. comm.), echinoderms (Eppley and Lasker, 1959; Favorov and Vaskovsky, 1971) and crustaceans (Kristensen, 1972a). Even though activity was found in mussels (Seiderer *et al.*, 1982) and the surf clam (Jacober *et al.*, 1980), Kristensen (1972a) and Favorov and Vaskovsky (1971) demonstrated that alginate lyase activity was higher in gastropods than bivalves.

Less evidence exists for the digestion of carrageenin. Digestion has been demonstrated in the gastropods, *Strombus gigas* (Horiuchi and Lane, 1966), *Haliotis midae* (Erasmus, 1996), *Turbo sarmaticus* (G.G. Foster, Rhodes University, Pers. Comm.), while Kristensen (1972a) also found weak activity in the sea star, *Asteria rubens*.

Hydrolysis of xylan is slightly more widespread than for carrageenin, and activity has been demonstrated in molluscs, crustaceans, annelids and echinoderms (Kristensen, 1972a; Alexander *et al.*, 1979; Liu *et al.*, 1984; Sweijd, 1990; G.G. Foster, Rhodes University, Pers. Comm.). Once again, activity tends to be higher in gastropods than filter feeders (Kristensen, 1972a).

Activity on fucoidan has been found mainly in grazing gastropods (Huang and Giese, 1958; Galli and Giese, 1959; Alexander *et al.*, 1979; G.G. Foster, Rhodes University, Pers. Comm.). Surprisingly, higher α-fucosidase activity was found in invertebrates from hydrothermal vents which harbour chemoautotrophic bacteria in their tissue, than any other marine invertebrate (Boetius and Felbeck, 1995). These authors suggest that, because this enzyme is usually only found in herbivores, and algae are not a likely food source at the thermal vents, this enzyme hydrolyses an undescribed substrate.

Sweijd (1990) found slight inulinase activity in the sea urchin *Parechinus angulosis*, and Van Weel (1961) made a brief reference to inulinase activity in molluscs. No further evidence for the digestion of inulin has been reported. The only evidence for the digestion of mannan has been found in the freshwater crayfish, *Paranephrops zealandicus* (Musgrove, 1988).

Carbohydrates digested:

The digestion of cellulose and cellulose derivatives, such as CMC and cellobiose, has been demonstrated in a wide range of marine invertebrates (Crosby and Reid, 1971; Elyakova, 1972; Gianfreda *et al.*, 1979). The complete digestion of cellulose requires three enzymes:

1) C_1 cellulase (β-1,4-glucanase), which digests crystalline cellulose;
2) C_x cellulase (poly-β-glycosidase) which digests soluble derivatives of cellulose, such as CMC;
3) cellobiase (β-glycosidase) which hydrolyses cellobiose to glucose (Owen, 1974; Morton, 1983; Newell and Langdon, 1986).

Even though Owen (1974) described C_1 cellulase activity as rare in bivalves, it has been found in the digestive glands of several bivalves (Kristensen, 1972a; Mathers, 1973; Stark and Walker, 1983; Teo and Sabapathy, 1990), and less commonly in their styles (Nair, 1955;

Kristensen, 1972a). C_x cellulase activity has been found in the digestive glands, styles and stomach contents of several bivalves (Payne *et al.*, 1972; Mathers, 1973; Stark and Walker, 1983), and gastropods (Horiuchi and Lane, 1966; Alexander *et al.*, 1979; Gómez-Pinchetti and Garcia-Reina, 1993). In particular, C_x cellulase activity has been demonstrated in the styles of several mytilids (eg. Seiderer *et al.*, 1982; Lucas and Newell, 1984; Langdon and Newell, 1990). Cellobiase is produced more commonly by the digestive glands than by the crystalline styles of various molluscs (Nair, 1955; Horiuchi and Lane, 1966; Kristensen, 1972a; Mathers, 1973; Stark and Walker, 1983; Teo and Sabapathy, 1990).

Digestion of CMC by *Perna perna* and *Choromytilus meridionalis* was very low, and both followed the same pattern of digestion (i.e. the specific enzyme activity fluctuated, but with a general upward trend; Fig. 2.2b). This pattern was exhibited in five of six assays, suggesting that this was a true reflection of the pattern of digestion, and not caused by an experimental error. A possible explanation for this phenomenon is based on negative feedback inhibition, where an increase in the products of digestion inhibits further digestion of the substrate (Bohinski, 1987). In the animal, cellulose and CM-cellulose is hydrolysed to produce cellobiose. The cellobiose is then absorbed by the digestive cells of the digestive gland, where it is further hydrolysed to glucose. During digestion, the enzyme (E) and substrate (S) bind to form an enzyme-substrate (ES) complex, which then dissociates, releasing the enzyme and products (P):

$$E + S \rightleftharpoons ES \rightleftharpoons E + P$$

Usually the enzyme and substrate are in equilibrium with the ES complex, while the reverse reaction of the complex reforming from the enzyme and products is usually negligible (Bohinski, 1987). However, it is possible that because the product (i.e. cellobiose) is not being removed from the experimental system, the enzyme-substrate complex is reformed, leading to the drop in specific enzyme activity at 8 minutes (Fig. 2.2b).

The digestion of refractory cellulose has been assayed in bivalves living in water with a high cellulose content (Lucas and Newell, 1984; Newell and Langdon, 1986; Langdon and Newell, 1990). These researchers found that although the oyster *Crassostrea virginica* and the mussel *Guekensia demissa* produced enzymes that could digest detrital cellulose, it was unlikely that detrital carbon could fulfil a significant proportion of these bivalves' carbon

requirements. Under specialised conditions, such as an increase in gut passage time, digestion of detrital cellulose is enhanced, but even under these conditions, the animals still have to supplement their diets with plankton and bacteria (Kreeger *et al.*, 1990).

Amylase activity in bivalves was documented as early as 1900 by Coupin (Bailey and Worboys, 1960) and is ubiquitous in marine invertebrates (eg. Huang and Giese, 1958; Kristensen, 1972a; Elyakova *et al.*, 1981). Both *Perna perna* and *Choromytilus meridionalis* digested amylose and glycogen extensively (Fig. 2.2a & c).

Laminarinase (β-1,3-glucanase) activity has been found in coelenterates, annelids, crustaceans, molluscs and echinoderms (eg. Sova *et al.*, 1970; Kristensen, 1972a; Elyakova *et al.*, 1981). Sova *et al.* (1970) and Elyakova *et al.* (1981) found that activity was highest in molluscs, especially bivalves. In the present study, laminarin was digested extensively by both species (Fig. 2.2d).

Association between enzyme activity and diet

There is not always a very strong relationship between the presence of an enzyme and the animal's diet (Van Weel, 1961; Alexander *et al.*, 1979; Gianfreda *et al.*, 1979; Boetius and Felbeck, 1995). For example, Gianfreda *et al.* (1979) found cellulase activity in squid, even though these animals do not feed on plant matter. On the other hand, Kristensen (1972a) showed that even though detritivores from 5 taxonomic groups had similar enzyme suites, the relative importance of the enzymes differed, and that these differences could be linked to diet. It is therefore possible to deduce, from the relative importance of the different enzymes, which food particles within the seston are important in the diets of the animals.

Activity on inulin and mannan has not been widely tested for, and when tested for, activity was usually absent (Mathers, 1973; Stark and Walker, 1983; Payne and Thorpe, 1993; G.G. Foster, Rhodes University, Pers. Comm.; present study). This suggests that these carbohydrates are not widely used by either grazers or filter feeders. The literature strongly suggests that activity on alginic acid, carrageenin, fucoidan and xylan is greater in gastropods, particularly grazers, than in filter feeders (Galli and Giese, 1959; Horiuchi and Lane, 1966; Favarov and Vaskovsky, 1971; Kristensen, 1972a; Seiderer *et al.*, 1982; Liu *et al.*, 1984; Erasmus, 1996; G.G. Foster, Rhodes University, Pers. Comm.; present study). It therefore

seems that there is a strong relationship between diet and the ability to digest structural carbohydrates.

In the present study, CMC was the only structural carbohydrate digested by *Perna perna* and *Choromytilus meridionalis*. Furthermore, digestion of CMC was very low. This suggests that cellulase activity by *P. perna* and *C. meridionalis* acts primarily as a "tool", causing partial hydrolysis of cell walls, releasing the digestible storage carbohydrates (Kristensen, 1972a), and is only secondarily involved in the digestion of cellulose as a food source. Furthermore, cellulase may actually aid the digestion of other nutrients, and not cellulose (Newell and Langdon, 1986). Newell and Langdon (1986) also suggested that cellulose digestion by *Crassostrea virginica* may be enhanced if the animals consistently ingested large quantities of cellulose. This may also apply to the digestion of other structural carbohydrates that are digested by only a limited number of animals. The limited ability of *P.perna* and *C.meridionalis* to digest structural carbohydrates supports the belief that bivalves derive limited carbon through direct utilisation of structural carbohydrates (Kreeger *et al.*, 1990), and that indirect utilisation of detrital carbon, through the bacterial loop, is more significant. In this model, bacteria which digest detritus are consumed by nanociliates and flagellates, which are then consumed by filter feeders (Kreeger *et al.*, 1990; Langdon and Newell, 1990). Another possibility is that bacteria found on the detritus and in the bivalves' alimentary canals may digest the structural carbohydrates, making the products of digestion available to the bivalve (Huang and Giese, 1958; Crosby and Reid, 1971; Fong and Mann, 1980; Sweijd, 1990). This will be explored in the next chapter.

While the link between enzyme activity and diet is not always strong, one can draw tentative conclusions about the importance of the dietary components on the basis of enzyme efficiency. *Perna perna* exhibited maximum digestion of laminarin, followed by glycogen and amylose (Fig. 2.3a). The relative digestion of laminarin and glycogen was also significantly higher than that of amylose ($P<0.05$, Fig. 2.3b). This may imply that laminarin and glycogen are relatively more important than amylose in the diet of *P. perna*. Brown algae, which contain mainly laminarin as a storage carbohydrate, are the least abundant algal type on this coast (Seagrief, 1988), and it is therefore likely that laminarinase acts primarily on chrysolaminarin, derived from diatoms (Beattie *et al.*, 1961). Implicit support for this is

presented by Elyakova *et al.* (1981), who demonstrated that laminarinase activity was highest in filter feeding bivalves. The high digestion of glycogen further implies that microzooplankton (and possibly bacteria) form a considerable part of *P. perna's* diet. It therefore appears likely that *P. perna* relies more heavily on phyto- and microzooplankton than on detritus. This supports Mann (1988), who concluded that most invertebrates acquired more carbon from microalgae than macrophytes.

Specific and relative digestion of laminarin, glycogen and amylose by *Choromytilus meridionalis* were very similar (Fig. 2.4). This implies that these sugars are equally important to *C. meridionalis*, and that it relies equally on phyto- and microzooplankton and detritus (and possibly bacteria). On the west coast, the importance of detritus, phytoplankton and bacteria in the diet of *C.meridionalis* depends on up- or downwelling conditions, but in mussels from areas not exposed to up- and downwelling, phytoplankton, bacteria and detritus were equally important as a carbon and nitrogen source (Seiderer and Newell, 1985). This is supported by the findings of the present study.

Seiderer *et al.* (1982) showed that in *Perna perna* and *Choromytilus meridionalis*, α-amylase activity was higher than laminarinase activity. In addition, laminarinase activity was relatively more important in *C. meridionalis* than in *P. perna*. This was attributed to the fact that *C. meridionalis* lived in kelp beds, where laminarin, originating from the kelp, will be more abundant than in the plankton-rich water inhabited by *P. perna*. The specimens of the two species used in the present study were collected approximately 4km apart, and the organic particulate content of the water is not as disparate as in the study by Seiderer *et al.* (1982). Yet there was a difference in preferential digestion of the different substrates by the two species. Although they generally show habitat separation (Marshall and McQuaid, 1993), *P. perna* and *C. meridionalis* can co-occur, and it is possible that the differential digestion found in this study is a reflection of preferential selection from a common food source (Shumway *et al.*, 1985). It is also possible that these animals are capable of selecting certain food particles based on food value (Navarro and Iglesias, 1993).

An important consideration which should be kept in mind is that invertebrates have the ability to change their enzymes in response to their diets (Stuart *et al.*, 1985; Harris *et al.*, 1986a; Erasmus, 1996). The abalone, *Haliotis midae*, digests carrageenin more efficiently than alginic acid when it has been fed red algae, while the reverse is true for abalone which have been fed brown algae (Erasmus, 1996). More specifically, Seiderer *et al.* (1982) found

very weak alginate lyase activity in the styles of *Perna perna* and *Choromytilus meridionalis* found in, or close to, the west coast kelp-beds. These animals are exposed to higher concentrations of alginic acid (the main structural carbohydrate in brown algae) in their diets than the animals used in the present study which did not digest alginic acid. This suggests that the activity found by Seiderer *et al.* (1982) was a result of preconditioning to alginic acid.

It is therefore possible that the activity detected in the present study was a better reflection of the contents of the seston, than the animals' endogenous enzyme activity. This is quite possible, as bivalves are capable of changing their digestive enzymes in response to changes in their food environment over a time-scale of days (Bayne, 1993). This problem could be resolved by conducting laboratory-based experiments, or by collecting enough animals with which to complete the entire experiment, at the same time. The latter is, however, not always possible.

Differences in enzyme activity:

Equal volumes of style extract, from equal numbers of styles, from *Perna perna* released significantly more glucose from a given concentration of substrate than extracts from *Choromytilus meridionalis* ($P<0.05$, Fig. 2.5a-d). Except for CMC, *P. perna* had a significantly lower specific enzyme activity than *C. meridionalis* for all the digested sugars ($P<0.05$, Figs. 2.2a-d). *P. perna* style extracts consistently had a protein concentration (mean 4.14mg.ml^{-1}, ± SD 1.65) an order of a magnitude greater than *C. meridionalis* (mean 0.44mg.ml^{-1}, ± SD 0.20). Consequently, even though *P. perna* had a lower specific enzyme activity, it produced so much protein, that it was able to release more glucose from the substrates than *C. meridionalis*. The opposite trend was found by Seiderer *et al.* (1982).

In the present study, the protein concentrations of the enzyme extracts varied greatly, ranging from 1.27 - 7.34 mg.ml^{-1} for *Perna perna* and 0.16 - 0.75 mg.ml^{-1} for *Choromytilus meridionalis*. Langton and Gabbot (1974) and Langton (1977) demonstrated that style protein concentrations of *Ostrea edulis* and *Mytilus edulis*, respectively, fluctuated with the tide. In the present study, samples were collected at low tide, but the exact time after the onset of the tide was not standardised. It is therefore probable that the fluctuation in protein concentration and consequently, α-amylase activity, of the styles was related to a tidal rhythm.

Different authors have found different relationships between protein concentration of

styles and α-amylase activity: it was positive in *Strombus gigas* (Horiuchi and Lane, 1966) and *Ostrea edulis* (Langton and Gabbot, 1974), while there was no relationship in *Mytilus edulis* (Langton, 1977). There is a weak positive correlation in *Perna perna* (Table 2.4), and a larger sample size is needed to confirm these results. A strong negative correlation was found in *Choromytilus meridionalis*. This unexpected relationship has been found in *M.edulis*, for lysozyme activity (McHenery *et al.*, 1983). These authors proposed that the enzyme is released more easily at low protein concentrations.

In conclusion, this study has shown that *Perna perna* and *Choromytilus meridionalis* are both capable of digesting the storage carbohydrates, amylose, glycogen and laminarin, and the structural carbohydrate, CMC, but not alginic acid, carrageenin, fucoidan, inulin, mannan or xylan. This suggests that neither species relies heavily on structural carbohydrates as a source of carbon. *P. perna* digested laminarin and glycogen preferentially over amylose and CMC, suggesting that it relies more on phyto- and microzooplankton (and possibly bacteria) than detritus as a food source. *C. meridionalis* digested amylose, glycogen and laminarin with equal efficiency, suggesting that it is a more "generalist" feeder than *P. perna*. This suggests that there may be some degree of resource partitioning of the available seston by the two species. Styles from *P. perna* has a lower specific enzyme activity, but higher protein content than *C. meridionalis*. This resulted in the release of more glucose from a given concentration of amylose, glycogen and laminarin by *P. perna* than *C. meridionalis*. This indicates that *P.perna* is both more specialised and more efficient than *C.meridionalis*.

CHAPTER 3

THE DIGESTIVE ROLE OF THE ENTERIC BACTERIA IN *PERNA PERNA* AND *CHOROMYTILUS MERIDIONALIS*

INTRODUCTION

Detritus forms the bulk of the biomass of particulate organic matter (POM) in the water column, followed by phytoplankton and finally, bacteria (eg. Fenchel and Jørgensen, 1977; Cliff, 1982; Talbot and Bate, 1988). Phytoplankton production is very variable and erratic (Griffiths, 1980; Schleyer, 1981; Cliff, 1982; Talbot and Bate, 1988; Asmus and Asmus, 1993). On the coast of South Africa, the contribution of detritus also shows seasonal variation (Cliff, 1982), but always exceeds the phytoplankton biomass. Bacteria show very little seasonal variation on the Natal coast of South Africa, though there is a marginal increase in the proportion of attached bacteria in summer (Schleyer, 1981). It is therefore expected that during periods of low phytoplankton concentrations, filter feeders will rely more heavily on detritus, with its associated bacteria, as well as free-living bacteria, as a food source.

This idea is not a new one, and it has been tested from various angles, for many decades now. As has been discussed in the previous chapter, marine invertebrates generally have a limited ability to utilise the refractory components of detritus. This has led to extensive studies examining the possible contributions to digestion made by bacteria on seaweed, within the water column, and in invertebrate guts. These studies have led to the conclusion that bacteria have the potential to perform several functions:

1) the production of supplementary digestive enzymes (eg. Lasker and Giese, 1954; Huang and Giese, 1958; Prim and Lawrence, 1975; Musgrove, 1988; Erasmus, 1996);
2) fixing nitrogen (Waterbury *et al.*, 1983);
3) the production of essential amino acids (Fong and Mann, 1980; Guerinot and Patriquin, 1981) and vitamins (Phillips, 1984; Austin, 1988);
4) acting as a food source for the host (eg. Zobell and Landon, 1937; Zobell and Feltham, 1938; Newell, 1965; Reiswig, 1975).

The first 2 functions can actually occur within the water column, as bacteria increase the nutritional value of detritus by digesting the refractory material (which would otherwise be unavailable to filter feeders) and, at the same time, take up dissolved nitrogen, increasing the protein content of the detritus (Cliff, 1982; Mann, 1988).

Marine bacteria are usually Gram negative rods, which are motile, and facultative anaerobes, with more Gram positive bacteria associated with the sediment (Rheinheimer, 1985; Austin, 1988). Throughout the world, the genera of bacteria within the guts of

invertebrates are consistent, and these include *Pseudomonas, Vibrio, Aeromonas, Flavobacterium, Achromobacter, Micrococcus* and *Alcaligiensis* (eg. Colwell and Liston, 1962; Beeson and Johnson, 1967; Murchelano and Brown, 1968; Unkles, 1977; Sochard *et al.*, 1979; Mohan *et al.*, 1986; Prieur *et al.*, 1990; Erasmus, 1996; Ward-Rainey *et al.*, 1996). These bacteria are also found within the water column, but selection occurs within the gut, resulting in the dominance of mainly *Vibrio* and *Pseudomonas* in the gut (eg. Sochard *et al.*, 1979; Unkles, 1977; Prieur, 1981). Colwell and Liston (1960) found that the bacteria in the oyster, *Crassostrea gigas*, were similar in animals from different areas, and they proposed that these animals housed a commensal flora. This idea was later echoed by Kueh and Chan (1985).

Collectively, bacteria isolated from invertebrate guts and from seaweed fronds have been demonstrated to digest starch, laminarin, carrageenin, alginic acid, agar, chitin, cellulose, fucoidan and inulin (eg. Yaphe, 1963; Prim and Lawrence, 1975; Quatrano and Cladwell, 1978; Harris *et al.*, 1986b; Sweijd, 1990; Gacesa, 1992; Erasmus, 1996). If the host lacks enzymes to digest any of these substrates, it has to rely on enzymes from microorganisms, or microbial "conditioning" of the food, to make it more palatable and digestible (Baker and Bradnam, 1976; Hanson and Tenore, 1981; Musgrove, 1988). This ability of gut bacteria to digest a range of carbohydrates does not, however, necessarily mean that they do so for the benefit of their hosts (Erasmus, 1996). Furthermore, the type of diet determines the type of association: co-operation between host and bacteria will occur when the diet has a high carbohydrate and fibre content, while competition between the bacteria and host is more likely when the diet contains easily digested food (Hungate, 1961, in Plante *et al.*, 1990). One can, however, assume that if the bacteria produce enzymes which the hosts cannot, that the hosts benefit from this activity. The fact that marine invertebrates from different localities house very similar bacteria, while bacteria in different invertebrates digest very different substrates suggests that the actual identity of the bacteria is not as important as the digestive function which the bacteria may perform.

The ability of invertebrates to utilise bacteria as a food source is a contentious issue. In general, bacteria are considered to have a low nutritional value (eg. Phillips, 1984; Matthews *et al.*, 1989; Langdon and Newell, 1990). In spite of this, several authors have shown that some invertebrates do feed on bacteria to varying degrees (Zobell and Landon,

1937; Zobell and Feltham, 1938; Newell, 1965; Fenchel, 1970; MacGinitie, 1978; Rieper, 1978; Lopez and Cheng, 1983; Amouroux, 1986).

Filter feeders are, however, also limited by their ability to filter particles of bacterial size from the water column. Stuart and Klumpp (1984) showed that 3 mussel species on the west coast of South Africa retained particles of 0.6-1.6μm diameter (upper size range of marine bacteria) with an efficiency ranging from 20-90%, and they concluded that, given the bacterial concentrations in the water column, these bacteria could provide only 6-20% of the mussels' carbon requirements. There is, however, evidence that other factors, such as locality and temperature, play an important role in the ability of an animal to filter bacteria. Lucas *et al.* (1987) showed that estuarine and coastal populations of the mussel, *Mytilus edulis*, retained 2μm diameter bacterial particles at an efficiency of 82 and 47.9% respectively - the former locality having a higher bacterial concentration than the latter. Birkbeck and McHenery (1982) proposed that *M.edulis* living close to faecal outfalls would utilise bacteria relatively more efficiently than populations that are exposed primarily to marine bacteria. The mussel, *Geukensia demissa*, which lives in salt marshes, has a small gill mesh-size, which allows it to filter bacteria relatively efficiently (Wright *et al.*, 1982). A change in temperature changes the ability of *Cerastoderma edule* (McHenery and Birkbeck, 1985) and *Choromytilus meridionalis* (Muir *et al.*, 1986) to filter and utilise bacteria.

Two further factors will influence an animal's ability to filter bacteria. Firstly, 20-30% of bacterioplankton form aggregates or microcolonies of 4-6μm diameter (Sorokin, 1973; Prieur, 1981), a size range retained by mussels with 100% efficiency (Stuart and Klumpp, 1984). Secondly, detritus may contain up to 5 billion cells/1g wet weight (Zhukova, 1963) or 5×10^8 - 10^{10} cells/1g dry weight (Fenchel and Jørgensen, 1977). This implies that filter feeders are able to access considerably more bacteria in association with detritus than by filtering individual cells.

An alternative way of determining whether or not bivalves are able to use bacteria as a food source would be by screening their enzymes for bacteriolytic activity. Bacteriolytic activity has been found in the styles and digestive glands of several bivalves (McHenery *et al.*, 1979; Jamieson and Wardlaw, 1989), the gills and haemolymph of bivalves (McDade and Tripp, 1967; Hardy *et al.*, 1976; McHenery *et al.*, 1979) and the coelomic fluid of sea urchins (Wardlaw and Unkles, 1978). The presence of bacteriolytic activity in the style and digestive glands suggest that the enzyme has a nutritional function, while the presence in the

gills, haemolymph and coelomic fluid suggests that the enzyme might function primarily in defence. Plante and Mayer (1994) proposed that the lytic activity in the midgut of the polychaete, *Arenicola marina* may function in the removal of potential competitors for its gut contents, and for nutritive gain. Similarly there is also evidence that some oysters may destroy bacteria in the gut without assimilating them (Mori *et al.*, 1980, in Amouroux, 1986).

Berry and Schleyer (1983) demonstrated that *Perna perna* on the Natal coast of South Africa were able to filter free-living bacteria from the water column, and proposed that *P.perna* relied at least partly on bacteria as a food source. Seiderer *et al.* (1984) and Muir *et al.* (1986) demonstrated that *Choromytilus meridionalis* on the west coast of South Africa produces a bacteriolytic enzyme, and increases its absorption efficiency of bacteria during upwelling, when plankton and detritus concentrations are low.

This chapter therefore addresses the following questions:

1) Are mixed cultures of bacteria from the guts of *Perna perna* and *Choromytilus meridionalis* capable of digesting a range of substrates which are likely to be available in the water column?
2) Do *P.perna* and *C.meridionalis* produce bacteriolytic enzymes which enable them to utilize bacteria as a food source?

METHODS AND MATERIALS

A. ENZYME ACTIVITY BY ENTERIC BACTERIA:

Collection of specimens:

Similarly sized (3.5 - 4.5 cm) *Perna perna* (n=6) and *Choromytilus meridionalis* (n=6) were collected from Rufane's River Mouth($33^0$34'S/$26^0$56'E) during July and September 1996. The animals were transported back to the laboratory and processed within 1.5 to 2.5hrs.

Preparation of gut material:

The guts were dissected out, using flame sterilised instruments, and treated individually. The style was removed and discarded, and the rest of the gut chopped finely, suspended in 15ml Nutrient Broth (Appendix 2), and mixed vigorously. The suspensions were incubated overnight in a shaking water bath at 25^0C.

Bacterial counts:

Ten-fold serial dilutions (10^{-1} - 10^{-6}) were made of the overnight cultures, and 0.1ml of the 10^{-2}, 10^{-4} and 10^{-6} dilutions were plated out on a seawater plate medium (Appendix 2). Colony forming units (CFU's) were counted after incubating the plates at 25^0C for 3-5 days.

Enzyme assays:

Enzyme activity was measured, using the Nelson-Somogyi assay, as outlined in Chapter 2, with the following amendments:

1ml Bacterial suspension was mixed with 1ml substrate solution. Aliquots of 0.4ml were removed immediately, and the reaction stopped. The rest of the mixture was incubated at 20^0C for 90 minutes, when 0.4ml was removed, and the reaction stopped. The increase in the reducing sugars released was calculated from the standard curves prepared previously (Table 2.2). Two sets of assays were performed, each assay having three replicates.

B. BACTERIOLYTIC ACTIVITY BY MUSSELS:

Style enzymes:

The style enzyme extracts were prepared as described in Chapter 2. For *Choromytilus meridionalis*, the concentration of styles was increased to 3 styles/1.5ml buffer, because the protein concentration of 20 styles/12ml buffer was too low. The extracts were then standardised to a concentration of 1mg protein/ml.

Isolation and preparation of bacteria:

Ten of the most commonly occurring colony types were picked from the mixed plate cultures of bacteria from each of *Perna perna* and *Choromytilus meridionalis*. The bacteria were isolated by restreaking the isolates on fresh agar plates, and incubated at 25°C for 3 days. This was repeated twice. The isolates were stored on agar slants, at 4°C.

The bacteria were prepared for the assays according to the method of Plante and Mayer (1994). Nutrient broth was inoculated with the isolated bacteria, and grown for 14-16hrs at 25°C in a shaking waterbath. The bacteria were pelleted out by centrifuging the liquid cultures at 4000 rpm for 10 minutes, at 5°C. The pellets were then washed in 0.066M phosphate buffer (pH 6.2), repelleted and resuspended in the buffer.

Enzyme assay:

The bacteria were screened for susceptibility to the style enzymes from the host mussel species as follows:

1) Test: 650µl bacterial suspension + 100µl style enzyme (n=5)
2) Control: 650µl bacterial suspension + 100µl hen's egg white (HEW) lysozyme (n=1)
3) Control: 650µl bacterial suspension + 100µl buffer (n=1)

Lysozyme is a specific enzyme which digests bacteria, and its main diagnostic feature is its ability to digest the bacterium *Micrococcus lysodeikticus* (Salton, 1957). Lysozyme activity was tested for as follows:

4) Test: 650µl *Micrococcus lysodeikticus* + 100µl style enzyme (n=5)
5) Control: 650µl *M.lysodeikticus* + 100µl HEW lysozyme. (n=3)
6) Blank: phosphate buffer.

Digestion of the bacteria was measured spectrophotometrically, at 450nm. A decrease in absorbance represents a decrease in bacterial density through the lysis of bacteria resulting from digestion. Readings were taken at 0, 5, 10, 20, 30, 40, 50 and 60 minutes. A preliminary study, testing for susceptibility of the bacteria, was performed, using duplicate enzyme extracts. Only the bacteria that appeared to be susceptible to the enzymes (i.e., those that showed a decrease in absorbance of more than 30%) were considered for further testing. The actual test runs were repeated using 5 replicates for each mussel species.

RESULTS

Plate counts.

Table 3.1 lists the numbers of viable bacteria or colony forming units (CFU's) obtained from *Perna perna* and *Choromytilus meridionalis*. The values are comparable with each other as the mussels were similarly sized.

Table 3.1 CFU's from whole-gut homogenates.

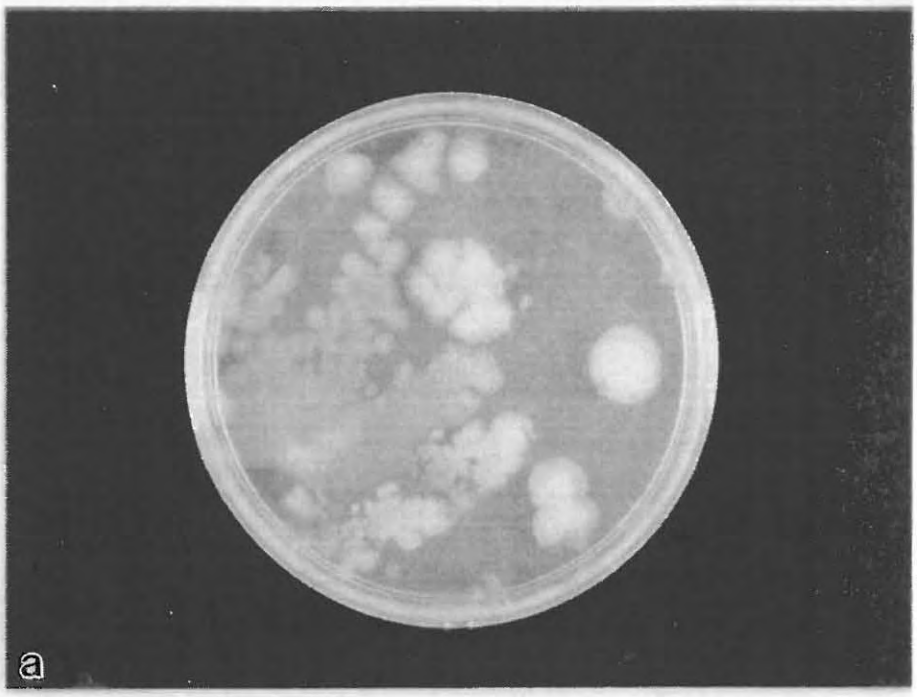
	CFU's/ml ± SD (n)
P. perna	$6.8x10^8 \pm 3.7x10^8$ (6)
C. meridionalis	$1.1x10^9 \pm 1.7x10^9$ (5)

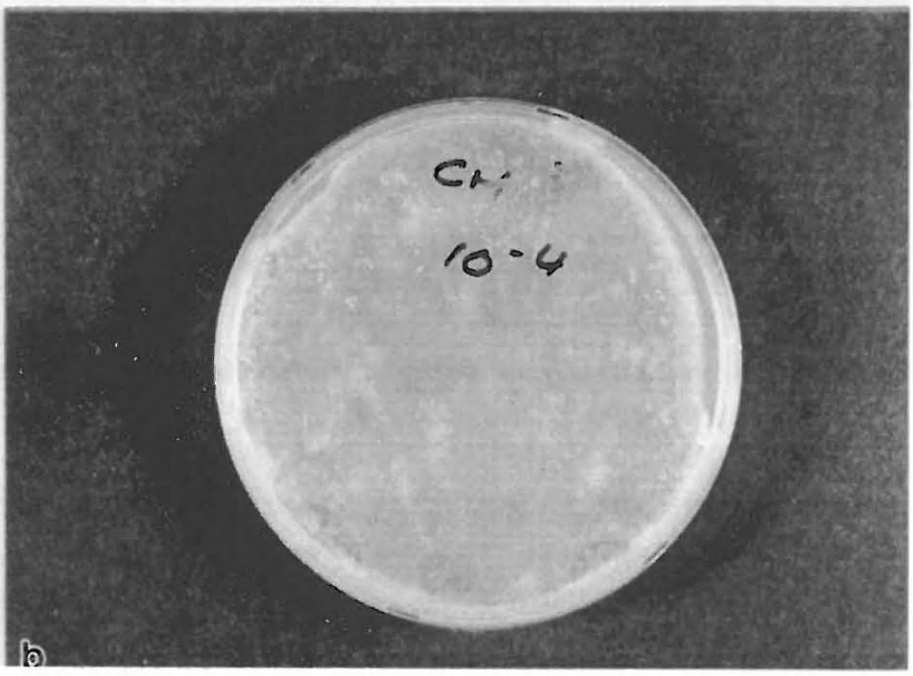
The mixed plate cultures were characterised by 17 and 18 colony types for *P.perna* and *C.meridionalis*, respectively (Fig. 3.1 a & b). Gram stains were only done on the bacteria that were isolated for the bacteriolytic experiments. Collectively, 50% were Gram negative rods, 30% were Gram negative cocci and the rest Gram positive cocci. Agar digesting bacteria (forming pits in the agar) were uncommon, and were found only in the lowest dilutions.

A. Bacterial enzyme assays:

Table 3.2 lists the carbohydrates digested by bacteria from *Perna perna* and *Choromytilus meridionalis* during the preliminary study. Figs. 3.2 a-c illustrates the digestion of the substrates by the gut bacteria in the actual test assays. The reaction was accepted to be positive if the mean was significantly different from 0, using a One-tailed t-Test, ($P<0.05$).

The bacteria from *Choromytilus meridionalis* consistently digested amylose, glycogen, and laminarin more efficiently than those from *Perna perna* (Figs. 3.2 a-c), but this difference was only statistically significant for glycogen (Two-tailed t-Test, $P<0.05$), for the


a


10-4
b

pooled data. Activity on the storage carbohydrates, amylose, glycogen and laminarin by the bacteria from *P.perna* was variable, while activity by the bacteria from *C.meridionalis* was

Table 3.2. Preliminary results of digestion by bacteria isolated from *P.perna* and *C.meridionalis*.

Substrate	*P.perna*	*C.meridionalis*
STORAGE		
Amylose	+	+
Glycogen	+	+
Laminarin	+	+
Inulin	-	-
STRUCTURAL		
Alginic acid	-	-
Carrageenin	+	+
CMC	+	+
Fucoidan	-	-
Mannan	-	-
Xylan	-	-

more consistent (see error bars, Figs. 3.2 a-c). When present, activity on the structural carbohydrates was consistently low, and with very little variability (Figs. 3.2 a-c). Activity on xylan occurred for bacteria from *P.perna* but not *C.meridionalis* in the first set of assays (Fig. 3.2a), and for neither species in the second (Fig. 3.2b). No activity on inulin and alginic acid was detected.

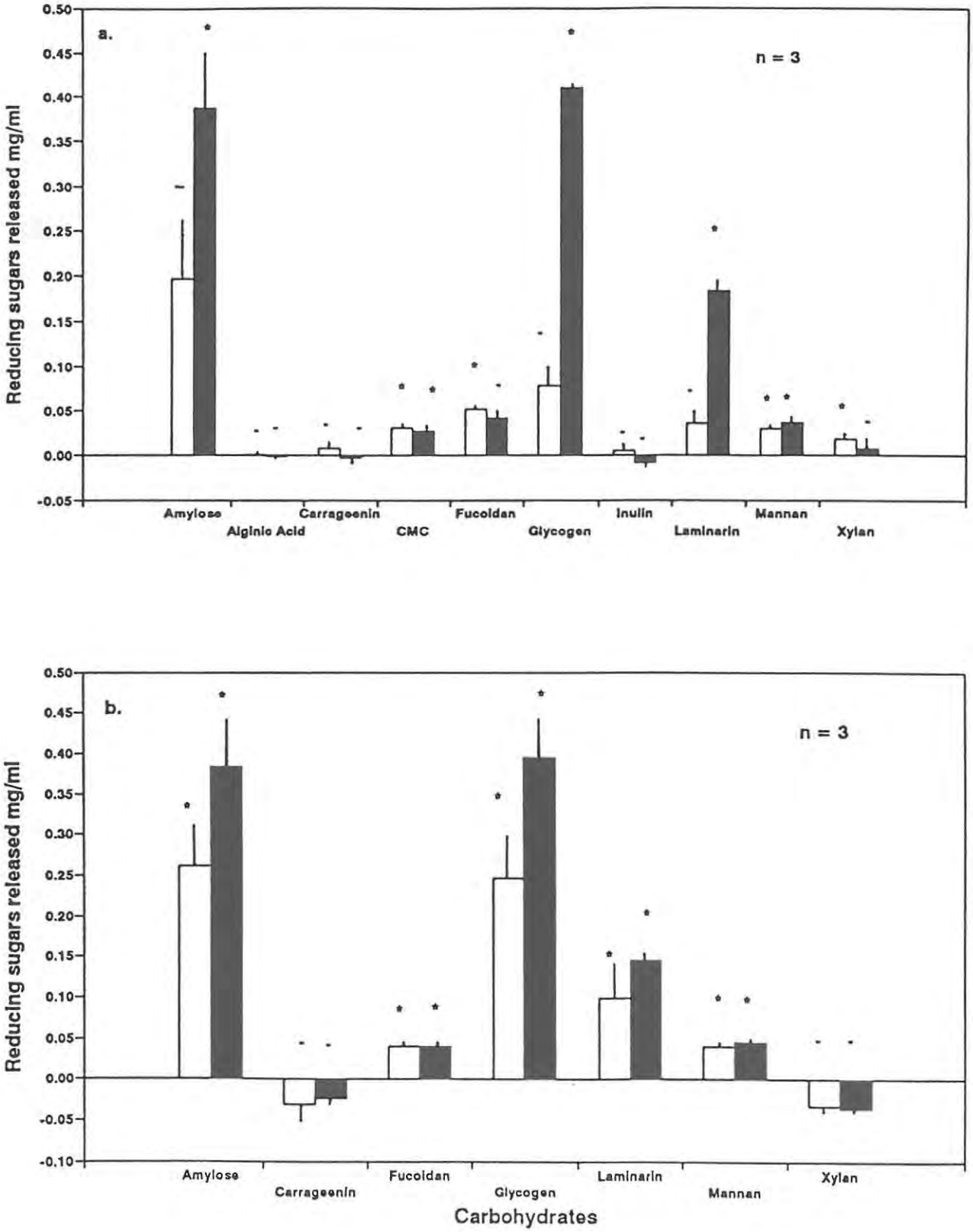


Fig. 3.2 Digestion of carbohydrates by bacteria: a) Assay 1, b) Assay. (□ = *P.perna*; ■ = *C.meridionalis*; *, positive reaction; -, no reaction)

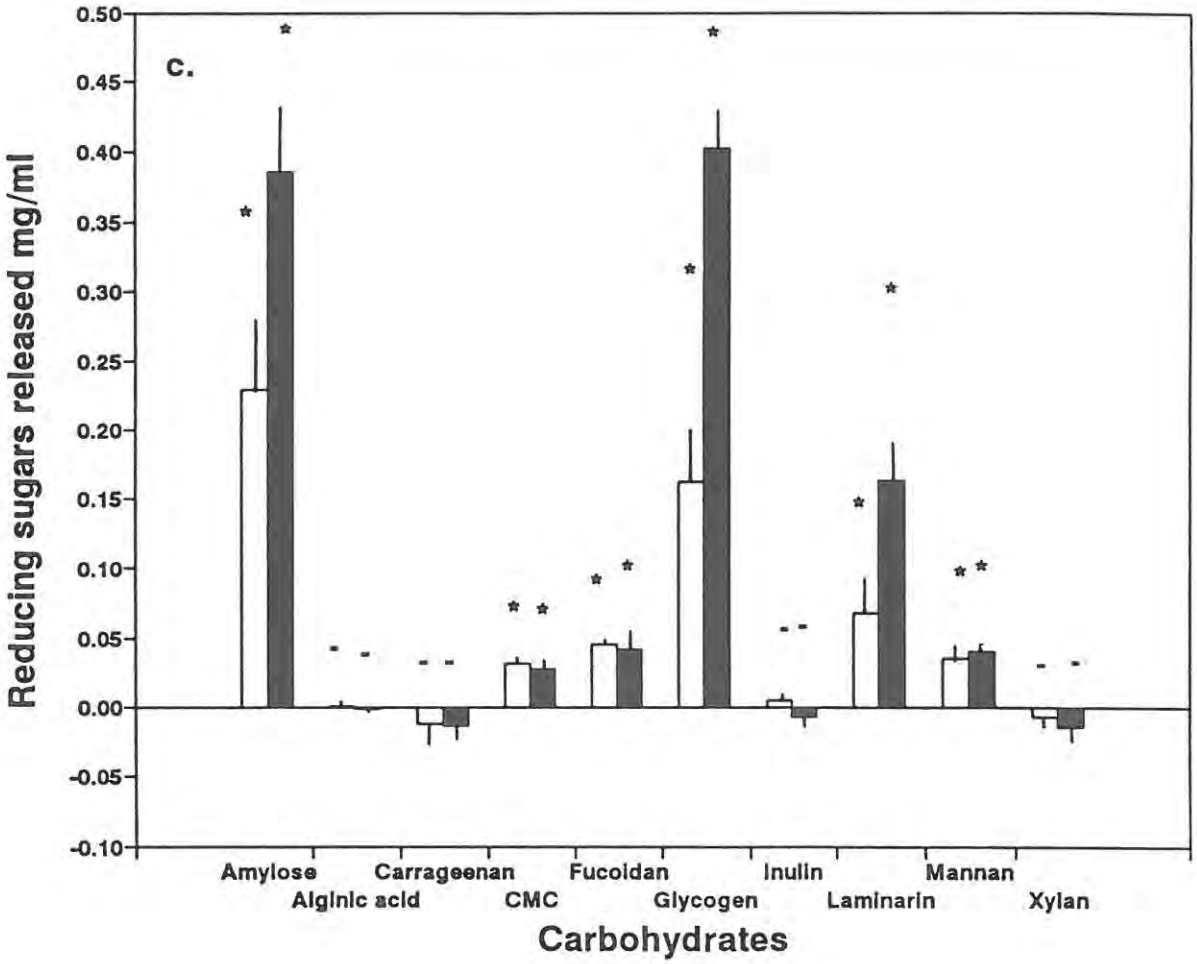


Fig. 3.2c Pooled data of Assays 1 and 2. Legend as for Fig. 3.2 a and b.

B. Bactericidal activity:

The 10 bacteria isolated from each species were screened for susceptibility to the host mussel's style enzymes. Four of the bacterial strains (strains P3, P4, P8 and P9) from *Perna perna* and 1 (strain C3) from *Choromytilus meridionalis*, respectively, appeared to be susceptible to the style enzymes. These bacteria were then tested further for susceptibility.

Perna perna digested 3 (P3, P4 and P8) of the 4 bacterial strains tested (Fig. 3.3 a-d), resulting in a reduction in absorbance of between 35 - 60%. The change in absorbance in the test sample was considerably greater than the change in the controls. All 4 isolates were resistant to HEW lysozyme.

Enzymes from *Choromytilus meridionalis* caused a reduction in absorbance of about 45%, and the bacterium C3 was resistant to HEW lysozyme (Fig. 3.4).

Three of the enzyme extracts from *Perna perna* digested *Micrococcus lysodeikticus*, to varying degrees, while the two remaining extracts showed no lysozyme activity. The mean activity (including all the extracts) was equivalent to activity by HEW lysozyme (Fig. 3.5). Enzyme extracts from *Choromytilus meridionalis* showed no activity on *M.lysodeikticus* (Fig. 3.5). This indicates that some of the extracts from *P.perna* contained a true lysozyme, while those from *C.meridionalis* did not.

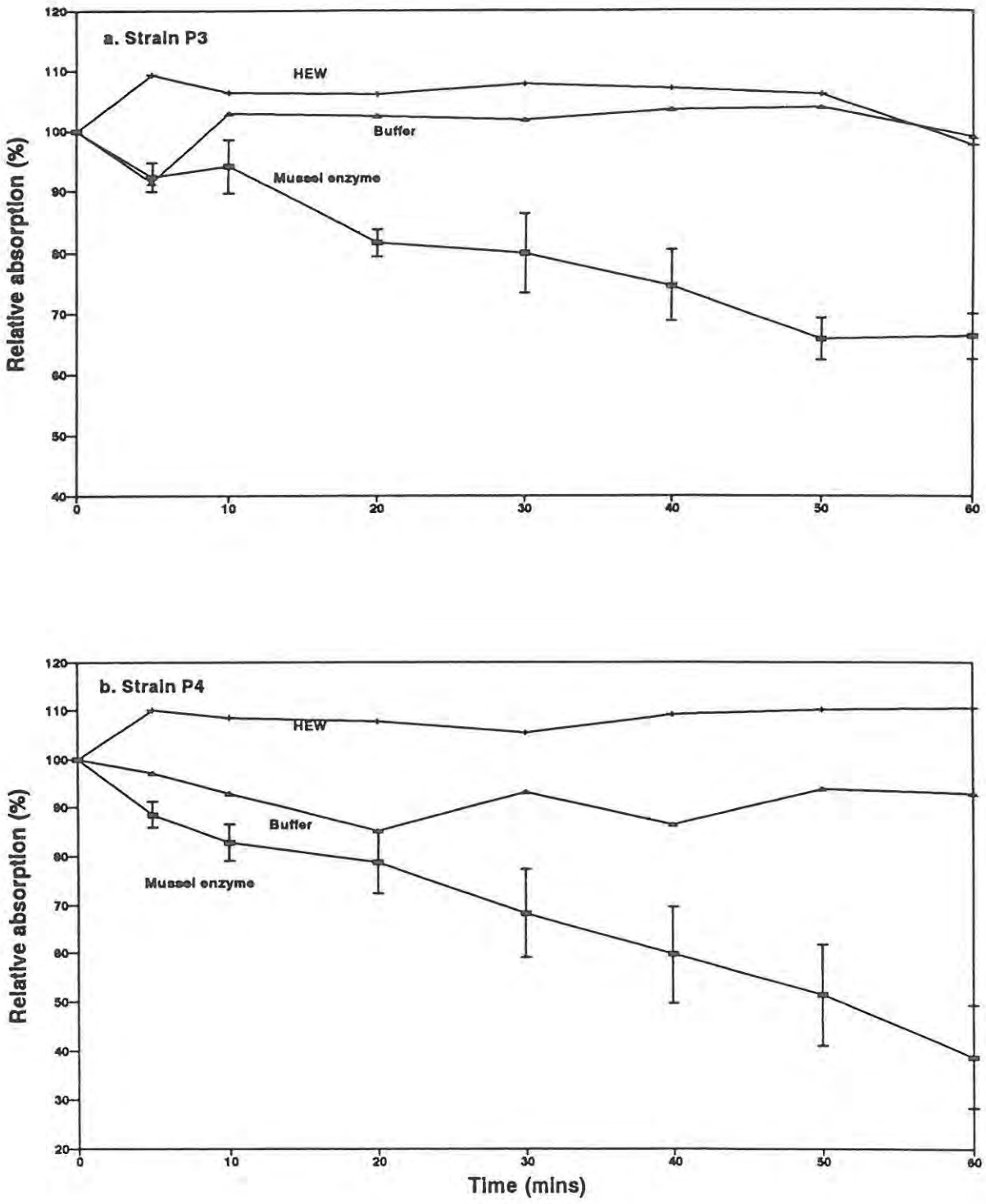


Fig. 3.3 Bacteriolytic activity by *P.perna* on strains a) P3, b) P4.

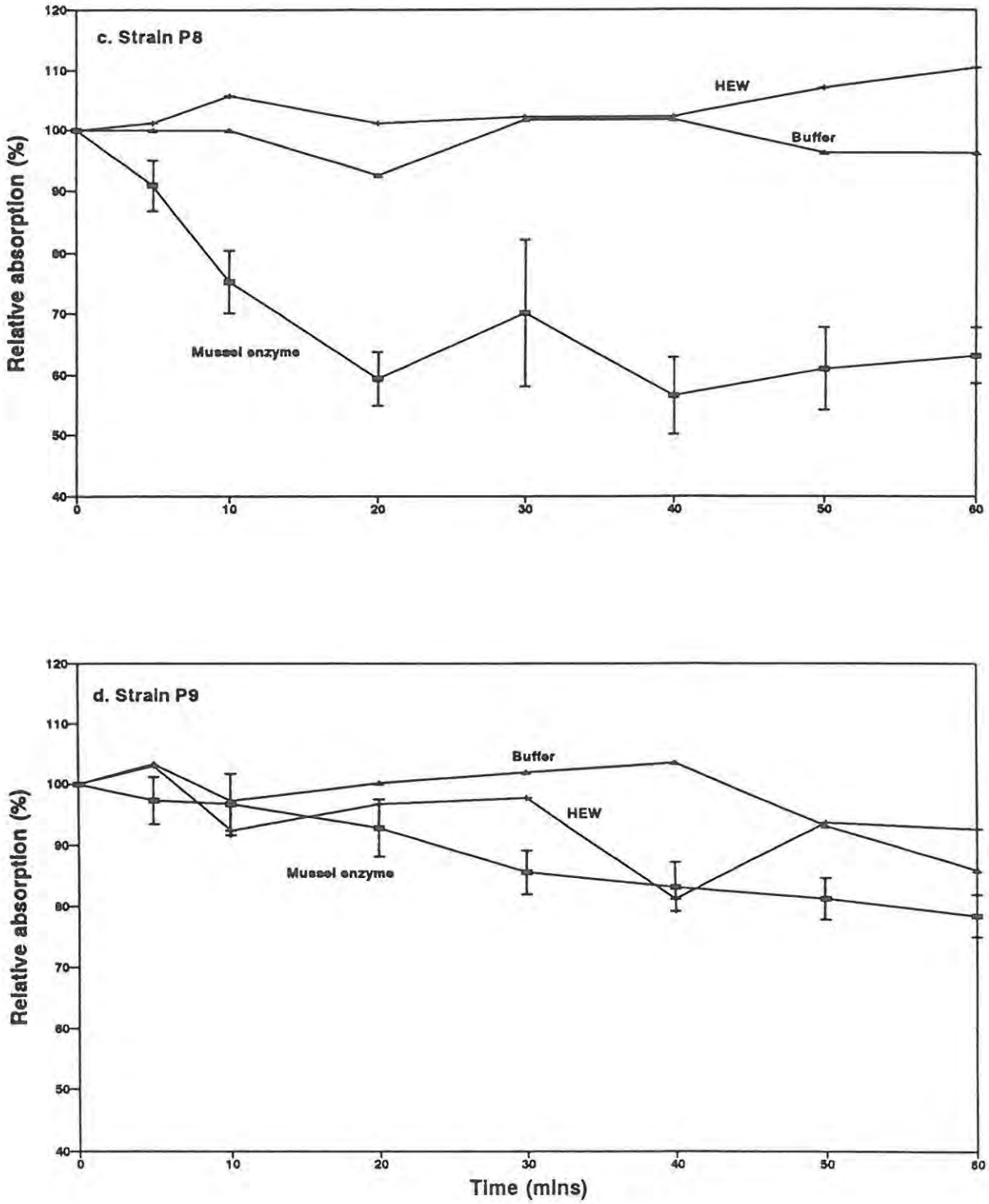


Fig. 3.3 Bacteriolytic activity by *P.perna* on strains c) P8, d) P9.

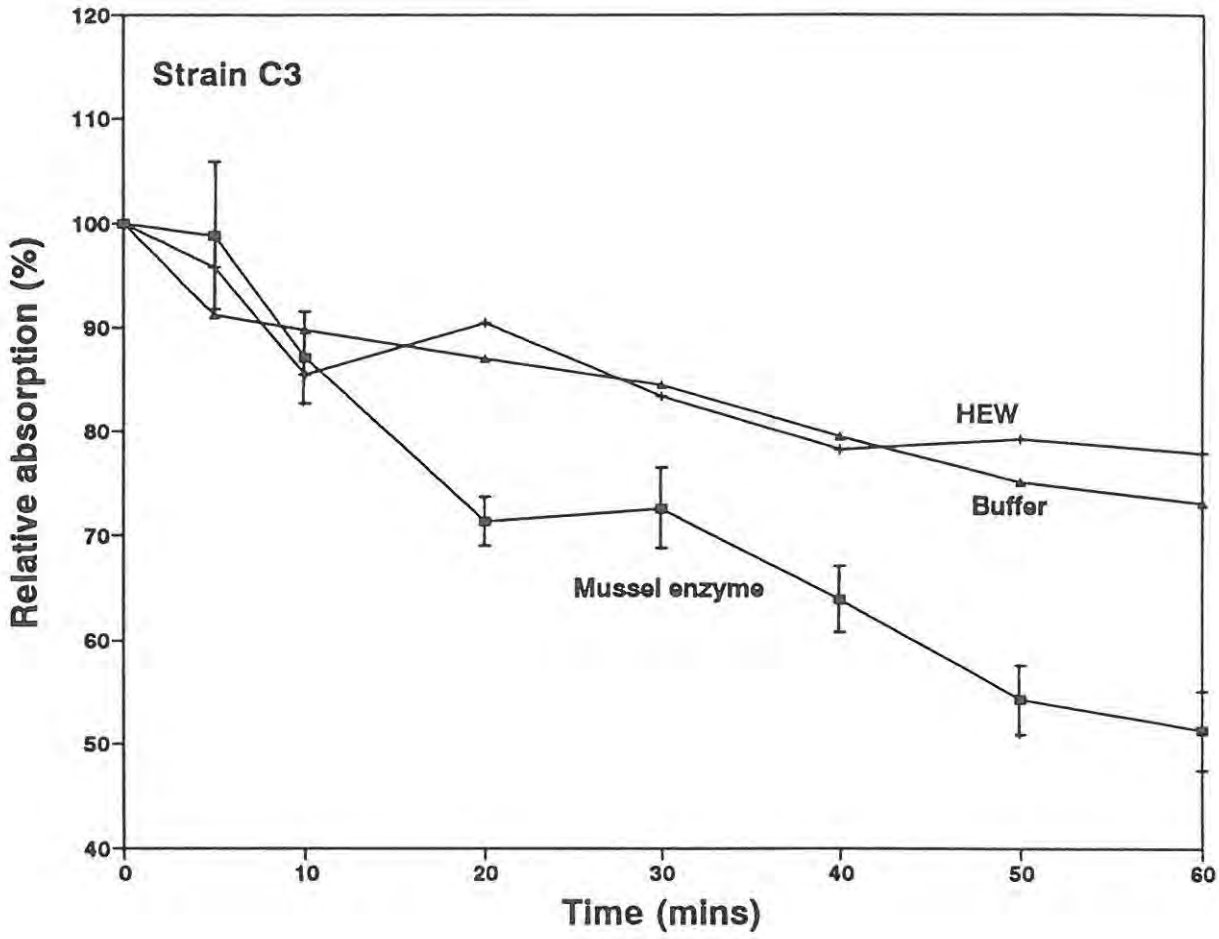


Fig. 3.4 Bacteriolytic activity by *C.meridionalis* on strain C3.

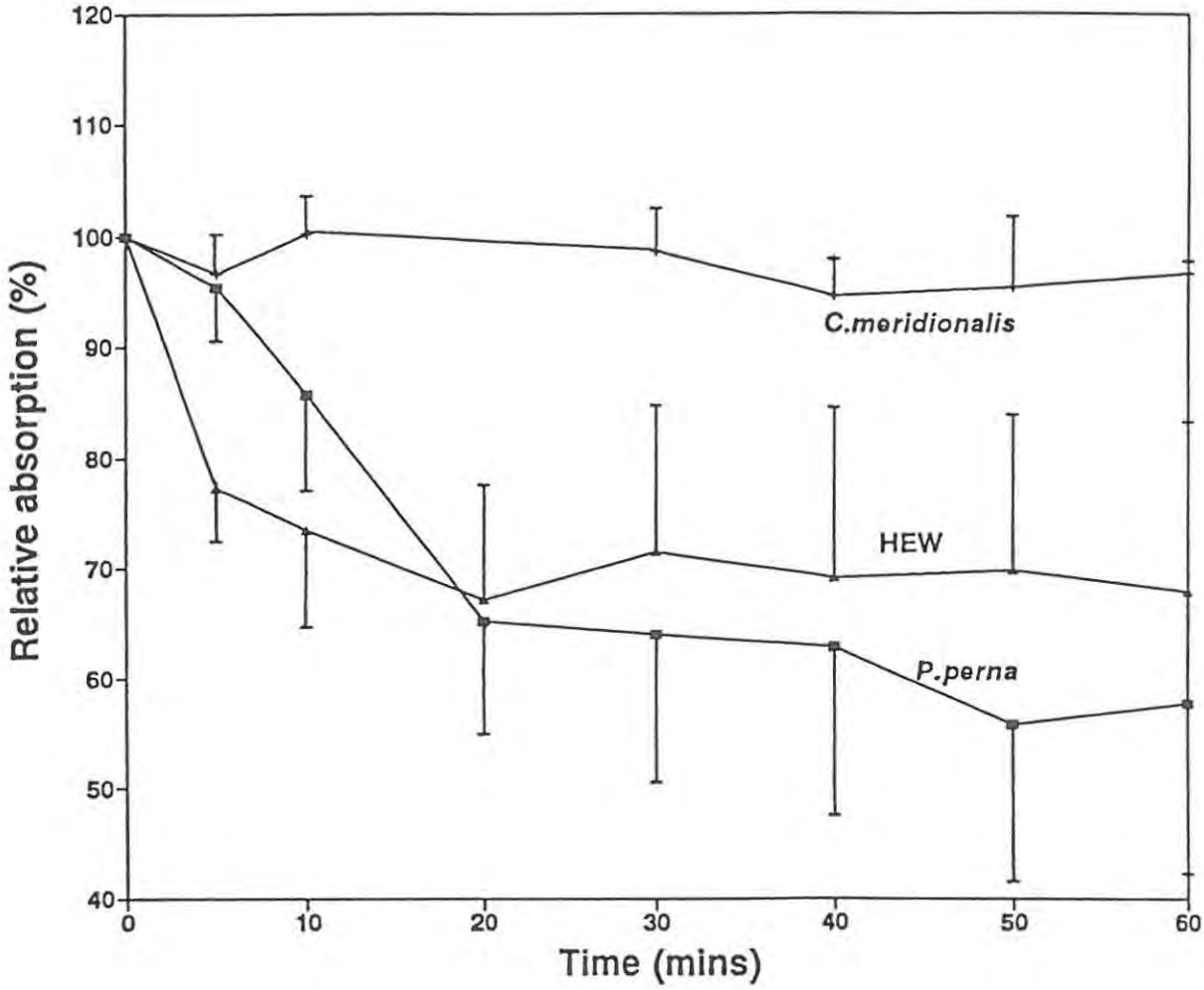


Fig. 3.5 Bacteriolytic activity on *M.lysodeikticus*. (+ or - 1 SE).

DISCUSSION

Both species supported extensive bacterial populations, and as expected, most of the bacteria were Gram negative rods. The viable counts were at least an order of a magnitude higher than in the sea urchin, *Parechinus angulosus* (Sweijd, 1990). Numbers were also higher than in the abalone, *Haliotis midae* (Erasmus, 1996), although a direct comparison is difficult, as different units of counting were used. These high numbers in the mussels could be attributed to the higher numbers of bacteria which are associated with detrital particles than with intact algae on which grazers such as urchins and abalone feed.

Direct counts of bacteria give one a more accurate estimate of bacterial numbers, as they include dead and living cells. If one wants to use bacterial numbers as a rough measure of digestive activity, it is, however, preferable to use viable counts, or colony forming units (CFU's). The solid and broth media used for growth of bacteria were the same. This means that the bacteria present in the broth cultures, and contributing to digestion in the assays, would probably be present on the plate media. *Choromytilus meridionalis* consistently housed populations that were an order of a magnitude higher and less variable than in *Perna perna*, though this difference was not statistically significant ($P>0.05$, Two-tailed t-Test).

Bacterial enzyme activity:

a) Storage carbohydrates:

The digestion of most of the substrates used in this study has been demonstrated in a wide range of bacteria. Bacteria digesting amylose, glycogen and laminarin are especially widespread. These bacteria have been isolated from the guts of sea urchins (Huang and Giese, 1958; Prim and Lawrence, 1975; Sweijd, 1990), crustaceans (Dempsey and Kitting, 1987; Musgrove, 1988) and gastropods (Vitalis *et al.*, 1988; Muthu, 1990; Šyvokienė, 1990; Erasmus, 1996), as well as from seaweed fronds (Quatrano and Caldwell, 1978). Surprisingly few bacteria digesting laminarin were isolated from *Laminaria* fronds (Ushida, 1995). Furthermore, laminarinases have also been detected in yeasts and fungi (Chesters and Bull, 1963) and starved free-living bacteria (Davis, 1992). It appears that even though laminarinase activity is relatively common, it is not as widespread, or strong, as amylase activity.

Both *Perna perna* and *Choromytilus meridionalis* housed bacteria which digested these 3 compounds. Digestion was always higher, and less variable, in *C.meridionalis* bacteria

than in bacteria from *P.perna*. In both species, activity on laminarin was at most half the activity on amylose and glycogen. The level of activity on glycogen, amylose and laminarin appears to be related to bacterial numbers. Bacterial numbers were consistently higher and less variable in *C.meridionalis* than in *P.perna*, which probably led to the lower, and more variable activity in *P.perna*.

The only storage carbohydrate not digested by bacteria from either species was inulin. Digestion of this carbohydrate has not been widely tested for, and was absent in bacteria from *Fucus* fronds (Quatrano and Caldwell, 1978), but present in bacteria from the gut of the urchin, *Parechinus angulosus* (Sweijd, 1990).

b) Structural carbohydrates:

Detritus is composed mainly of refractory carbon, and the ability of bacteria to digest structural carbohydrates is therefore very important in the detrital food chain, as well as to animals which ingest detritus. The digestion of cellulose, especially, has received a great deal of attention, due to its presence in all vascular plants and algae (Percival, 1968; Price, 1990), and the belief that most animals have limited ability to digest this carbohydrate adequately. Teredinids (wood-boring bivalves) have an obligate, symbiotic relationship with cellulolytic bacteria which they house in special structures associated with their gills (eg. Morton, 1978; Waterbury *et al.*, 1983; Griffin *et al.*, 1987), while some house additional cellulolytic bacteria in the gut lumen (Crosby and Reid, 1971). In other invertebrates, cellulolytic bacteria have been both present (eg. Harris *et al.*, 1986b; Dempsey and Kitting, 1987; Lésel *et al.*, 1990; Šyvokienė, 1990; Erasmus, 1996) or absent (eg. Horiuchi and Lane, 1966; Prim and Lawrence, 1975; Vitalis *et al.*, 1988; Sweijd, 1990; Harris *et al.*, 1991), with no clear phylogenetic tendencies. Sometimes the bacteria may not be associated with the gut contents, but instead, with the exoskeleton or burrows of the animal, as in the case of wood-boring isopods (Boyle and Mitchell, 1981; El-Shanshoury *et al.*, 1994). Cellulolytic bacteria are also, quite naturally, associated with seaweed fronds (Quatrano and Caldwell, 1978; Ushida, 1995). Both *Perna perna* and *Choromytilus meridionalis* housed bacteria with only low cellulolytic activity.

Bacteria with alginolytic activity are relatively common in the marine environment (see Gacesa, 1992, for review). Alginases have been found in both free-living bacteria

(Davis, 1992), and bacteria attached to seaweed fronds (Quatrano and Caldwell, 1978; Doubett and Quatrano, 1984; Ushida, 1995). In spite of this, very few enteric bacteria seem to posses alginolytic activity, and if it is present, it is very weak (Sweijd, 1990; Harris *et al.*, 1991). Erasmus (1996) has, however, isolated a *Pseudomonas* sp from the abalone *Haliotis midae* with very high activity. Brown algae, of which alginic acid is the major structural carbohydrate (Percival, 1968; Price, 1990), are not abundant at the sample site of the present study, so the absence of alginolytic activity in *Perna perna* and *Choromytilus meridionalis* is not surprising.

Fucoidan is a secondary structural carbohydrate in brown algae, and mannan and xylan are secondary structural carbohydrates in red and green algae (Percival, 1968; Price, 1990). Bacteria digesting fucoidan have been found in sea urchins (Huang and Giese, 1958), and on *Fucus* and *Laminaria* fronds (Quatrano and Caldwell, 1978; Ushida, 1995). Digestion of mannan has not been tested for widely, but has been found in bacteria from the gut of the crayfish, *Paranephrops zealandicus* (Musgrove, 1988). I could find no evidence in the literature of xylanolytic activity in gut bacteria. Bacteria from *Perna perna* and *Choromytilus meridionalis* were able to digest fucoidan and mannan, while only *P.perna* showed activity on xylan, though this occurred in only the first set of assays (Fig. 3.2a). This means that even though there are bacteria capable of digesting xylan, they are not always present in the guts of these animals.

Bacteria capable of digesting carrageenin have been isolated from *Fucus* fronds (Quatrano and Caldwell, 1978) and the guts of sea urchins (Prim and Lawrence, 1975), the seahare, *Aplysia juliana* (Vitalis *et al.*, 1988) and the abalone *Haliotis midae* (Erasmus, 1996). Carrageenin is the major structural carbohydrate in red algae (Percival, 1968; Price, 1990), which are the most common algae on the south east coast of South Africa (Seagrief, 1988). It was therefore expected that bacteria within the water column, or associated with detrital particles, which could digest carrageenin, would be relatively common.

Bacterial digestion of carrageenin occurred in *Perna perna* and *Choromytilus meridionalis* in the preliminary assay (Table 3.1) but not in either species, in the two test assays (Figs. 3.2 a-c). The mussels used in the preliminary assays had been kept under laboratory conditions for a month, without the food being replenished, while freshly caught

mussels were used in the actual test assays. This suggests that during the period of starvation in the laboratory, bacteria which digest carrageenin persisted, implying that they were performing an endosymbiotic role. This is in keeping with the suggestion that the importance of potential symbiotic bacteria increases during periods of food paucity, when the gut passage time increases (Lasker and Giese, 1954; Prim and Lawrence, 1975; Moriarty, 1990).

c) Effects of starvation:

An experiment was subsequently performed to test this hypothesis, testing for activity in freshly caught mussels, and mussels that had been starved for 21 days. The bacteria from fresh and starved *Perna perna* digested carrageenin, while activity was absent in *Choromytilus meridionalis* in both samples (Fig. 3.6). After 21 days, activity in *P.perna* dropped slightly, but not significantly (Two-tailed t-Test, $P<0.05$). These results indicate that bacteria which are able to digest carrageenin are not constantly present in the guts of these mussels, but that when they are, they are capable of persisting in the gut, but that the importance of these bacteria do not increase during starvation (cf. Lésel *et al.*, 1990).

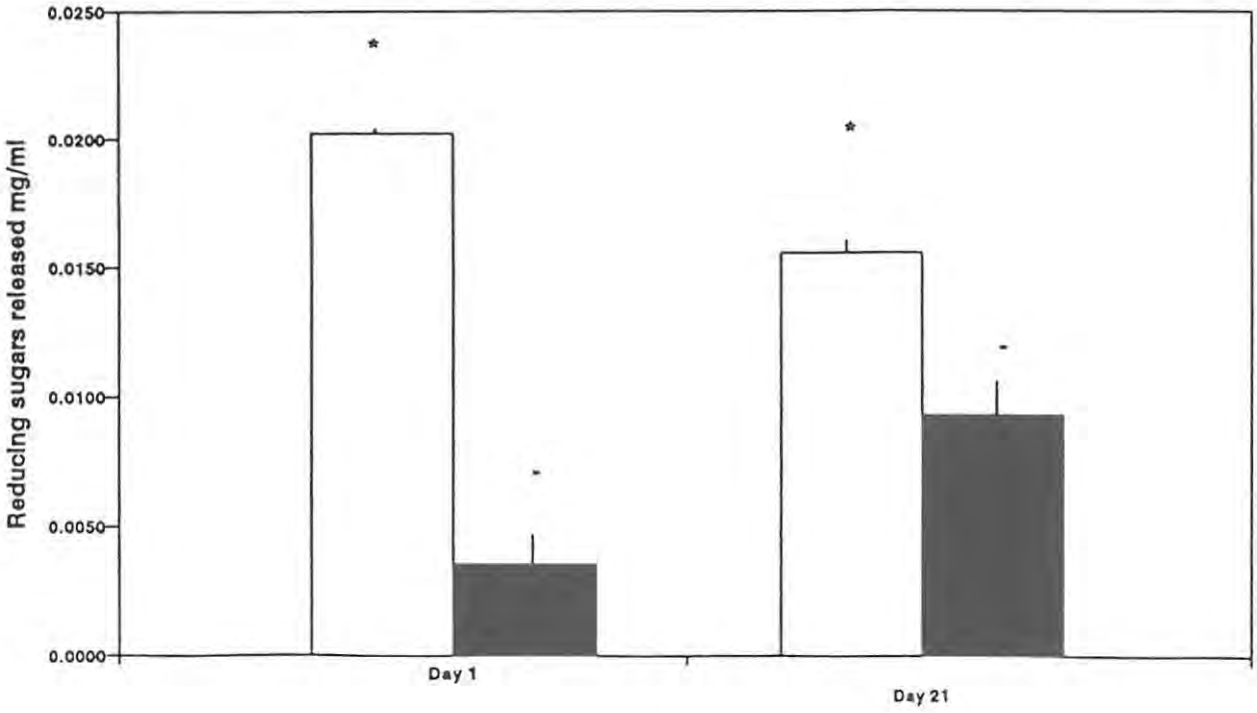


Fig. 3.6 Digestion of carrageenin at Day 1 and after 21 days (□ = *P.perna* and ■ = *C.meridionalis*; * = positive digestion, - = no digestion).

One cannot, however, conclude that because bacteria capable of digesting mannan and fucoidan were present in both test assays, that these bacteria are always present in fresh mussels. It is possible that mussels housing these bacteria were sampled by chance. The absence of these bacteria in the starved mussels further suggests that these bacteria do not persist during periods of food paucity. It is, however, also possible that these mussels did not house bacteria which digested mannan and fucoidan at the time of collection.

It would be safe to conclude that bacteria which digest amylose, glycogen, laminarin, and CMC are always present in the guts of these mussels, as activity on these carbohydrates was detected in all the assays. These bacteria also tend to persist during starvation (Šyvokeinė, 1990).

Even though agarase activity was not tested for, bacteria digesting this carbohydrate were detected in both *Perna perna* and *Choromytilus meridionalis*, on the solid growth media. This occurred in the form of pits in the agar around the colony, and the partial liquification of the agar. Agarolytic activity has been found in bacteria housed in gastropods (Harris *et al.*, 1986b; Erasmus, 1996) and in bacteria attached to algae (Quatrano and Caldwell, 1978).

Digestion of bacteria:

Bacteria are generally considered to have a low nutritional value in terms of carbon. They are poor sources of polyunsaturated fatty acids and sterols, and contribute little to the hosts' overall carbon budget (eg. Cammen, 1980; Phillips, 1984; Stuart and Klumpp, 1984; Matthews *et al.*, 1989; Langdon and Newell, 1990). Several authors have, however, shown that some deposit feeders assimilate the bacteria associated with the detritus, while egesting the intact detrital particles (eg. Newell, 1965; Fenchel, 1970), or that bacteria are assimilated more efficiently than algae (Dye and Lasiak, 1987). Some researchers have maintained mussels (Zobell and Landon, 1937; Zobell and Feltham, 1938), worms (MacGinitie, 1978) and copepods (Rieper, 1978) on a bacterial diet for extended periods, while Reiswig (1975) contended that bacteria alone could satisfy the food requirements of two sponges. Furthermore, some invertebrates are able to assimilate between 40 and 72% of the bacteria ingested (Lopez and Cheng, 1983; Amouroux, 1986). Indirect evidence for the utilisation of bacteria is presented by Baker and Bradnam (1976) and Kueh and Chan (1985), who demonstrated a decrease in bacterial numbers from the fore- to the hindguts of aquatic insects

and bivalves, respectively. Bivalves which rely completely on bacteria as a food source are found at hydrothermal vents and in reducing sediments, and the bivalves ingest the chemoautotrophic bacteria housed in their gills (eg. Herry *et al.*, 1989; Boetius and Felbeck, 1995; Johnson and Le Pennec, 1995).

Many researchers have shown that bacteria are an important source of nitrogen (Lucas and Newell, 1984; Seiderer *et al.*, 1984; Crosby *et al.*, 1990; Landgon and Newell, 1990). Bacteria are able to supply their hosts with essential amino acids which they produce themselves (Fong and Mann, 1980; Guerinot and Patriquin, 1981) or the bacteria may increase the release of essential amino acids from algae (Vitalis *et al.*, 1988). In addition, bacteria may also supply their hosts with B-complex vitamins (Phillips, 1984). So, even though bacteria do not contribute much to the hosts' carbon requirements, they do supply the hosts with supplementary nutrients, and may even fulfil the host's nitrogen requirements (Seiderer *et al.*, 1984).

Unless the bacteria release these nutrients into the host's gut lumen, the host has to digest the bacteria to gain access to the nutrients. Several different enzymes are responsible for the digestion of bacteria: lysozyme (muramidase), N-acetyl-ß-glucosaminidase (chitobiase), protease and peptidase (Boetius and Felbeck, 1995). Many researchers refer to bacteriolytic activity as lysozyme or lysozyme-like activity (eg. McHenery *et al.*, 1979; Muir *et al.*, 1986). According to Salton (1957), the main characteristic feature of a lysozyme is its ability to digest the bacterium *Micrococcus lysodeikticus* (= *M.luteus*). Not all bacteriolytic enzymes are lysozymes, and one therefore has to be sceptical of data generated by using only *M.lysodeikticus* as a substrate, instead of bacteria from the host (Sweijd, 1990). One also has to be careful of naming the enzyme. Bacteriolytic activity or enzyme is the most general, and perhaps the most accurate term to use.

Bacteriolytic enzymes have been found in a wide range of invertebrates, including polychaetes (Plante and Mayer, 1994), sea urchins (Wardlaw and Unkels, 1978) and bivalves (McHenery *et al.*, 1979; Jamieson and Wardlaw, 1989). In bivalves, the highest incidence of bacteriolytic activity has been found in the crystalline style, with considerable activity also present in the digestive gland (McHenery *et al.*, 1979; Jamieson and Wardlaw, 1989). The presence of the enzyme in these organs suggests that the enzyme has a nutritive function, in contrast to defence, when the enzyme is concentrated in the haemolymph of the bivalve (McDade and Tripp, 1967; Hardy *et al.*, 1976). Jamieson and Wardlaw's (1989) experiments

showed that the enzyme from the digestive glands of the bivalves *Mya arenaria, Modiolus modiolus, Cerastoderma edule, Mytilus edulis* and *Pectin maximus* digested only the polysaccharide and not the lipid component of the lipopolysaccharide which makes up the bacterial cell wall. This led them to believe that only the polysaccharide contributed to the nutrition of the bivalves. It is also possible that the enzyme breaks the cell wall, exposing the contents to the host (Seiderer *et al.*, 1984).

Perna perna has never been tested for bacteriolytic activity, but it can filter bacteria from the water column on the Natal coast of South Africa (Berry and Schleyer, 1983). *Choromytilus meridionalis* on the west coast of South Africa produces a bacteriolytic enzyme during periods of upwelling, when phytoplankton and detritus concentrations are low (Seiderer *et al.*, 1984; Muir *et al.*, 1986).

Perna perna digested 3 of the 10 bacteria tested, while *Choromytilus meridionalis* digested only 1. This relatively low incidence of susceptibility to the enzymes actually does not have negative implications in terms of the animals' ability to utilise bacteria as a food source. Muir *et al.* (1986) found that 79% of the bacteria isolated from the guts of *C.meridionalis* were resistant to lysis by the mussels' enzymes. Seiderer *et al.* (1984) found that free-living bacteria were far more susceptible to digestion than enteric bacteria, suggesting that free-living bacteria contribute more to the nutrition of the mussel than "resident" bacteria. This also suggests that there is rapid and selective digestion of bacteria in the stomach (and possibly digestive gland) (eg. Deming and Colwell, 1982; Prieur, 1981), followed by the proliferation or stabilisation of the resistant bacteria in the hindgut. These bacteria are possibly responsible for supplying the host with supplementary digestive enzymes (Prieur, 1987).

Not only did *Perna perna* digest relatively more bacterial species than *Choromytilus meridionalis*, but it was also enzymatically more active. After 60 minutes, the difference between the relative absorbances of the test assays and the controls was greater for *P.perna* than for *C.meridionalis* (Figs. 3.3 a-c and Fig. 3.4), although this was not tested statistically. This could explain why *P.perna* usually had lower bacterial numbers in the gut than *C.meridionalis*. Even though all the mussels used in these experiments were collected at the same tide, there was a high degree of individual variability in enzyme activity. This seems to be a common characteristic of bacteriolytic activity (McHenery *et al.*, 1979; McHenery *et al.*, 1983).

None of the bacterial isolates were susceptible to hen's egg white lysozyme. This is actually not surprising, as lysozyme is generally not very active against Gram negative bacteria unless the bacteria have been pretreated with EDTA (Salton, 1957). All these bacteria were Gram negative.

None of the enzyme extracts from *Choromytilus meridionalis* were able to digest *Micrococcus lysodeikticus* (Fig. 3.5), supporting Muir *et al.*'s (1986) finding that *C.meridionalis* does not produce a true lysozyme. In *Perna perna*, two of the enzyme extracts were not active on *M.lysodeikticus*, even though they were active on the susceptible bacteria. The other three extracts resulted in changes in relative absorbance ranging from 40-80%. This means that these 3 extracts contained a true lysozyme. If these extracts contained true lysozyme, how can one explain the fact that these extracts were active on the isolated bacteria, while the bacteria were resistant to commercial lysozyme (Figs. 3.3 a-c)? The only explanation is that the enzyme extract contains a mixture of different enzymes which function together in digesting the bacteria. This has been found in *Mytilus edulis*, which has a true lysozyme in its style (McHenery and Birkbeck, 1979), and a non-lysozyme bacteriolytic enzyme produced by rod-shaped bacteria found in its outer style matrix (Seiderer *et al.*, 1987). It is not known if the different enzymes in *P.perna* are all released from the style, or if there is a bacterial contribution.

To summarise, these experiments have shown that the enteric bacteria of *Perna perna* and *Choromytilus meridionalis* are able to digest 8 of the 10 substrates tested. These bacteria are active on some, or all of the structural and storage carbohydrates found in red, green and brown algae. Bacteria digesting all these substrates were not always present, and their presence is most likely a reflection of the detrital material and their attached bacteria present at the time of sampling (Sugita *et al.*, 1981; Šyvokienė *et al.*, 1986; 1988; Moriarty, 1990). One can therefore conclude that different bacteria, digesting different substrates, occur incidentally in the guts of the mussels.

Both *Perna perna* and *Choromytilus meridionalis* produce bacteriolytic enzymes, though *C.meridionalis* digests fewer strains. This gives them the potential to utilize bacteria as a supplementary food source. The results also suggest that bacteriolytic activity in *P.perna* is the result of a mixture of different enzymes, which act differently on different bacteria. Some of these individuals also possessed a true lysozyme. It is possible that bacteriolytic

activity in *C.meridionalis* is also due to a mixture of enzymes, but this is not evident from the results.

CHAPTER 4

A MICROSCOPIC EXAMINATION OF THE ALIMENTARY CANAL OF *PERNA PERNA* AND *CHOROMYTILUS MERIDIONALIS*

INTRODUCTION

Initial studies of the gut bacteria of marine invertebrates used mainly microbiological techniques (eg. Colwell and Liston, 1960; Liston and Colwell, 1963; Zhukova, 1963), allowing the researchers to characterise the bacteria. In addition, the bacteria were often isolated from whole gut homogenates (Colwell and Liston, 1960; Sochard *et al.*, 1979; Crosby and Peele, 1987), so that bacteria associated with the gut contents could not be distinguished from bacteria associated with the gut lining. This method could give no indication of the colonisation sites of the bacteria along the gut. Some have shown that while bacteria are found along the complete length of the gut, different parts of the gut may harbour different populations (Unkles, 1977; Kueh and Chan, 1985; Harris, 1993a; Erasmus, 1996).

Electron microscopy is very useful in studying bacteria within the alimentary canal, and can shed some light on such problems. It has been used successfully to describe fermentative bacteria associated with the gut tissue of ruminants (eg. McCowan *et al.*, 1978; McCowan *et al.*, 1980) and terrestrial invertebrates, such as termites (Breznak and Pankratz, 1977), cockroaches (Cruden and Markovetz, 1981) and millipedes (Green and Baker, 1995). The results of these studies led to the conclusion that in mammals and terrestrial arthropods, attachment of bacteria to gut tissue is facilitated by the specialised structures within the gut (McBee, 1971).

Savage (1977) also proposed that in mammals bacteria have to fulfil several criteria before they can be considered to be part of the hosts' normal gut flora:

1) they must be able to grow anaerobically,
2) they must always be found in normal adults,
3) they must colonise particular areas of the digestive tract,
4) they must colonise their habitats during succession in infants,
5) they must maintain stable population levels in adults,
6) they may be associated intimately with the mucosal epithelium in the area colonized.

These criteria (or at least some of them) can easily be extended to help define the normal gut flora of invertebrates.

Bacteria have been detected in marine invertebrates, from a wide range of phylogenetic groups (see Chang *et al.*, 1984; Harris, 1993a; Cavanaugh, 1994 for reviews). Factors which affect the presence of bacteria in aquatic invertebrates include gut structure, the nature of the

gut lining, resistance to the mechanical activity of the gut, gut passage time, diet, temperature and pH, physiological properties of the bacteria and seasonality (Harris, 1993a). Of these, gut structure (Juilfs and Wägele, 1987; Moriarty, 1990; Harris, 1993a), nature of the gut lining (Boyle and Mitchell, 1978; Harris, 1993b)and the mechanical action of the gut (Garland *et al.*, 1982) seem to be the main factors influencing the actual attachment of bacteria to the gut tissue.

In molluscs, electron microscopic studies seem to be biased in favour of species with specialised living requirements (eg. Cavanaugh, 1983; Felbeck, 1983; Southward, 1986; Herry *et al.*, 1989; Johnson and Le Pennec, 1995), while non-specialised species, such as herbivores, scavengers and filter feeders have been neglected (eg. Garland *et al.*, 1982; Harris *et al.*, 1986b; Charrier, 1990). One association that has been well documented is that between bivalve styles and spirochaete bacteria (eg. Bernard, 1970; Judd, 1979; Lawry *et al.*, 1981; Tall and Nauman, 1981; Lawry, 1987). In spite of this long standing interest, very little is known about the nature of this association (Johnson, 1977; Breznak, 1984; Brock and Madigan, 1991).

Collectively, studies of the gut bacteria of invertebrates have led to the conclusion that the bacteria can be divided into two broad groups: 1) bacteria associated with the food, and 2) bacteria attached to, or closely associated with specific areas of the intestinal tract (Sieburth, 1976). Furthermore, these groups of bacteria also perform different functions: bacteria attached to the food produce enzymes which supplement those of the host (eg. Musgrove, 1988; Muthu, 1990; Harris, 1993a), while there is direct nutrient flux between the attached bacteria and the gut tissue (Plante *et al.*, 1990; Harris, 1993a). Bacteria associated with the food are usually accepted as being transient (or coincidental), while attached bacteria are resident. This distinction can become a bit vague, as many "resident" bacteria originate from the environment (Harris, 1993a). Many hosts are also able to maintain a relatively constant population of bacteria that may actually not be attached to the tissue (Colwell and Liston, 1960). Furthermore, starved molluscs house bacterial populations similar in composition to those in fed animals, though bacterial numbers are lower in starved animals (Šyvokienė, 1990).

This study proposed to answer the following questions:

1) Do *Perna perna* and *Choromytilus meridionalis* house bacteria which are associated

with their food and/or with their gut lining?

2) If the bacteria are associated with gut tissue, are there specific colonisation sites?

METHODS AND MATERIALS

Specimen collection:

Samples of *Perna perna* were collected from Port Alfred (33°36'S/26°54'E) and Rufane's River Mouth (33°34'S/26°56'E), and *Choromytilus meridionalis* was collected at Rufane's River Mouth. The animals were transported to the laboratory and processed within 1.5-2.5hrs.

Preparation of tissue:

The alimentary canals were dissected out using flame-sterilized instruments. Sections from the gills, stomach, mid- and hind guts and styles were fixed in 2.5% gluteraldehyde in 0.2 µm filtered sea water. The samples were stored at 4°C (for no longer than 2 months), until processed further.

Scanning Electron Microscopy:

The tissue was post-fixed in 1% OsO_4 in Sodium Cacodylate buffer (pH 7.2), for 1 hour and further processed according to Cross (1987; Appendix 3a). The amyl-acetate step was omitted to prevent the food particles from disintegrating in the tissue used to examine the gut contents (Sweijd, 1990). The tissue was then Critical Point Dried, using CO_2 as the transitional fluid. The stomach and mid- and hindgut sections were slit longitudinally, to expose the contents or linings. The tissue was then mounted on copper specimen stubs, coated with 19.4nm gold on a sputter coater and examined on a Jeol JSM 840 Scanning Electron Microscope.

At least three individuals of each species were examined.

Transmission Electron Microscopy:

The tissue was prepared according to Cross (1987; Appendix 3b). To examine the mid- and hindgut linings, the sections were gently scraped, slit longitudinally and washed in 0.2µm filtered sea water, to remove the gut contents, before fixing. Ultrathin sections (100nm thick) were cut on an RMC MT-7 ultramicrotome, using a diamond knife. The sections were mounted on copper grids, and stained with uranyl acetate (30 mins) and lead citrate (10 mins). The grids were viewed on a Jeol 1210 Transmission Electron Microscope.

At least three individuals were examined for each species, and for each section, at least three grids were viewed.

Phase contrast microscopy:

Intact styles of at least 5 individuals per species, per site, were stained in 0.5% methylene blue in 0.2µm filtered sea water (Lawry *et al*, 1981) for 10 mins. Styles were viewed on an Olympus BX40 phase contrast microscope.

RESULTS

Gills:

Associated with the gill surfaces of *Perna perna* were coccoid bodies, ranging from 1 - 2µm in diameter (Fig. 4.1a), and rod-shaped organisms (2 x 0.5 µm), which sometimes formed strings of approximately 4µm long (Fig. 4.1b). The rods were always covered with extracellular debris. Only coccoid bacteria (0.3 -1µm in diameter) were found in *Choromytilus meridionalis* (Fig. 4.1c). In both *P.perna* and *C.meridionalis*, the cocci occurred in strings, but more often they occurred singly (Figs. 4.1a, c & d).

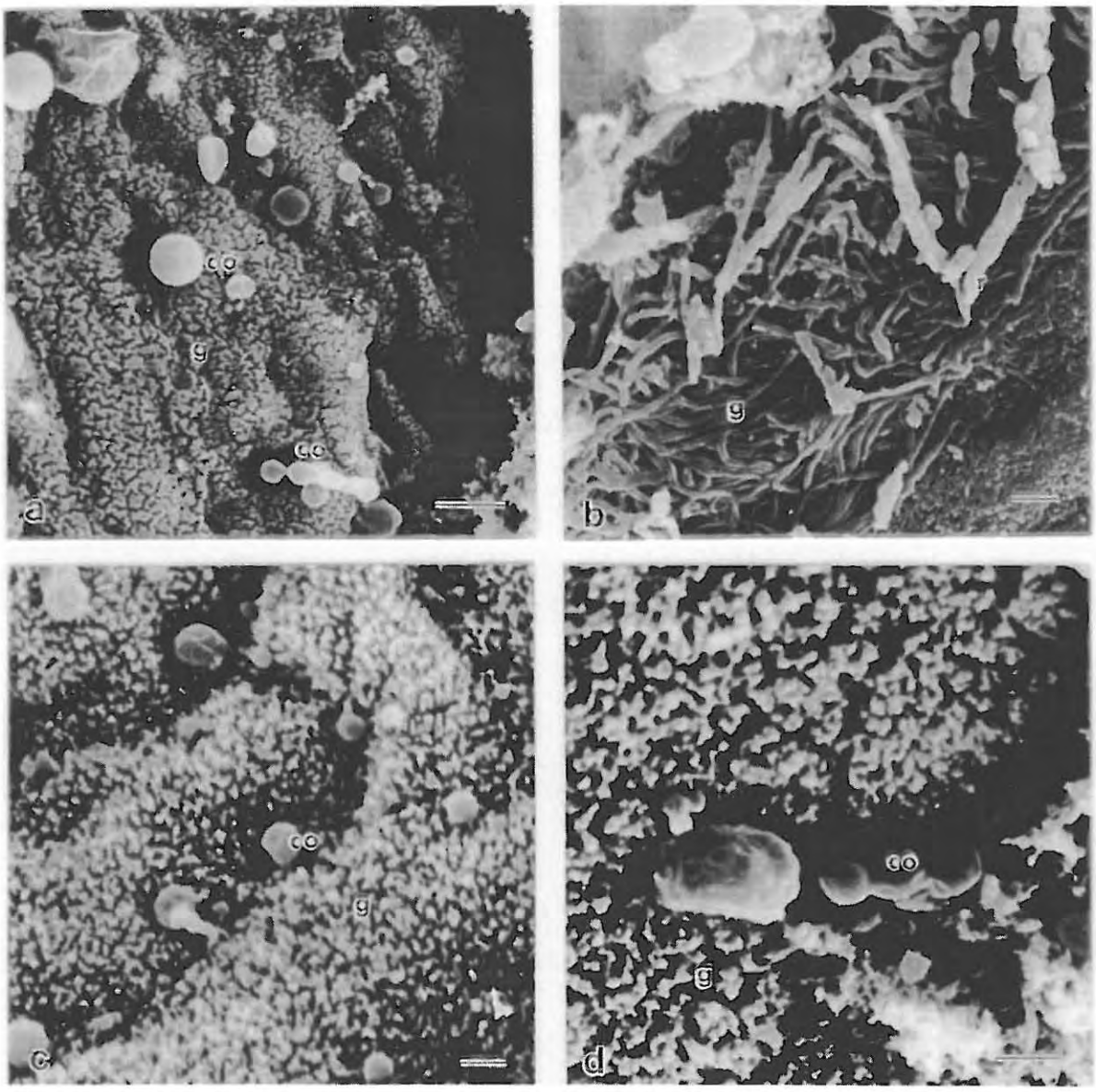
Styles:

Perna perna:

Unwashed styles were covered with paddle cilia, diatom frustules, mucus, food particles and coccoid bodies (Fig. 4.2a). Styles that had been washed in sterile sea water revealed an even, but porous surface. Associated with the surfaces of the styles were irregular shaped bacteria (spirochaetes, 5 - 19µm long), covered with extracellular debris (Fig. 4.2b). Styles examined by phase contrast microscopy were densely covered with large numbers of live spirochaetes (Fig. 4.2c), though the degree of colonisation varied among individuals.

In section, the styles had two layers. The outer matrix harboured high numbers of spirochaetes (Fig. 4.2d), in their spiral configuration; at high magnification, the characteristic axial fibrils and three layered cell wall can be seen (Fig. 4.2d, inset). These bacteria were found in clear areas within the matrix. On average, the spirochaetes were approximately 0.25 µm in diameter, and reached lengths of up to 160µm, by measuring the live bacteria by phase contrast microscopy. The bacteria found with SEM were obviously partly embedded in the style matrix, exposing only part of the cell.

The inner matrix was characterised by spindle-shaped clear areas approximately 8 x 3 µm in size. Often there were spiral "tails" leading from one or both ends of these spaces (Fig. 4.2e). These areas were sometimes empty, but more often they contained circular bodies ranging from 0.7 - 1 µm in diameter. The multilayered wall indicated that these spheres were bacterial in origin (Fig. 4.2e, inset). These spaces were distributed evenly throughout the matrix, in concentric circles. Part of one such circle is shown in Fig. 4.2f.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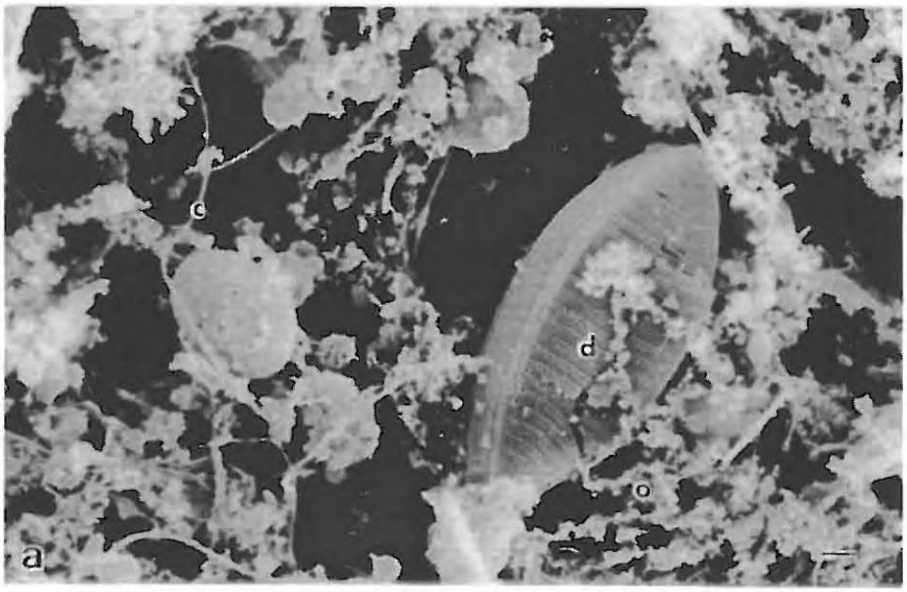
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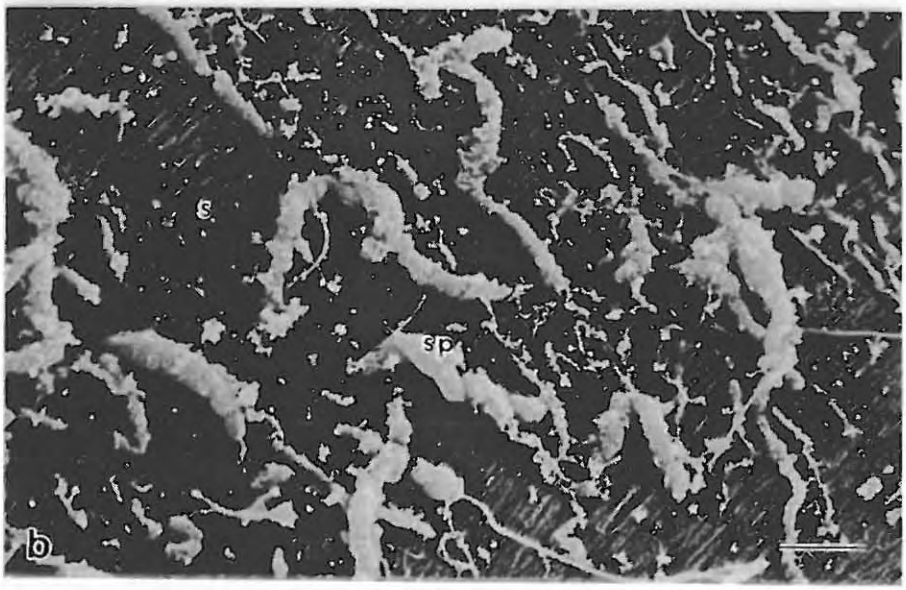
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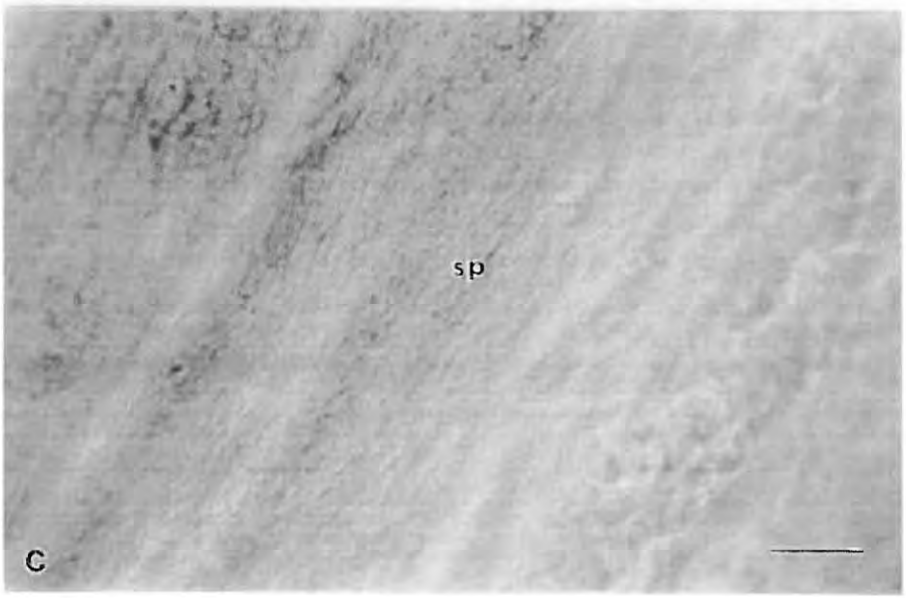
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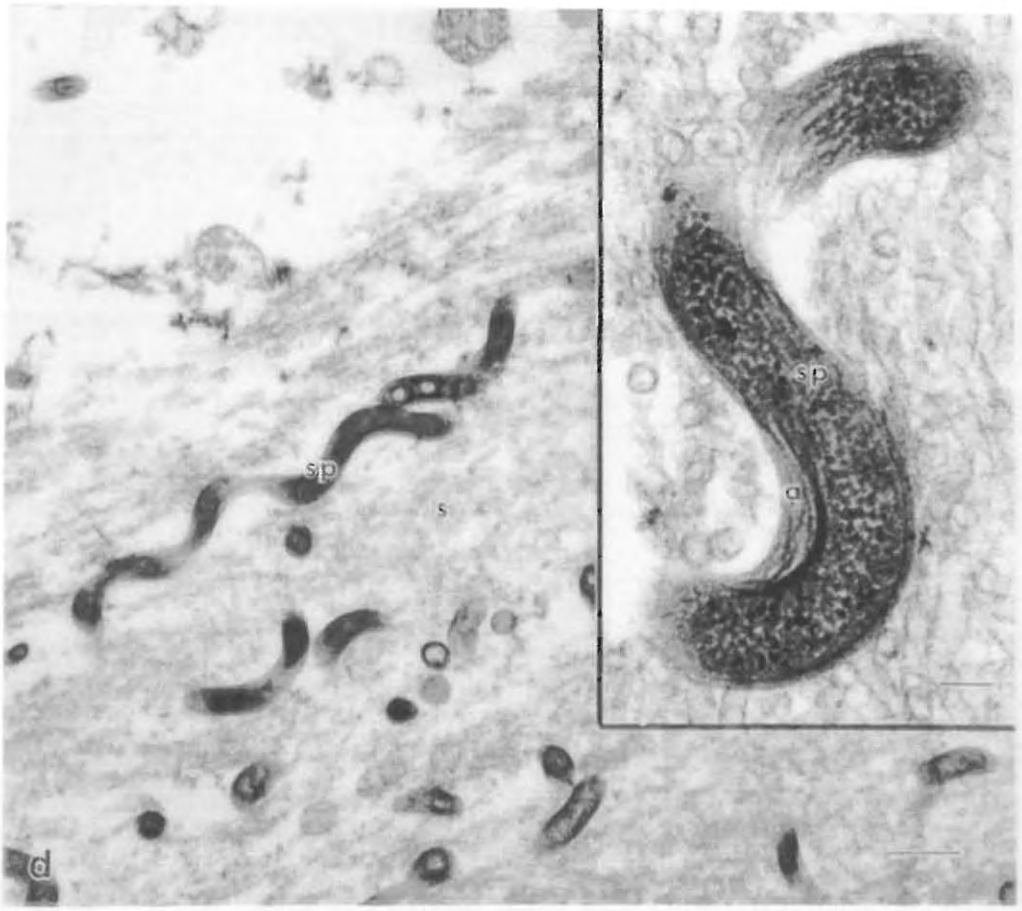
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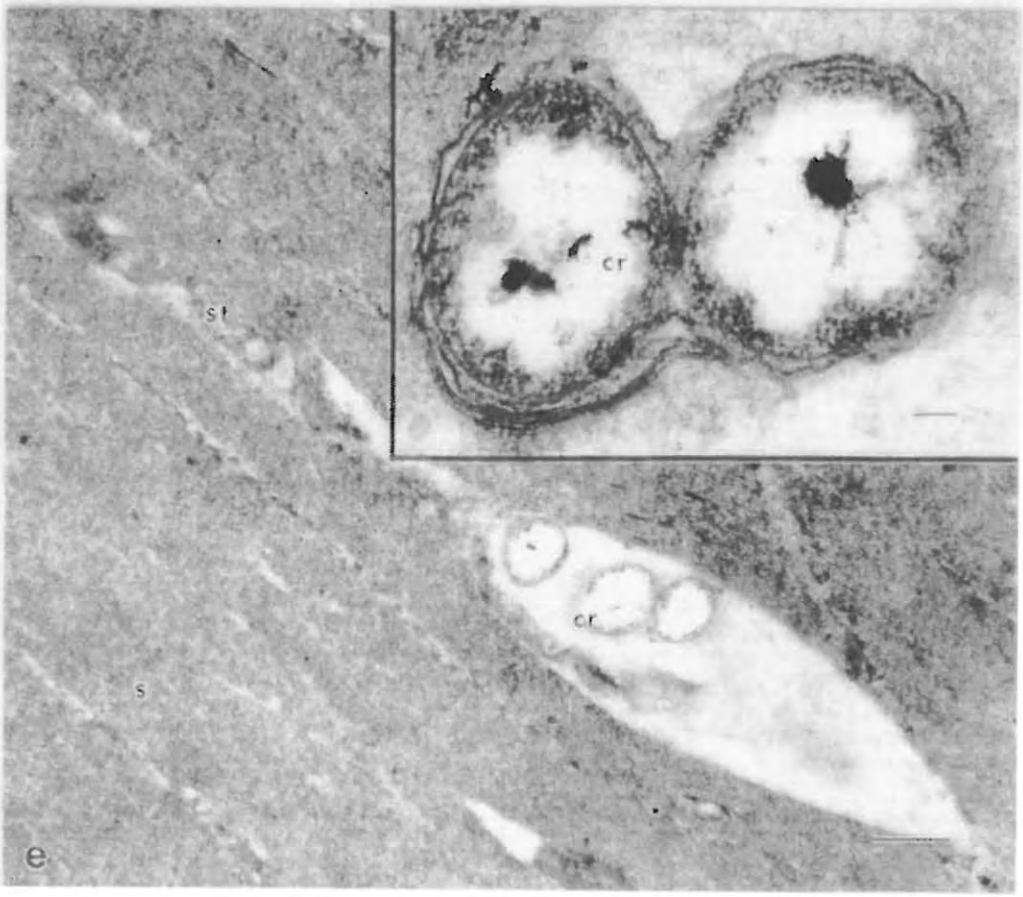
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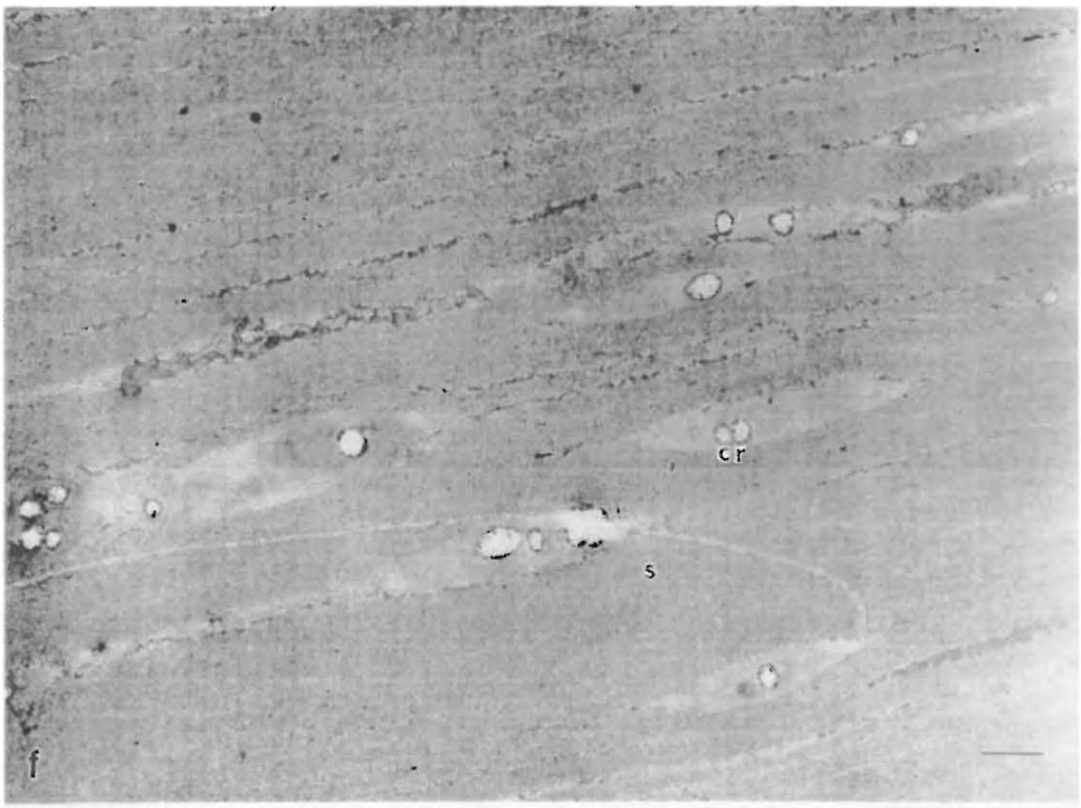
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Choromytilus meridionalis:

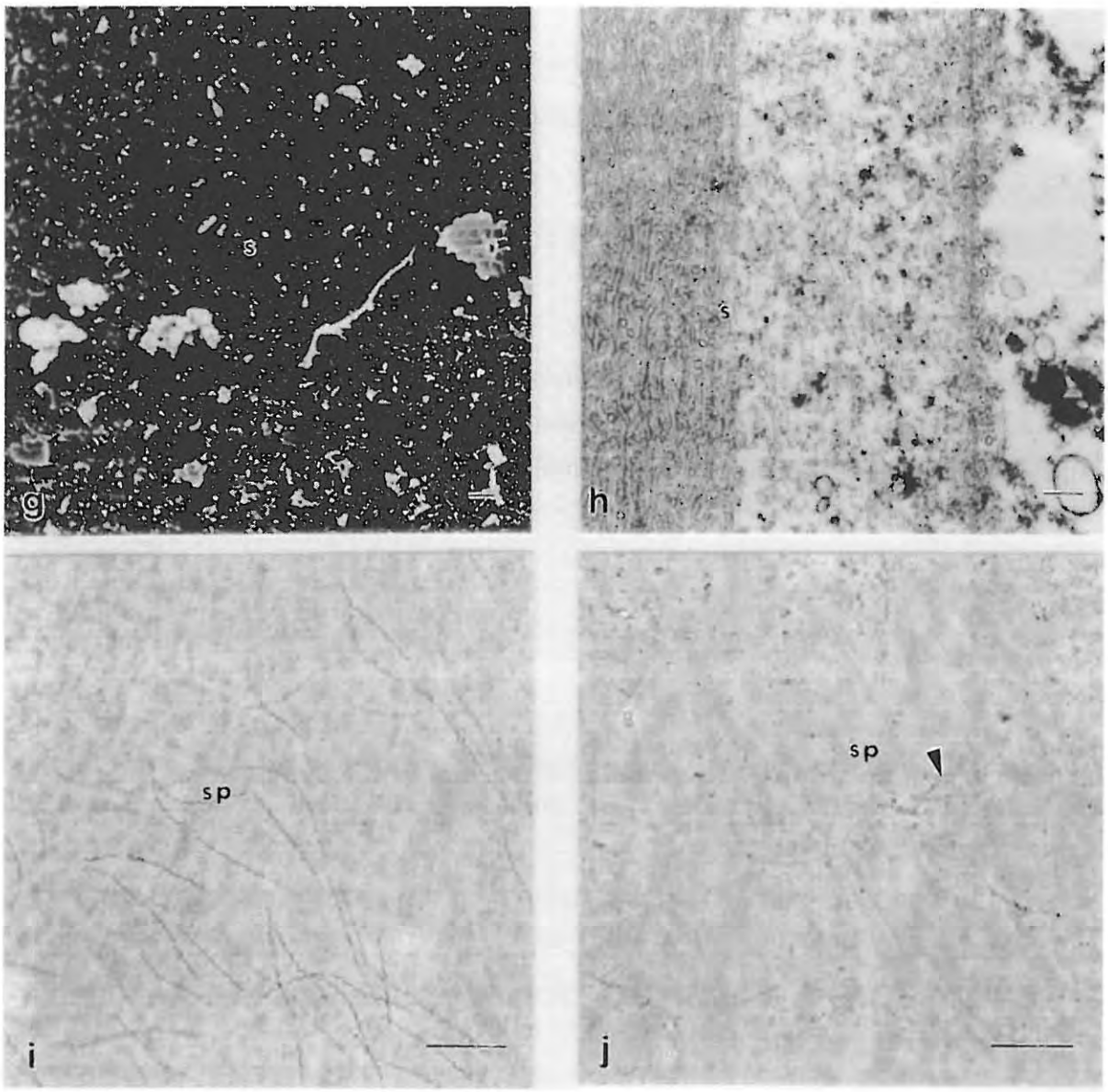
The spirochaete bacteria were absent when examined by SEM and TEM (Figs. 4.2g &h). However, phase contrast microscopy demonstrated that the styles were colonised by two types of spirochaetes: one that was shorter but thicker than the previous species (0.5 x 30 µm, Fig. 4.2i), and another which was morphologically similar to the bacteria found in *Perna perna* (Fig. 4.2c and j). All the individuals examined (n=10)were colonised by the shorter species, while only some (approximately 50%) of the individuals were colonised by both species. These bacteria were present in much lower numbers than in *P.perna.*

Stomach and gut contents:

In both *Perna perna* and *Choromytilus meridionalis,* the gut contents were made up of mixtures of food particles, mucus globules, diatom frustules, bacteria and cilia (Figs. 4.3a-i). Attached to many of the mucus globules and food particles were "flaccid" spheres (0.2 - 2 µm diameter, Fig. 4.3b & d), and three types of bacteria. These were cocci (0.1 - 3µm diameter, Figs. 4.3a, b, e, g & h), filaments (0.25-0.9µm diameter, Fig. 3b & d), that were covered with extracellular debris, and rod-shaped bacteria (approximately 2 x 0.7µm, Fig. 4.3f). The exact lengths of the filaments were impossible to ascertain, as complete filaments were never found.

Gut wall:

The mid- and hindgut walls were lined with cilia and microvilli (Figs. 4.4a-h). Associated with the cilia of the hindguts were mucus globules, spherical bodies (0.2-0.4µm diameter) and fine organic matter (Figs. 4.4e & g). The TEM micrographs clearly show that there were no bacteria closely associated with the gut walls (Figs. 4.4b, d, f & h).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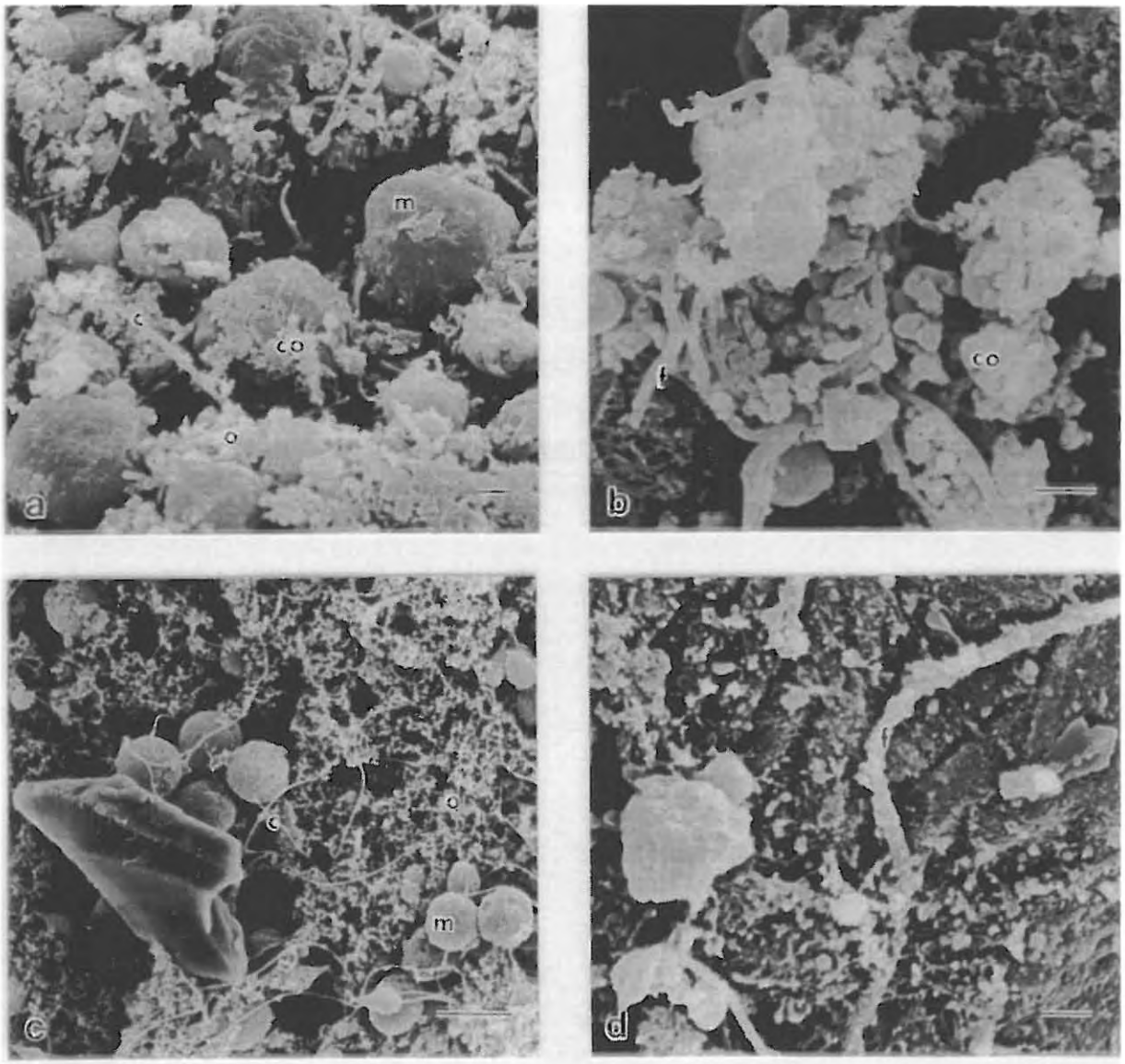
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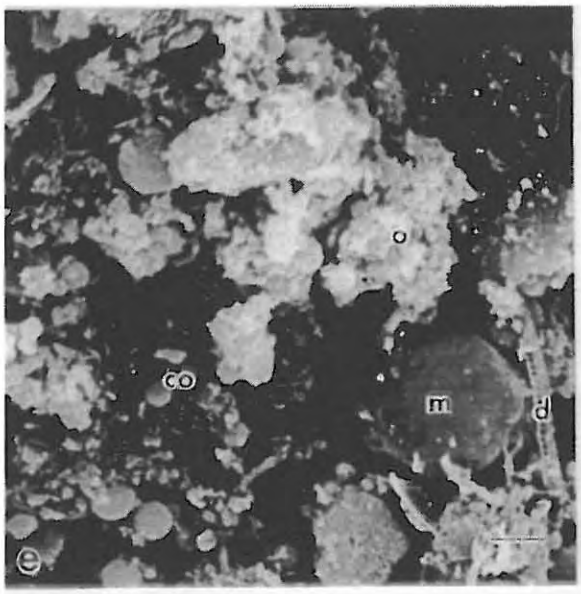
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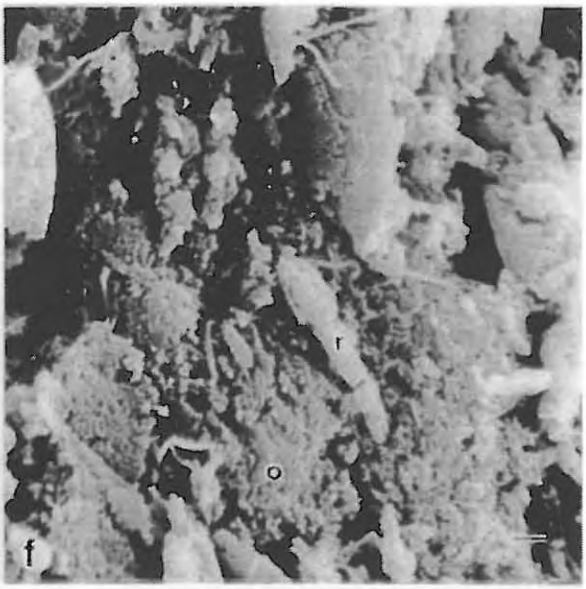
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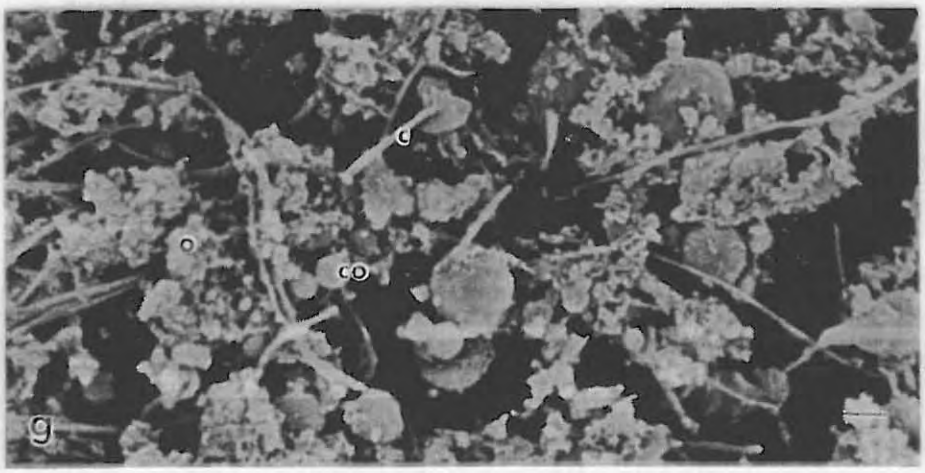
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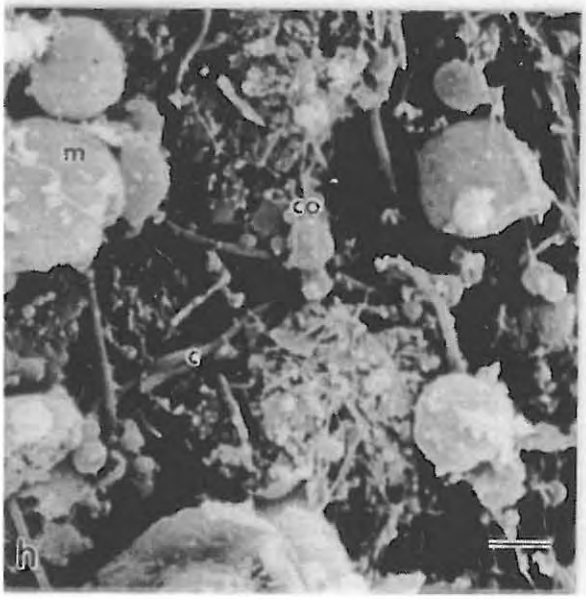
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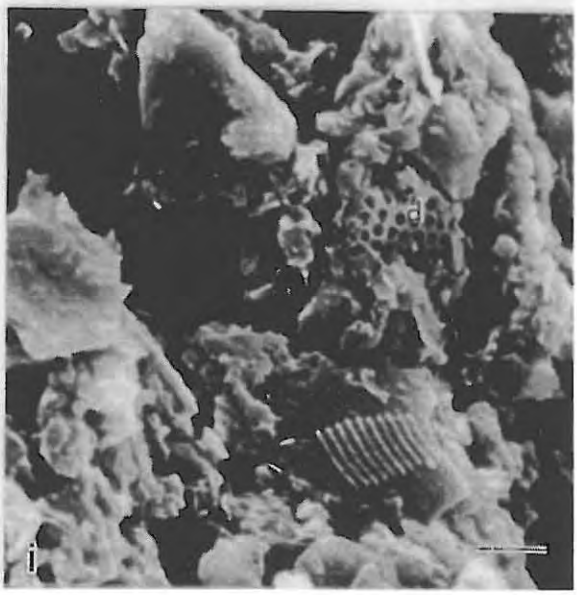
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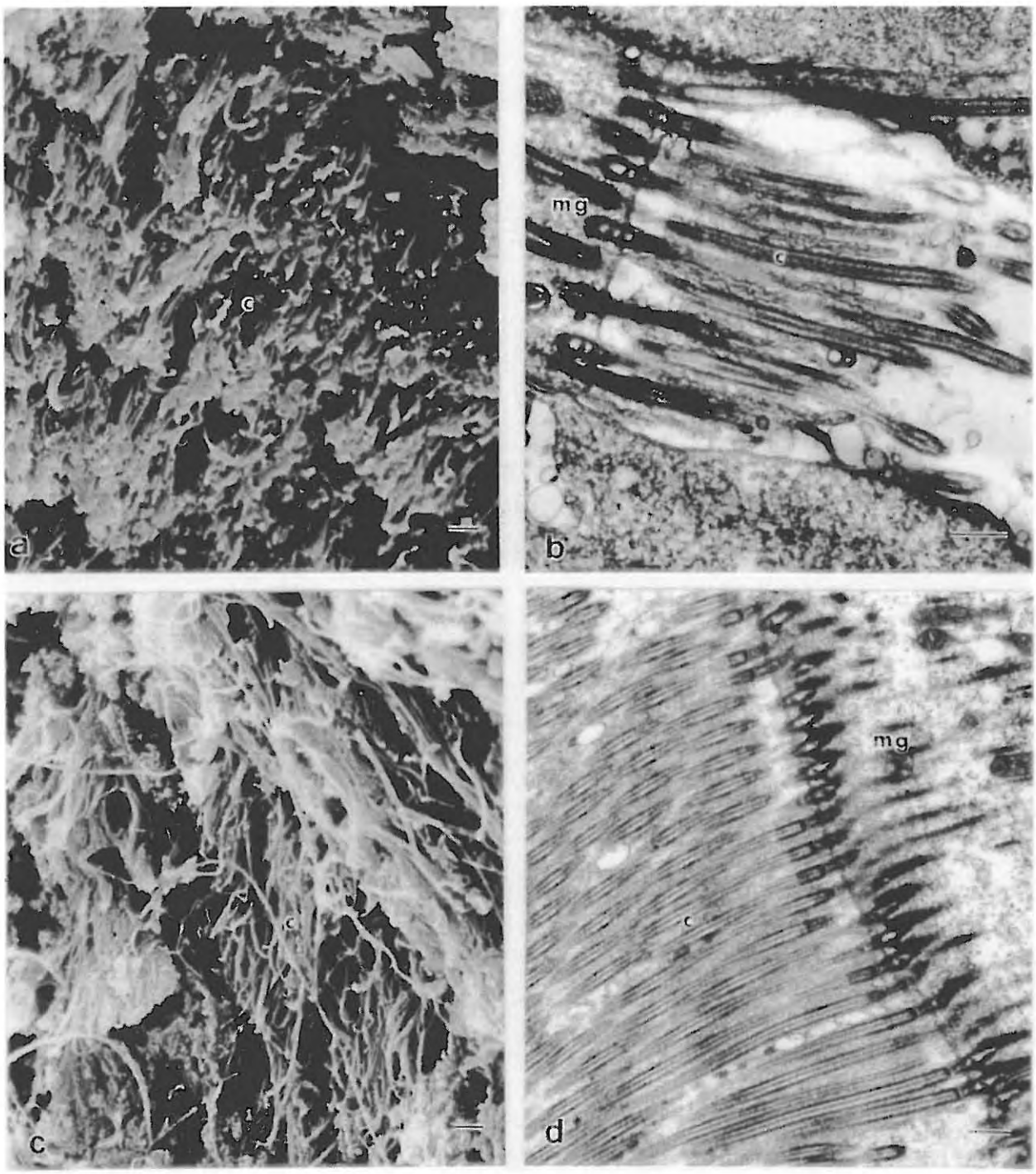
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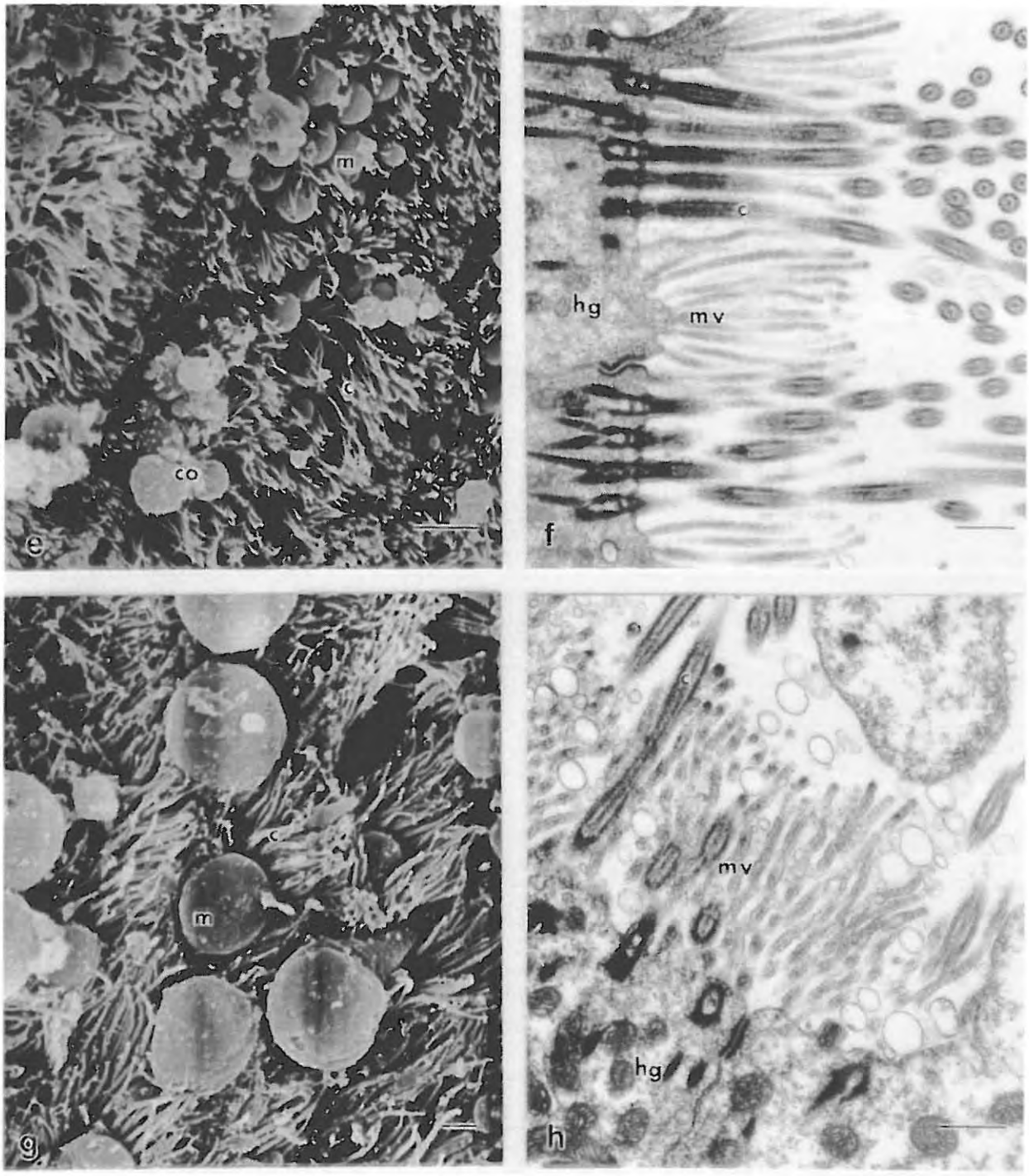
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DISCUSSION

Bacteria are associated with particular areas of the gut, gut contents and external surfaces of many invertebrates (see Tables 4.1 and 4.2). In particular, bacteria have been found in the style, style sac, gland of Deshayes, oesophagus, stomach, intestine, mid- and hindgut and liver of bivalves (Harris, 1993a). The present study concentrated on the gills, mid- and hindgut walls and stomach and gut contents of the mussels. The stomach wall was not examined, due to difficulty experienced during sectioning. Furthermore, the stomach wall is unlikely to house surface-associated bacteria (Plante *et al.*, 1990). The different gut sections, together with associated literature, will be discussed separately.

Gills:

The first point of contact between the animal and its food are the gills, and one would therefore expect bacteria to be found here. Intracellular gill bacteria are commonly found in the families, Solemyidae, Lucinidae, Thyasiridae and Teredinidae (Table 4.1). Solemyids, thyasirids and lucinids inhabit rift and fault vents, reducing sediments and other habitats that have high sulphur concentrations (Southward, 1986; Reid and Brand, 1987; Herry *et al.*, 1989), although thyasirids also inhabit areas that do not have high free sulphur concentrations. Their bacteria are found in bacteriocytes, in their thickened gill filaments (eg. Cavanaugh, 1983; Reid and Brand, 1987; Herry *et al.*, 1989). Solemyids and lucinids usually have large and fleshy gills, which may comprise up to half the animals' wet weight (Herry *et al.*, 1989), and the gut may be reduced (Reid and Brand, 1987; Herry *et al.*, 1989), or completely absent (Felbeck, 1983). These animals are nourished almost entirely by reduced carbon and nitrogen compounds synthesised by chemoautotrophic bacteria (Felbeck, 1983), which may sometimes be supplemented by reduced filter feeding, as in *Solemya velesiana* (Reid and Brand, 1987), and some thyasirids (Southward, 1986).

Teredinids are wood-borers, and house their bacteria in the organ or gland of Deshayes, which is analogous to a salivary gland (Popham & Dickson, 1973). These bacteria aid in the digestion of the wood, (see Morton, 1978 for a review) and they fix nitrogen (Waterbury *et al.*, 1983). The only example of ectocommensal bacteria described to date involves an unidentified limpet found at hydrothermal vents (de Burgh and Singla, 1984), the gill surfaces of which are colonised by large numbers of filamentous bacteria. *Perna perna*

Table 4.1. The occurrence of bacteria in invertebrates.

Phylum	Site of association	Habitat	Diet	Reference
Porifera				
Pericharax heteroraphis	Freeliving, in cyanocytes, in digestive vacuoles	Intertidal to subtidal	Filter feeder	Wilkinson, 1978a, b.
Jaspis stellifera				
Neofibularia irata				
Ircinia wistaria				
Nemetoda				
Eubostrichus cf. parasitiferus	Body surface	Oxic-anoxic inter- faces, Marine sand	Bacteriovore	Polz, *et al*, 1992, in Cavanaugh, 1994
Pogonophora				
Riftia pachyptila	Trophosome	Hydrothermal vent	Bacteriovore	Cavanaugh *et al*., 1981
Unknown vestimentiferan	Trophosome	Fault vent	Bacteriovore	Cavanaugh, 1983
Annelida				
Nerillidae				
Micronerilla brevis	Epithelium, cuticle	Interstitial, subtidal	Probably bacteriovore	Tzetlin & Saphonov, 1995
Throchonerilla mobilis	Epithelium, cuticle	Bottom gravel	Diatoms, bacteria	Tzetlin & Saphonov, 1995
Oligochaeta				
Inanidrilus leukodermatus	Subcuticular space	Coralline sands	Bacteriovore	Giere, 1989, in Cavanaugh, 1994
Olavius planus	Subcuticular space	coralline sands	Bacteriovore	Giere, 1989, in Cavanaugh, 1994

Mollusca				
Gastropoda				
Haliotis midae	Gut wall and contents	Intertidal	Grazer	Erasmus, 1996
Unidentified limpet	Gill surface	Hydrothermal vents	Bacteriovore	de Burgh & Singla, 1984
Helix aspersa	Gut contents	Terrestrial	Omnivore	Charrier, 1990
Patella vulgata	Gut contents?[a]	Intertidal	Grazer	Bush, 1988
Bivalvia				
Crassostrea gigas	Gut contents, shell	Intertidal	Filter feeder	Garland *et al.*, 1982
Solemya vellum	Gill	Reducing sediments	Bacteriovore	Felbek, 1983
S. reidi	Gill	Reducing sediments	Bacteriovore	Reid & Brand, 1987
S. velesiana	Gill	Reducing sediments	Bacteriovore	Reid & Brand, 1987
S. australis	Gill	Reducing sediments	Bacteriovore	Cavanaugh, 1983
S. occidentalis	Gill	Coral reef sediments	Bacteriovore	Krueger *et al.*, 1996
Calyptogena magnifica	Gill	Hydrothermal rift vent	Bacteriovore	Cavanaugh, 1983
C. pacifica	Gill	Fault vent	Bacteriovore	Cavanaugh, 1983
Lucinoma annulata	Gill	Reducing sediments	Bacteriovore	Cavanaugh, 1983
Loripes lucinalis	Gill	Reducing sediments	Bacteriovore	Herry *et al.*, 1989
Bankia australis	Gland of Deshayes	Wood	Wood	Popham & Dickson, 1973
Teredo navalis	Gland of Deshayes	Wood	Wood	Popham & Dickson, 1973
Lyrodus pedicellatus	Gland of Deshayes	Wood	Wood	Popham & Dickson, 1973
L. medilobata	Gland of Deshayes	Wood	Wood	Popham & Dickson, 1973
Thyasira flexuosa	Gill	Shallow water, burrower	Bacteriovore	Southward, 1986
T. sarsi	Gill	Shallow water, burrower	Bacteriovore	Southward, 1986
T. gouldi	Gill	Shallow water, burrower	Bacteriovore	Southward, 1986
T. equalis	Gill	Shallow water, burrower	Bacteriovore/Filter Feeder	Southward, 1986
Thyasira sp T1	Gill	Deep sea, burrower	Bacteriovore	Southward, 1986
Thyasira sp T2	Gill	Deep sea, burrower	Baceriovore	Southward, 1986
Mytilus edulis	Style[b]	Intertidal	Filter feeder	Seiderer *et al.*, 1987
Bathymodiolus thermophilus	Gill	Hydrothermal vent	Bacteriovore	Le Pennec *et al.*, 1985, in Southward, 1986
Unnamed Mytillid	Gill	Hydrothermal vent	Bacteriovore	Cavanaugh, 1983
Cephalopoda				
Loligo peali (♀)	Accessory gland	Pelagic	Carnivore	Bloodgood, 1977
Euprymna scolopes	Accessory gland	Pelagic	Carnivore	Boettcher, *et al.*, 1996

Arthropoda				
Crustacea				
Isopoda				
Limnoria tripunctata	Exoskeleton	Wood	Wood	Boyle & Mitchell, 1978; 1981
	Gut lining	Creosote-treated wood	Wood	Zachary & Colwell, 1979
L. lignorium	Exoskeleton	Wood	Wood	Boyle & Mitchell, 1978; 1981
Oniscus asellus	Exoskeleton	Wood	Wood	Boyle & Mitchell, 1978; 1981
Gnathia calva	Rectal vesicle	Fish parasite	Blood	Juilfs & Wägele, 1987
Amphipoda				
Chelura terebrans	Exoskeleton	Wood	Wood	Boyle & Mitchell, 1978, 1981
Boeckosimus affinis	Gut contents	Scavenger	Omnivore	Atlas *et al*, 1981
Decapoda				
Upogebia pugettensis	Hindgut lining	Coastal mudflat	Detritivore	Harris, 1993b
U. africana	Hindgut lining	Saltmarsh mudflat	Detritivore	Harris *et al*., 1991
Callianassa australiensis	Hindgut lining	Mangrove sandflat	Detritivore	Harris, 1993b
C. californiensis	Hindgut lining	Coastal sandflat	Detritivore	Harris, 1993b
C. Kraussi	Hindgut lining	Salfmarsh sandflat	Detritivore	Harris *et al*., 1991
Sesarma catenta	Hindgut lining	Saltmarsh, estuary	Detritivore/Scavenger	Harris, 1993b
S. cinereum	Hindgut lining	Saltmarsh, coastal	Detritivore/Carnivore	Harris, 1993b
S. mesa	Hindgut lining	Mangrove forest	Detritivore	Harris, 1993b
Uca pugnax	Hindgut lining	Saltmarsh, coastal	Detritivore	Harris, 1993b
U. minax	Hindgut lining	Saltmarsh, coastal	Detritivore	Harris, 1993b
U. pugilator	Hindgut lining	Saltmarsh, coastal	Detritivore	Harris, 1993b
Myctiris longicarpus	Hindgut lining	Mangrove sandflat	Detritovre	Harris, 1993b
Panopeus herbsii	Hindgut lining	Saltmarsh, coastal	Detritivore	Harris, 1993b
Callinectes sadipus	Hindgut lining	Saltmarsh, coastal	Predator/Scavenger	Harris, 1993b
Ovalipes punctatus	Hindgut lining	Sandy subtidal, coastal	Carnivore	Harris, 1993b
Scylla serrata	Hindgut lining	Muddy subtidal, estuary	Carnivore	Harris, 1993b
Insecta				
Reticulitermes flavipes	Hindgut lining	Terrestrial	Wood	Breznak & Pankratz, 1977
Captotermes formosanus	Hindgut lining	Terrestrial	Wood	Breznak & Pankratz, 1977
Eublaberus posticus	Hindgut lining	Terrestrial	Omnivore	Cruden & Markovetz, 1981
Diplopoda				
Spirostreptid millipede	Hindgut lining	Terrestrial	Omnivore	Green & Baker, 1995
Echinodermata				
Holothuroidia				
Psychropotes sp	Gut lining	Abyssal	Detritivore	Deming & Colwell, 1982
Echinoidia				
Echinocardium cordatum	Gut contents	Intertidal	Detritivore	De Ridder *et al*., 1985
Parachinus angulosus	Gut contents	Intertidal	Grazer	Sweijd, 1990

[a]: The author was not looking for bacteria, but micrographs of the hindgut indicate that the epithelium was not colonised by bacteria.
[b]: Non-spirochaete bacterium.

and *Choromytilus meridionalis* have typically mytilid digestive tracts, with thin pale gills, and gain their nutrition entirely through filter feeding. They are therefore not likely to house endosymbiotic bacteria within their gill tissue. Their gills were therefore only examined for superficially attached bacteria.

Low numbers of coccoid and rod-shaped bacteria were associated with the gill surfaces of both species (Figs. 4.1a-d). These low numbers are corroborated by Garland *et al.* (1982), who found no bacteria associated with the gills of the oyster, *Crassostrea gigas*, when examined by SEM. In addition, Kueh and Chan (1985) found that the gills of the oyster, *C. gigas*, the mussel, *Perna viridis*, and the clam, *Anadara cornea*, all had very low numbers of bacteria associated with them, and that the bacterial densities were close to those of the surrounding water.

Gut contents:

Conflicting data exist concerning the changes in the density of bacteria along the alimentary canal. There is usually a sharp decrease in bacterial numbers in the foregut or stomach as the bacteria are subjected to selective digestion (eg. Prieur, 1981; Deming and Colwell, 1982). In the hindgut and rectum there may be an increase in the surviving bacteria (Prieur, 1981; 1987; Charrier, 1990; Prieur *et al.*, 1990; Watkins and Simkiss, 1990; Ward-Rainey *et al.*, 1996) or a steady decrease as the bacteria are presumably digested and assimilated (Hyllerberg and Gallucci, 1975; Baker and Bradnam, 1976; Deming and Colwell, 1982).

The gut contents of *Perna perna* and *Choromytilus meridionalis* consist of mixtures of mucus globules, detached cilia, diatom frustules, bacteria and fine organic matter (Figs. 4.3a-i). Neither of these species showed a remarkable increase or decrease in bacterial numbers along the gut. Bacteriolytic activity was detected in *P.perna* and *C.meridionalis* (refer to Chapter 3). This could explain why most of the bacteria observed seemed to be partially digested (Figs. 4.3d & f). This could also account for the low numbers of bacteria observed. An alternative reason for the low numbers of bacteria is that the SEM technique employed did not preserve the bacteria adequately, even though this technique had previously been used successfully (Sweijd, 1990).

Gut Wall:

Plante *et al.* (1990) proposed that for detrivorous invertebrates, the costs of bacterial associations in the fore- and midguts outweigh the benefits, while there is little obvious cost to the detritivore in allowing bacterial attachment to the hindgut lining. For these reasons they predicted that fore- and midgut walls are seldom utilized by potential symbionts, while microbes will attach to the hindgut lining.

The mid- and hindgut walls of both *Perna perna* and *Choromytilus meridionalis* were heavily lined with cilia and microvilli, through which mucus globules protruded (Figs. 4.4a-h). Attached to the mucus in the hindgut of *P. perna* were spherical organisms with fuzzy surfaces (Fig. 4.4e). These could be bacteria, though the larger of these bodies (5µm diameter) were larger than the documented size of most marine bacteria (Prieur, 1981; Austin, 1988). No bacteria were associated with the surface of the epithelium, cilia or within the epithelial cells (Figs. 4.4b, d, f & h) of either species. It therefore appears that any association between the cilia and bacteria is incidental.

As can be seen from Table 4.1, the absence of surface-associated bacteria has been shown in other animals, such as the oyster, *Crassostrea gigas* (Garland *et al.*, 1982), the limpet, *Patella vulgata* (Bush, 1988), the snail, *Helix aspersa* (Charrier, 1990), the sea urchin, *Parechinus angulosus* (Sweijd, 1990) and the amphipod, *Boeckosimus affinis* (Atlas *et al.*, 1982). Garland *et al.* (1982) proposed that the rapid pumping rate of water through the gut due to ciliary movement, does not allow sufficient time to allow for significant bacterial attachment and growth. This is supported by the fact that *C.gigas*, *P.vulgata*, *H.aspersa* and *P.angulosus* (Garland *et al.*, 1982; Bush, 1988; Charrier, 1990; Sweijd, 1990, respectively), all have highly ciliated gut epithelia, while bacteria have been found closely associated with the gut walls of the abalone, *Haliotis midae* (Erasmus, 1996), the walls of which appear to lack cilia (J. Erasmus, University of Cape Town, pers. comm.).

Some bivalves have been demonstrated to house intracellular bacteria that belong to the same genera that cause diseases in humans and domestic animals (Table 4.2). These bacteria have been found in gill and gut tissue, and as yet their effect on their hosts is unknown. A high load of these bacteria in the gill tissue may have an adverse effect on gill function, which may impair energy intake and waste removal (Fries and Grant, 1991). These bacteria have not resulted in mass mortality of clams in their natural setting (Fries and Grant, 1991), and there are even signs that the host may utilise the bacteria as a nutrient source

Table 4.2 Bivalves housing intracellular pathogenic bacteria.

Species	Tissue	Bacterial type	Reference
Crassostrea virginica	Gut goblet cells	Mycoplasmas	Harshbarger *et al.*, 1977
Loripes lucinalis	Digestive gland, gill, oesophagus	Chlamydia-like	Johnson & Le Pennec, 1995
Mercenaria mercenaria	Gills	Rickettsiae	Fries & Grant, 1991
	Gills	Erhlichia-like	Fries & Grant, 1992
	Digestive tubules	Chlamydia	Harshbarger *et al.*, 1977
Mya arenaria	Gills	Rickettsiae	Fries *et al.*, 1991
	Digestive tubules	Rickettsiae	Harshbarger *et al.*, 1977

(Johnson and Le Pennec, 1995).

Style:

Spirochaetes were first observed in bivalves as early as 1882, though the discoverer thought that they were Trypanosomae (Certes, 1882, in Lawry *et al.*, 1981). To date, no one has succeeded in growing these bacteria in pure culture (eg. Tall and Nauman, 1981; Breznak, 1984; Brock and Madigan, 1991). The genus name, *Cristispira*, has therefore been assigned to these bacteria by virtue of their site of colonisation and gross morphology, rather than biochemical and physiological requirements.

The occurrence of *Cristispira* infection is widely distributed among marine and freshwater molluscs (see Breznak, 1984, for a review). Not all bivalve species harbour the bacterium (Bernard, 1970; Breznak, 1984), and the levels of colonisation can differ between species, and among individuals (Mayasich and Smucker, 1987). Furthermore, a comprehensive study of Canadian bivalves showed no clear patterns of occurrence of the bacterium, except that intertidal species are more readily infected than subtidal species (Bernard, 1970).

In *Perna perna* the spirochaetes were seen in their spiral form, in clear zones, in the outer matrix of the style (Fig. 4.2d). The clear zones present around the bacteria are produced by enzymatic action, which forms channels through which the bacteria move (Lawry, 1987).

Towards the centre of the style, circular structures, found within spindle-shaped areas, were more common (Figs. 4.2e & f). These spindle-shaped areas were arranged parallel to each other, and perpendicular to the longitudinal axis of the style.

Although it has not been confirmed that these circular structures are "polymorphs" of *Cristispira*, the following description by Breznak (1984) strongly suggests that they are: "A bundle of 100 or more periplasmic flagella.....is intertwined with the protoplasmic cylinder and may distend the outer sheath to form.....the so-called "cristae" on the protoplasmic cylinder.....When removed from its habitat, degenerative changes readily occur and accompany the loss of motility. Cristae may become markedly distended, multiple swellings may appear on the cell body, and cells may lyse or form spherical bodies."

The absence of spirochaetes in the electron microscopic study, and their detection using phase contrast microscopy when examining the styles of *Choromytilus meridionalis* is puzzling. The most likely explanation is that the spirochaetes in *C.meridionalis* are

superficially attached to the styles (cf. Bernard, 1970), and not embedded in the matrix, as in *Perna perna*. Washing the styles would have removed most of the bacteria, and fixing might have caused the remaining bacteria to rupture. This would also explain why so few bacteria were seen on the surface of *P.perna* styles, when prepared for SEM. In general, spirochaete numbers were lower in *C.meridionalis* than in *P.perna*, and in both species, numbers varied between individuals.

The two *Cristispira* species measured 0.25µm by 160µm and 0.5µm by 30µm. The diameter of the first species is half the documented size, while the second species is much shorter (Breznak, 1984; Brock and Madigan, 1991), suggesting that these are previously undescribed species.

Bernard (1970) demonstrated that, within species, infection may change according to locality. Additional styles from *Perna perna* and *Choromytilus meridionalis* collected from Rufane's River and Old Woman's River, were therefore examined for infection, to determine whether the infection was locality specific. Colonisation levels in *P.perna* collected from Rufane's River and Port Alfred, which are 4km apart, were consistently high, while levels in animals collected from Old Woman's River, which is 27km north, were much lower. In *C.meridionalis*, infection levels in animals collected at Old Woman's River were much lower than in those collected at Rufane's River. This indicates that, in both *P.perna* and *C.meridionalis*, infection levels depend on locality.

From the data condensed in Tables 4.1 and 4.2, as well as the results obtained in this study, one can attempt to draw some conclusions about the presence of obligate symbiotic bacteria in invertebrates. There do not seem to be any definite rules governing the occurrence of endosymbiotic bacteria in arthropods. Wood-eating insects house endosymbiotic bacteria (Breznak and Pankratz, 1977). While bacteria are usually absent from wood-eating crustaceans (Boyle and Mitchell, 1978), bacteria are present in isopods inhabiting creosote-treated wood (Zachary and Colwell, 1979). Aquatic detritivores, however, seem to be more likely to house extensive bacterial populations than members of other feeding groups (Harris, 1993b). Also, terrestrial animals tend to have specialised structures in which to house bacteria (Breznak and Pankratz, 1977; Cruden and Markovetz, 1981; Green and Baker, 1995), while these structures are absent in aquatic crustaceans (Harris, 1993b). An exception is the fish parasite, the isopod, *Gnathia calva*, which houses its bacteria in a rectal vesicle (Juilfs

and Wägele, 1987).

Molluscs seem to be less prone to the development of intimate associations with bacteria. Nevertheless, two types of associations are commonly found among bivalves: the non-obligatory spirochaete-style association, and the bacteria found in the gills of certain species. Even though molluscs do not have specialised organs in which to house endosymbiotic bacteria (Morton, 1978), gills housing bacteria are usually greatly enlarged (eg. Cavanaugh, 1983; Reid and Brand, 1987; Herry *et al.*, 1989). The evidence suggests that, with the exception of the possible pathogens, only molluscs in highly specialised habitats, or with highly specialised diets, have bacteria closely associated with the epithelial tissue (Tables 4.1 and 4.2).

We must be cautious, as there are few data for molluscs that do not have specialised needs. However, bacteria in the water column digest plant polymers, releasing compounds that detritivores (and filter feeders) can digest, thus reducing the necessity for endosymbiotic fermentative bacteria (Moriarty, 1990). In addition, the difference between the internal and external environments in marine environments is much smaller than in terrestrial or freshwater environments. This similarity means that the opportunity for facultative or relatively weak associations between microbes and detritivores (and filter feeders) is greater in marine than in terrestrial or freshwater environments (Plante *et al.*, 1990).

Finally, it can be concluded that both *Perna perna* and *Choromytilus meridionalis* have a facultative symbiotic relationship with spirochaetes which are closely associated with the animals' styles. No bacteria were found to be intimately associated with the gill filaments or the gut lining, using the present methods. All other bacteria present in the alimentary canal are associated with the gut contents, and have been ingested incidentally with the food.

CHAPTER 5

DISCUSSION

Filter feeders are exposed to a wide range of potential food particles, and consequently, carbohydrates, within the water column. The concentrations of these particles vary seasonally (Schleyer, 1981; Cliff, 1982), and the animals have to adapt to a varied and sometimes, unpredictable, food source (MacDonald and Ward, 1994).

The present study attempted to determine whether two intertidal mussel species are able to digest the wide range of carbohydrates available within the water column, and whether these mussels have different enzyme suites which would allow them to utilise different components of the seston. The enteric bacteria of these mussels were also tested for enzyme activity, to determine whether or not the bacteria have the potential to perform a symbiotic function by supplementing the mussels' digestive enzymes. The mussels were also tested for enzymatic activity on bacteria, which should give an indication of whether these mussels are able to exploit bacteria as an additional food source. Finally, the gut tissue of the animals were examined microscopically, to determine if the bacteria were associated with the gut contents or the gut tissue. This would further elucidate the nature of any relationship there may be between the bacteria and the host: bacteria associated with the gut contents are more likely to be transient or incidental, while bacteria attached to the gut tissue would be resident, and the relationship obligatory.

Digestion of Carbohydrates:

Researchers have attempted to determine the exact nature of the relationship between invertebrates and their associated gut bacteria. Many have found that enzymes produced by the bacteria do not contribute significantly to the overall digestion by the host (Galli and Giese, 1959; Horiuchi and Lane, 1966; Payne *et al.*, 1972; Newell and Langdon, 1986; Harris *et al.*, 1991), while others have found that bacteria either increase enzyme activity on the substrates (Crosby and Reid, 1971; Harris *et al.*, 1986b; Dempsey and Kitting, 1987; Musgrove, 1988; Muthu, 1990), or produce enzymes which the hosts do not, thereby increasing the digestive range of the host (Huang and Giese, 1958; Wainwright and Mann, 1982; Sweijd, 1990; Erasmus, 1996). Other studies have tested for enzyme activity by the bacteria alone, without comparing them with the endogenous activity of the animal (Prim and Lawrence, 1975; Vitalis *et al.*, 1988), although the latter study did show that antibiotic treatment reduced the growth rate of the sea hare, *Aplysia juliana*.

Perna perna and *Choromytilus meridionalis* both digested the storage carbohydrates

amylose, glycogen and laminarin, and the structural carbohydrate carboxymethyl cellulose (CMC). These animals showed no activity on the storage carbohydrate, inulin, or the structural carbohydrates, carrageenin, alginic acid, mannan, fucoidan or xylan. This supports the theory that filter feeders acquire limited carbon through the direct utilisation of refractory carbon (Brock, 1989; Kreeger *et al.*, 1990; Langdon and Newell, 1990). Instead, filter feeders assimilate refractory carbon indirectly, through the microbial loop. This can occur through three routes (Fig. 5.1):

1) Bacteria associated with the detritus are ingested by protozoans and microzooplankton, which are then, in turn, ingested by filter feeders;
2) The filter feeders assimilate the products of bacterial digestion; and
3) After processing the detritus, the bacteria are digested and assimilated directly by the filter feeders.

Only the second and third routes were investigated in this study.

In addition to the carbohydrates digested by the mussels, bacteria from the guts of these animals also digested carrageenin, fucoidan, mannan and xylan. The bacteria therefore have the potential to supplement the mussels' enzymes, allowing the mussels to exploit detrital particles arising from all macrophytes. The enzyme activity on the structural components was low (relative to activity on amylose, glycogen and laminarin). This suggests that the enzymes have a qualitative rather than a quantitative role, hydrolysing the cell walls to expose the digestible contents, rather than using structural carbohydrates as an energy source themselves. Furthermore, activity on all the above-mentioned substrates was not always present. This indicates that the bacteria digesting these substrates were not always present, and that enzymes produced by the bacteria depend on the nature of the ingesta (Moriarty, 1990; Seeto *et al.*, 1996). It is therefore obvious that, even though there is a potential for a symbiotic relationship between the bacteria and the mussels, this relationship is incidental. This supports Moriarty (1990), who stated that aquatic detritivores (or filter feeders) do not need an obligatory association with bacteria which digest the indigestible components of their diets, as this activity occurs within the water column. Even though this study demonstrated that mussels do maintain a bacterial population during starvation, there was little evidence to support the theory that the importance of symbiotic bacteria increases during periods of food paucity, when the gut passage time increases (Prim and Lawrence, 1975; Moriarty, 1990).

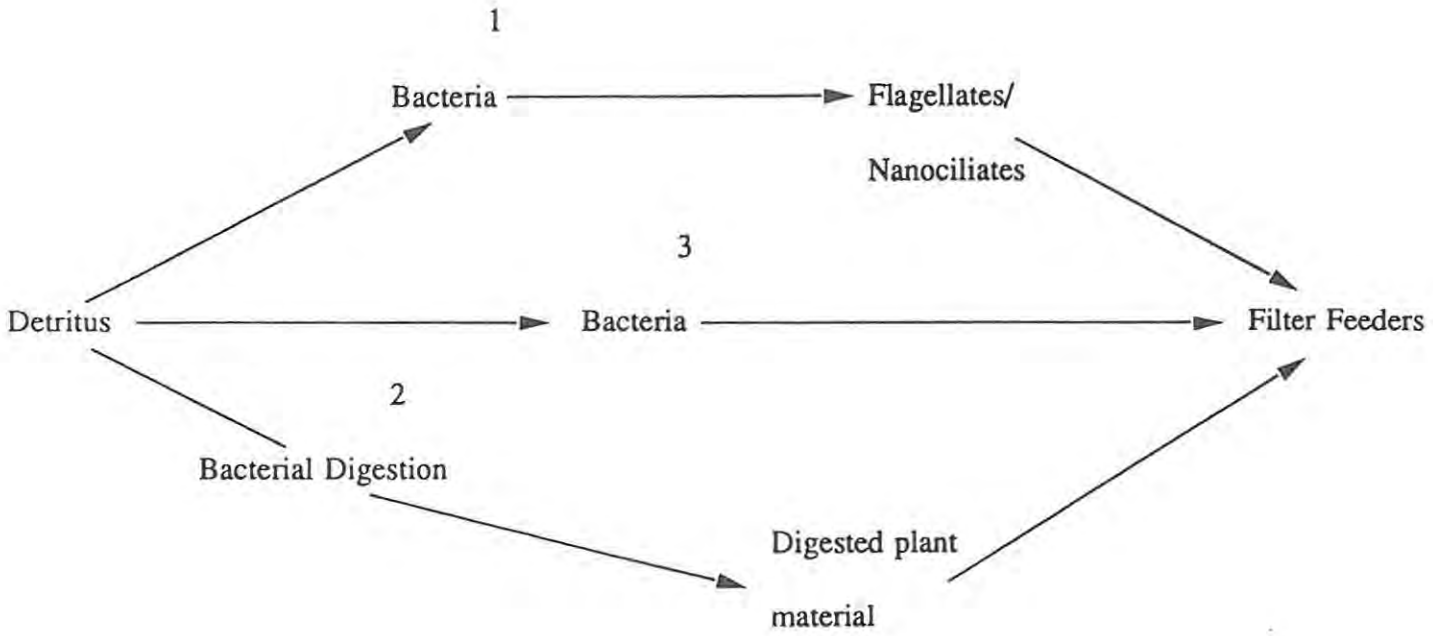

Fig. 5.1. A generalised illustration of the bacterial loop (adapted from McLachlan and Romer, 1990)

Digestion of Bacteria:

Bivalves have been demonstrated to supplement their diets by filtering and assimilating bacteria (eg. Lopez and Cheng, 1983; Harvey and Luoma, 1984; Amouroux, 1986). *Choromytilus meridionalis*, on the west coast of South Africa, produces a bacteriolytic enzyme and increases its assimilation efficiency of bacteria, during upwelling conditions (Muir *et al.*, 1986). This is in response to a decrease in phytoplankton and detritus concentrations and an increase in the relative importance of bacteria. Upwelling does occur on the east coast of South Africa, but is not as pronounced or predictable as on the west (Beckley, 1983). In addition, primary production decreases from the west to east, even though it is disproportionately high at Port Alfred (Brown, 1992; Bustamante *et al.*, 1995). The presence of bacteriolytic activity in *Perna perna* and *C.meridionalis* in the present study, during late winter/early spring, suggests that the relative importance of bacteria is higher on the east than west coast, due to the lower primary production.

Relationship between enzyme activity and diet:

These experiments have illustrated that *Perna perna* and *Choromytilus meridionalis*, together with their enteric bacteria, are able to digest all components of the detrital complex.

The relationship between enzyme activity and diet is demonstrated by the relative importance of amylase, cellulase and laminarinase to two gastropods. Amylase and cellulase activity was relatively more important than laminarinase activity in *Strombus gigas*, which feeds on macrophytes (Horiuchi and Lane, 1966). The reverse was found in *Telescopium telescopium*, which feeds mainly on diatoms (Alexander *et al.*, 1979). Furthermore, Erasmus (1996) found that the abalone, *Haliotis midae*, exhibited higher activity on carrageenin than alginic acid when fed on red algae, while the opposite was true for *H.midae* fed on brown algae.

Perna perna digested laminarin and glycogen more efficiently than it did amylose and CMC. This implies that *P.perna* relies more on phyto- and microzooplankton (and perhaps even bacteria; see Table 1.1) than detritus, as a food source. In addition, its enzymes were also highly active on bacteria, giving it the potential to utilise bacteria as an additional food source. *Choromytilus meridionalis*, however, digested amylose, glycogen and laminarin equally efficiently, suggesting that it relies equally on detritus and plankton (see also Seiderer *et al.*, 1984). Enzymes from *C.meridionalis* were also active on bacteria, but less so than

P.perna, suggesting that bacteria are more important to *P.perna* than to *C.meridionalis*.

These differences in the relative importance of enzymes suggest that there is some degree of resource partitioning of the seston. For resource partitioning to occur, filter feeders must be able to select certain particles from the seston preferentially over others. Stuart and Klumpp (1984) showed that filter feeding sponges, bivalves and ascidians exhibit resource partitioning by filtering different sized food particles, reducing the potential for interspecific competition for food. They also showed that bivalves all filtered the same sized particles with equal efficiency, indicating that within classes, there was some potential for competition. However, Shumway *et al.* (1985) showed that different bivalves selected different phytoplankton particles from a mixture of similar size phytoplankton particles, suggesting that there is the potential for resource partitioning of similar sized particles.

The differences may also be a reflection of the habitats that the mussels occupy: *Perna perna* lives on open rocks, while *Choromytilus meridionalis* is found in areas subject to sand inundation. The animals are therefore exposed to different turbulence regimes and different food particles in suspension.

One important factor which should be borne in mind when making these assumptions is that animals have the potential to increase their enzyme activity in response to the nature of their food (cf. Stuart *et al.*, 1985; Harris *et al.*, 1986a; Erasmus, 1996). For this reason, it is surprising that in the present study, neither *P.perna* nor *C.meridionalis* was able to digest carrageenin, the major structural component of red algae (the most abundant class of algae on the eastern coast).

The differences in endogenous activity were not reflected in the enzyme activity of the mussels' enteric bacteria. This could be due to seasonal differences in the seston composition.

Differences in Enzyme Activity:

The enzyme extracts from *Perna perna* consistently had a low specific activity, but a high protein concentration, which allowed them to release high concentrations of reducing sugars from a given concentration of substrate. The opposite relationship was found in *Choromytilus meridionalis*. In contrast, the enteric bacteria from *C.meridionalis* were present in higher numbers and were enzymatically more active than the bacteria from *P.perna*. This implies that *C.meridionalis* may actually be relying more on the enzymatic activity of its

enteric bacteria than *P.perna*. Alternatively, competition between *C.meridionalis* and its enteric bacteria may be greater than in *P.perna*, which affects the animal's overall activity.

There are several problems associated with measuring and comparing the enzyme activity of these animals. Style size, protein content and enzyme activity have tidal cycles (Langton and Gabbot, 1974; Langton, 1977) and this was probably the cause of the great variability in enzyme activity evident in the present study. It would have been preferable to complete all the replicates of each assay using enzymes from animals collected at the same tides. This would have reduced the variability due to differences in tidal and environmental conditions. There would, however, still be high variability, as there appears to be a naturally high degree of individual variability in enzyme activity (Langton and Gabbot, 1974; Langton, 1977; McHenery *et al.*, 1983).

In situ distribution of Bacteria:

The microscopic examination of the gut tissue of the mussels showed that the only tissue with which bacteria were closely associated, was the crystalline style. This was the first time that spirochaetes (*Cristispira*) were detected in these mussels, even though the infection of bivalve styles with spirochaetes has been well documented (Breznak, 1984). Infection with *Cristispira* differed according to species, locality and position on the shore. *Perna perna* was infected with high numbers of morphologically identical bacteria at Port Alfred and Rufane's River, while infection rates were much lower at Old Woman's River. *Choromytilus meridionalis* was infected with two morphologically different bacteria, but infection levels were much lower than in *P.perna*.

The intimate association of these bacteria with the style suggests that the bacteria are resident, and the relationship obligatory. The literature, however, suggests the opposite - in populations known to be infected by *Cristispira*, not all individuals house bacteria (Bernard, 1970; Mayasich and Smucker, 1987), and infected individuals do not seem to be visibly advantaged (or disadvantaged) by the relationship (Bernard, 1970; Breznak, 1984, Brock and Madigan, 1991).

The association between the bacteria and the mussels poses an additional problem when assaying the styles for enzyme activity. When the styles were originally assayed for activity, no attempt was made to sterilize the styles. This means that the activity detected is likely to be due to a combination of mussel and spirochaete enzymes.

Attempts were then made to remove the spirochaetes, and compare the enzyme activity of infected and uninfected styles. Mussels were allowed to filter water to which tetracycline, at 0.5g/l, was added. Tetracycline was chosen as it is effective against pathogenic spirochaetes (Brock and Madigan, 1991). After 6 days, the mussels showed signs of accumulating the antibiotic, without an accompanying reduction in bacterial numbers. *Cristispira* are also resistant to chloramphenicol (Mayasich and Smucker, 1987).

Spirochaetes dissociate from the style surface, and diffuse into the surrounding water when the styles are allowed to soak in sterile sea water. Unfortunately, the styles also dissolve when left in solution, releasing the enzymes into solution (Mayasich and Smucker, 1987). Allowing the style to dissolve completely and then filtering the solution was, however, not the answer, as spirochaetes disintegrate when they die (Berkeley, 1962), and will therefore also release their enzymes into solution.

Chemicals known to be toxic to spirochaetes are glucosone and plankton extracts (Berkeley, 1962). Glucosone is produced when certain food material is oxidised, so the spirochaetes are exposed to it, and plankton extracts, on a regular basis in the stomach of its host. The bacteria respond to this by moving away from the food tassel end of the style (Berkeley, 1962; Tall and Nauman, 1981). Using these bacteria-free tips in assays would not be feasible, as these sections are only a few millimetres long, and too many styles would have to be used to obtain a usable volume of enzymes.

Very few studies have attempted to quantify the role that *Cristispira* might play in digestion, possibly for the above reasons. For this reason, the exact nature of the relationship between the bivalves and the spirochaetes is still unclear. Lawry (1987) suggested that the relationship is most likely to be mutualistic, with the host concentrating food, and the spirochaete aiding with the digestion of the hosts' food. Morton (1978) proposed that the spirochaetes provide their hosts with cellulase enzymes. Implicit support for this theory is the fact that spirochaetes are found in the digestive tracts of ruminants and wood-eating insects (Johnson, 1977). Mayasich and Smucker (1987) found that the spirochaetes did not contribute to chitinase activity in the oyster, *Crassostrea virginica*. Even though they found a positive correlation between spirochaete numbers and chitobiase activity, they concluded that the contribution made by the bacteria was not significant. It therefore appears that, enzymatically, the host gains little from the association. Lawry (1987) has, however, also suggested that degrading spirochaetes may serve as a nutrient source to the host.

Unfortunately, the present study was unable to shed any light on the nature of the relationship between the spirochaetes and the mussels. The enzyme activity detected in the styles is present in most marine invertebrates (Huang and Giese, 1958; Sova *et al.*, 1970; Elyakova, 1972; Kristensen, 1972a; Gianfreda *et al.*, 1979; Elyakova *et al.*, 1981). More specifically, they have been detected in *Perna perna* and *Choromytilus meridionalis* (Seiderer *et al.*, 1982). It is therefore unlikely that the spirochaetes increase the range of substrates that the mussels can digest (unless they produce an enzyme not tested for). If they do contribute to enzyme activity, they probably increase the endogenous enzyme activity.

All other bacteria found in the alimentary canal were associated with the gut contents. Surprisingly few bacteria were found, especially in the hindgut, where bacteria were expected to proliferate (Prieur, 1981; 1987). The presence of bacteriolytic activity in both *Perna perna* and *Choromytilus meridionalis* would explain both the low numbers and the partially digested appearance of the bacteria found. The association of the bacteria with the gut contents also supports the conclusion that the bacteria are ingested incidentally with the food, and that the relationship between the mussel and the bacteria is facultative.

Conclusion:

It is clear that both *Perna perna* and *Choromytilus meridionalis* have a fortuitous symbiotic relationship with bacteria ingested incidentally with their food. The association of these bacteria with the gut contents but not the wall confirms that the relationship is transient and incidental (Harris, 1993a). The combination of mussels and bacterial enzymes allows the mussels to exploit most of the storage and structural carbohydrates found in macroalgae, phyto- and microzooplankton. This allows the mussels to utilise all the components of the particulate matter in the seston. Style enzymes of *C.meridionalis* released less glucose from a given concentration of carbohydrate, while its enteric bacteria released more glucose than *P.perna*. This suggests that *C.meridionalis* may rely more on digestive activity by enteric bacteria than *P.perna*. The mussels also house spirochaetes (*Cristipsira*) in their styles, but the exact nature of the relationship between the mussels and bacteria is still unknown. The mussels also produce enzymes which allow them to utilise bacteria as an additional food source. This means that, even though all the components of the seston are not consistently present in high concentrations, the mussels are probably still able to satisfy their carbon and nitrogen requirements. They do this by not only utilising the products of bacterial digestion,

but also by using bacteria as supplementary food.

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APPENDIX 1

Arsenomolybdate:

1) Dissolve 25g $(NH_2)_6Mo_7O_{24}.4H_2O$ in 450ml distilled water, then add 21ml concentrated H_2SO_4.
2) Dissolve 3g $Na_2HAs)_4.7H_2O$ in 25ml distilled water, and add to the acid molybdate.
3) Store in a brown bottle for 24hrs at 37^0C. The reagent should be yellow, with no green tint.

APPENDIX 2

Estuarine Salts (A. Jaffray, University of Cape Town, pers. comm.)

NaCl	30.0g
$MgCl.6H_2O$	2.3g
KCl	0.3g

Dissolve in 1l distilled water.

Nutrient broth medium (Muir *et al.*, 1986)

0.5% (w/v) Peptone

0.1% (w/v) Yeast extract

Dissolve in estuarine salts.

Nutrient plate medium (Muir *et al.*, 1986)

0.5% (w/v) Peptone

0.1% (w/v) Yeast extract

1.5% (w/v) Agar

Dissolve in estuarine salts.

APPENDIX 3A

Preparation of tissue for Scanning Electron Microscopy

(After Cross, 1987).

Reagent	Time (mins)
1% OsO_4 in 0.2M Na Cacodylate buffer, pH7.2	60
0.2M Na Cacodylate buffer. pH 7.2	15 (x 2)
30% Ethanol	5 - 10
50% Ethanol	5 - 10
70% Ethanol	5 - 10
80% Ethanol	5 - 10
90% Ethanol	5 - 10
100% Ethanol	5 - 10 (x 2)
75:25% Ethanol: Amylacetate	15 - 20
50:50% Ethanol: Amylacetate	15 - 20
25:75% Ethanol: Amylacetate	20
100% Amylacetate	20

APPENDIX 3B

Preparation of specimens for Transmission Electron Microscopy

(After Cross, 1987).

Reagent	Time (min)
0.2M Na Cacodylate buffer, pH 7	10 (x2)
1% OsO_4 in 0.2M Na Cacodylate buffer, pH 7	90
0.2M Na Cacodylate buffer, pH 7	10 (x2)
30% Ethanol	3 - 5
50% Ethanol	3 - 5
70% Ethanol	3 - 5
80% Ethanol	3 - 5
90% Ethanol	3 - 5
100% Ethanol	3 - 5 (x2)
Propylene oxide	15 (x2)
75:25% Propylene oxide: Resin*	60 - 90
50:50% Propylene oxide: Resin	60 - 90
25:75% Propylene oxide: Resin	60 - 90
Pure Resin	Overnight

* = Taab/Araldite mixture

After allowing the resin to infiltrate the tissue overnight, the tissue was transferred to moulds that had been 3/4 filled with clean resin. The prepared moulds were then placed in an oven at 60°C to polymerise for 36 hours.