BURROWING AND FEEDING ECOLOGY OF THE GHOST SHRIMP *BIFFARIUS ARENOSUS* (DECAPODA: CALLIANASSIDAE).

Thesis submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy
of Victoria University of Technology

by

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DECLARATION

The research in this thesis has not been previously submitted for a degree or diploma in any University. The thesis contains no material that has been previously published or written by another person, except where due reference is made in the thesis itself.

FIONA L. BIRD

ABSTRACT

The thalassinidean ghost shrimp, *Biffarius arenosus* (Decapoda: Callianassidae), is a dominant component of coastal soft sediment communities in temperate south-eastern Australia. This thesis investigated the burrowing and feeding ecology of a population of shrimps inhabiting an intertidal sandflat in Western Port, Victoria. *Biffarius arenosus* constructs dynamic, unlined burrows with a complex shape indicating that the shrimps exploit the subsurface food supply. A multiple stable isotope study revealed that the food source was sedimentary organic matter, primarily derived from decomposing seagrass and seagrass epiphytes. Feeding and burrowing activity of the shrimps resulted in comparatively larger particles being ejected from burrows to the sediment surface. An investigation of the impact of burrowing and feeding activity on physiochemical and microbial properties of the sediment revealed that the burrow wall sediments were relatively more oxidised (measured via platinum redox electrodes) and had higher microbial enzyme activity rates (quantified via the hydrolysis of fluorescein diacetate) than surrounding subsurface sediments. Grazing by *B. arenosus* appeared to affect bacterial abundance (estimated with DAPI epifluorescent counts) in the burrow wall. Other properties of the burrow, such as organic carbon content (measured using a wet digestion technique) and microbial community structure (studied by examining phospholipid fatty acid profiles), did not differ between burrow wall, surrounding subsurface and surface sediments. The combination of active and passive burrow irrigation and an increase in the water-sediment interface surface area (caused by the presence of irrigated burrows in usually anoxic sediments), enhanced the diffusive flux of water from the sediments (quantified using a non-reactive tracer) by 400%. This research indicated that *B. arenosus* has a significant impact on the biogeochemistry of the marine sediments of Western Port.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 General overview

Burrowing marine fauna, such as the thalassinidean ghost shrimp *Biffarius arenosus* (Poore 1975), have a major impact on the biological, physical and chemical characteristics of marine sediment. Subsurface activity of macrofauna results in the biological reworking of sediments during burrow construction and feeding (bioturbation), and the mixing of oxygen-bearing waters into sediments (bioirrigation). Both processes have a significant impact on sediment and nutrient dynamics of the marine environment.

Bioturbation and bioirrigation affect biological, physical and chemical properties of marine sediment. Composition, density and distribution of biological communities are influenced by sediment reworking, and physical properties of the sediment, such as porosity and stability, are altered. Bioturbation can lead to an increased organic content of sediment through burial of surface layers, the presence of a mucus burrow lining, and the storage of food and faeces in burrows. Bioirrigation introduces oxygen to sediment depths, therein promoting aerobic decomposition in otherwise anoxic sediments. Consequently, microhabitats are created in the sediments (rich in organic matter and subject to oxidising conditions) that promote the growth and activity of microorganisms. Microbes mediate both the decomposition of organic matter and the subsequent recycling of nutrients, so nutrient cycling in bioturbated sediments is significantly altered, compared with adjacent sediments devoid of shrimps. Bioirrigation also enhances diffusive flux of

solutes over the water-sediment interface, further affecting nutrient dynamics of the sediments.

1.2 Thalassinidean fauna

Thalassinidean ghost shrimps are a common component of many intertidal and subtidal benthic soft sediment communities. Thalassinideans are important bioturbators and bioirrigators because they often occur in high densities (> 1100 m^{-2}, *Lepidophthalmus sinuensis*, Nates and Felder 1997), construct deep irrigated burrows (> 2 m depth, *Lepidophthalmus louisianensis*, Felder and Rodrigues 1993) and process sediment at high rates (12 kg ww $m^{-2} d^{-1}$, *Callianassa kraussi*, Branch and Pringle 1987). Thalassinidean shrimp depend on their burrows for shelter, feeding and reproduction, and except for a brief pelagic larval stage, most species spend their entire life below the sediment surface (Griffis and Chavez 1988).

Members of the infraorder Thalassinidea are distributed globally, inhabiting intertidal, shelf and slope sediments (except in the polar seas), and are very diverse with 11 families and 73 genera having been described (Poore 1994). The infraorder comprises 3 superfamilies Thalassinoidea, Callianassoidea and Axioidea. The largest of these, Callianassoidea, contains 6 families: Laomediidae, Upogebiidae, Callianideidae, Thomassiniidae, Ctenochelidae and Callianassidae (Poore 1994). Australia has a very rich thalassinidean fauna dominated by the families Upogebiidae and Callianassidae. Together they contain over 50% of the total species present (Poore and Griffin 1979).

Callianassidae (Dana 1852) is one of the largest families within the infraorder Thalassinidea. The family contains burrowing species with a characteristic elongated body form, specialised fossorial pereopods and considerable flexibility between cephalothorax and first abdominal somite (Poore 1994).

1.3 *Biffarius arenosus*

Biffarius arenosus (Poore 1975), of the family Callianassidae, is common in the eastern states of Australia, ranging from Tasmania to southern Queensland (Poore and Griffin 1979). The species inhabits intertidal to shallow subtidal sand and mudflats (Poore 1975, Coleman and Poore 1980). A maximum density of 60 individuals m^{-2} has been found in Port Phillip Bay, Victoria (Poore 1975). This species was chosen as the topic of my study because it is abundant in coastal waters, especially in the two major embayments of Victoria, Western Port and Port Phillip Bay (Poore 1975, Coleman and Poore 1980). Surrounding lands of both bays are heavily urbanised, and the waters are subject to extensive municipal run off, the disposal of treated sewage effluent, and agricultural and industrial inputs (Harris *et al.* 1996, May and Stephens 1996). Local concern regarding the "health" of these coastal systems has sparked considerable interest in the role bioturbating fauna play in nutrient cycling (eg. Harris *et al.* 1996). Despite being abundant and widely distributed, resident thalassinidean fauna has been the subject of little previous research.

To date, studies of *Biffarius arenosus*, the dominant thalassinidean species in Western Port, have documented abundance and distribution (Poore 1975, Coleman and Poore 1980), and some effects of anoxic conditions on the behaviour and physiology of individuals in their burrows (Paterson and Thorne 1993). The overall aim of this thesis was to investigate burrowing and feeding ecology of *B. arenosus* in Western Port in an attempt to understand its interaction with the immediate biogeochemical environment.

1.4 The thalassinidean burrow

Thalassinidean ghost shrimps depend on their burrows for shelter, food and reproduction (Griffis and Chavez 1988). Burrow shape varies markedly among species (see review in Dworschak 1983) and even between populations of the same species (Rowden and Jones 1995). Analysis of the functional morphology of individual burrow components has resulted in formulation of a model used to predict feeding behaviour and burrow use by a shrimp species (Suchanek 1985,

Griffis and Suchanek 1991, Nickell and Atkinson 1995). The presence or absence of particular structural components indicates if a certain trophic mode is employed by a species. For example, presence of a surface mound around a burrow opening indicates that the resident ghost shrimp processes sediment for feeding or burrow expansion and repair, and this behaviour is often assumed to be consistent with the species being a deposit-feeder (Nickell and Atkinson 1995).

The major thalassinidean burrow components include burrow entrances, flow-through sections to assist with irrigation, horizontal and vertical tunnels and central or peripheral storage chambers. The number of openings in thalassinidean burrows varies, with a maximum of 17 per individual burrow recorded for *Calocaris macandreae* (Nash *et al.* 1984). For some species, the number of burrow entrances is related to organic content and porosity of sediments (Miller 1984, Rowden and Jones 1995). Shape of the burrow opening also varies, and the presence of a funnel or thistle-shaped entrance is thought to aid collection of organic material from the sediment surface (Dworschak 1987). The top section of the burrow is often U-shaped, to allow a uni-directional flow of water through the burrow. Even though ghost shrimps are well adapted to short-term survival of hypoxic conditions (Anderson *et al.* 1991, Paterson and Thorne 1993, Astall *et al.* 1997), irrigation is necessary to periodically replenish the oxygen supply and flush out wastes and metabolites. Ghost shrimps actively irrigate their burrows by fanning their pleopods (Atkinson and Taylor 1988), but passive water movement, caused by differential water pressure in the burrow shafts (the Bernoulli effect) (Vogel 1981), also assists with the exchange of burrow and overlying waters.

Structure of the main body of the thalassinidean burrow ranges from a simple unbranched tunnel (eg. *Upogebia pusilla*, Dworschak 1983) to a complex series of spiral tunnels and chambers (eg. *Axianassa australis*, Dworschak and Rodrigues 1997). Simple burrows are usually associated with filter-feeding and seagrass/algal-harvesting, and the more complex with the exhaustive exploitation of sediment by deposit-feeding species (Suchanek 1985). Chambers are often present in burrows, either for storage of plant material (eg. *Neotrypea* (as

Callianassa) *gigas*, Griffis and Chavez 1988) or because of sediments too large to eject from the burrows (eg. *Callianassa subterranea*, Rowden and Jones 1995).

1.5 Thalassinidean feeding

Three ecological types of thalassinideans have been identified: filter/suspension-feeders, seagrass/algae-harvesters and detritus/deposit-feeding species (Suchanek 1985, Griffis and Suchanek 1991, Nickell and Atkinson 1995). Filter-feeding thalassinideans such as *Upogebia pusilla* (Dworschak 1983) use specialised mouth parts to filter food particles from the water current passing through their burrows. In contrast, seagrass/algal harvesters collect material floating past their burrow openings (eg. *Corallianassa longiventris*, Dworschak and Ott 1993), which they either ingest directly, work into burrow walls or store in chambers to promote microbial growth for food (Dworschak 1987, Griffis and Chavez 1988, Dworschak and Ott 1993). Detritus/deposit-feeding species ingest a combination of decomposing organic matter and sediment particles, gaining additional nutrition from the microorganisms adhered to the material (Lopez and Levinton 1987).

There has been considerable debate as to how deposit-feeders gain adequate nutrition. Decomposing vascular plant material is often refractory and of low food quality (Fenchel and Harrison 1976), and the microbial fraction ingested is thought to be too small to sustain the carbon and energy demands of the consumer (Phillips 1984, Goedkoop and Johnson 1994). It is known however, that bacteria are able to utilise nutrients from sources inaccessible to deposit-feeders (such as dissolved mineral nutrients) (Fenchel and Jorgensen 1977, Jumars *et al.* 1990) which are passed on to the consumer during digestion. Deposit-feeders often selectively ingest smaller particles to maximise nutritional gain during feeding (eg. the polychaete *Lumbrineris* cf. *latreilli*, Petch 1986), because small particles have a proportionally greater amount of utilisable organic material and a relatively larger microbial population (Hargrave 1972).

1.6 Feeding and burrowing activity

During feeding and burrowing activity, especially in deposit-feeding ghost shrimp species, sediment is processed, sorted, moved around the burrow and ejected from the openings. Constant processing disturbs the vertical profile of the sediment. Some species of shrimp selectively eject fine particles from the burrows, but store coarse sediments deep in chambers (Suchanek 1983, Suchanek *et al.* 1986, Vaugelas *et al.* 1986). Intensive ghost shrimp activity on the Great Barrier Reef Australia, resulted in a 5 to 60 cm thick surface layer of gravel-free sediment above deeper zones of gravel-rich sediment (Tudhope and Scoffin 1984). Sediment particle size correlates with many chemical properties of sediment so any redistribution vertically or laterally may result in a change in biogeochemical characteristics (Aller 1982, Rashid 1985).

Resuspension of sediment during ejection from burrows aerates the surface layers, but may also reintroduce polluted sediment to the water-sediment interface (Colin *et al.* 1986, Nalepa and Landrum 1988, Officer and Lynch 1989). Sediment processing can affect sediment properties such as permeability (Meadows and Tait 1989, Meadows and Hariri 1991, Jones and Jago 1993), which are linked to the sediment biogeochemistry.

1.7 The burrow environment

A distinctive burrow environment is created by the feeding and irrigation activity of the resident ghost shrimp. Burrow irrigation creates oxidising conditions along the burrow wall (Forster and Graf 1992), and walls often contain a higher concentration of organic carbon than the surrounding sediment (Abu-Hilal *et al.* 1988, Vaugelas and Buscail 1990). Some thalassinidean burrows are lined with mucopolysaccharides (eg. *Callichirus laurae*, Vaugelas and Buscail 1990), and some species incorporate plant material (eg. *Neotrypea* (as *Callianassa*) *californiensis* and *Neotrypea gigas*, Griffis and Chavez 1988) and/or faecal pellets (eg. *Glypturus armatus*, Vaugelas *et al.* 1986) into tunnel and chamber walls. Microbial decomposition is promoted by the addition of these new reactive substrates, as well as mucus, to the burrow wall (Kristensen 1988).

Oxygen is introduced into usually anoxic sediments via irrigation of the burrow environment. Periodic ventilation of burrow waters causes variable oxygen conditions in the burrow walls, with oxygen being rapidly exhausted during periods of rest or low tide (Kristensen 1988). Fluctuating oxygen conditions create a mosaic of microenvironments in and around the burrow wall (Dobbs and Guckert 1988, Tomaszek 1995), thereby providing niches for a diverse microbial community, including both aerobes (eg. nitrifying bacteria) and anaerobes (eg. denitrifying bacteria). The presence of reactive organic material and the mosaic of redox conditions in the burrow wall appears to promote the growth and production of the resident microbial populations (Aller and Aller 1986, Aller 1988, Reichardt 1988, Steward *et al.* 1996).

Bacteria play an important role linking organic matter and higher consumers in aquatic environments (Azam *et al.* 1983). In coastal sediments especially, bacteria are intrinsically linked to the decomposition of organic matter and the recycling of essential nutrients (Pollard and Moriarty 1991, Craven and Jahnke 1992, Alongi 1995). Bacteria mediate the major reactions involved with nitrogen cycling, including the conversion of ammonia to nitrate/nitrite (nitrification) which in turn may be converted to nitrogen gas (denitrification) and lost from the system (Henriksen and Kemp 1988, Koike and Sørensen 1988), or reconverted back to ammonium and thus retained in the community (Boon *et al.* 1986). Burrow environments enlarge the zone of nitrification by increasing the surface area of the oxidised water-sediment interface (Henriksen *et al.* 1980,1983, Kristensen *et al.* 1985, Hüttel 1990, Mayer *et al.* 1995, Tomaszek 1995). Burrows also bring the products of nitrification, nitrate and nitrite, in close contact with adjacent anoxic zones of denitrification, thereby coupling the nitrification/denitrification process (Revsbech *et al.* 1988, Kemp *et al.* 1990, Jensen *et al.* 1993, Rysgaard *et al.* 1993, Seitzinger 1993, Tomaszek 1995). The reactions can occur concurrently because of oxidised micro-zones in the anaerobic layer and reducing micro-zones in the aerobic layer of sediments (Tomaszek 1995). Under anaerobic conditions, denitrification is an important sink for nitrogen, which results in the removal of nitrate and export of nitrogen or nitrous oxide gas out of the sediment (Law *et al.* 1991). A number of studies have shown that denitrification is enhanced in the

presence of burrowing animals (Sayama and Kurihara 1983, Kristensen *et al.* 1991, Law *et al.* 1991).

Benthic activity also enhances diffusive flux of nutrients over the water-sediment interface (Waslenchuk *et al.* 1983, Hopkinson 1987, Aller and Aller 1992, Clavero *et al.* 1992, Marinelli 1994). Burrows increase the surface area for solute exchange between the burrow and adjacent sediment, up to 16 times in the case of *Upogebia litoralis* (Ott *et al.* 1976). Moreover, the one-dimensional concept of diffusion from the sediment to the overlying water is complicated by the construction and irrigation of burrows by macrofauna. Solutes can diffuse along both lateral and vertical concentration gradients (Aller 1982, 1983), while pressure gradients, active irrigation and animal movement induce convection of water and solutes into the burrow cavity (Ebenhöh *et al.* 1995). Therefore, burrowing activity has important implications for modelling of diffusive fluxes of nutrients from sediments to the overlying water and thus for the control of phytoplankton growth and for patterns of nutrient cycling in coastal waters.

1.8 Aims of this study

The lack of information about *Biffarius arenosus* and the potential of this species to significantly alter its sedimentary environment provided the instigation for this study investigating the species' feeding and burrowing ecology. This study is divided into four parts: burrow structure, feeding, physical characteristics and microbial characteristics of the burrow environment. Specific aims were as follows:

1. To describe shape, size and functional morphology of the burrows, thereby providing the background information necessary to understand the specific processes studied in later sections.
2. To assess if *Biffarius arenosus* was primarily a deposit feeder, and to identify what food was ingested and assimilated, its source and the mechanism by which the shrimp obtained the material.

3. To investigate physiochemical characteristics of the burrow environment of *Biffarius arenosus* compared to surface sediments and surrounding anoxic subsurface sediments by:

(i) measuring organic carbon content to investigate if the burrow walls were enriched with organic material;

(ii) describing the physical nature of the burrow wall, including the detection of mucopolysaccharides as a lining material; and

(iii) documenting reducing/oxidising conditions to reveal if the burrow environment was as oxidising as the surface sediments, or anoxic similar to surrounding subsurface sediments.

4. To quantify the surface area of the burrows and to examine the effect of the burrows and burrowing activity of *Biffarius arenosus* on diffusive flux of water across the water-sediment interface.

5. To investigate various microbial properties of the wall of *Biffarius arenosus* burrows in comparison with the surrounding surface and subsurface sediments by:

(i) estimating bacterial abundance;

(ii) measuring microbial enzyme activity as an indicator of the potential of each community to decompose organic matter;

(iii) quantifying total microbial biomass; and

(iv) investigating any differences in microbial community structure.

1.9 Format of this thesis

This thesis is divided into a further five chapters. Chapter 2 describes structure of *Biffarius arenosus* burrows, including gross dimensions and functional morphology of specific structural components. Burrows are compared with those of other species of shrimp in order to predict what feeding habit (filter/suspension-feeding, detritus/deposit-feeding or seagrass/algal harvesting) was most likely employed by *B. arenosus*. Chapter 3 investigates feeding in more depth by identifying what *B. arenosus* eats and from where it obtains its food. The chapter also addresses the impact of feeding activity on sediment dynamics, including measurements of processing rates and the influence of activity on sediment particle size distributions. Chapter 4 describes the impact of a burrow of *B. arenosus* on the physiochemical

burrow environment, specifically, whether organic carbon is concentrated in the burrow walls, whether walls are lined with mucopolysaccharides, how burrow irrigation affects redox potential of the sediments, and whether burrows and burrowing activity influence diffusive flux of water over the water-sediment interface. Chapter 5 investigates microbial properties of the environment, by quantifying bacterial abundances, microbial activity, biomass and community structure of the microbial assemblages in the burrow wall in comparison with the surrounding surface and subsurface sediments. A final chapter summarises the findings and presents an overall view of burrowing and feeding ecology of this species of thalassinidean ghost shrimp.

CHAPTER 2

BURROW STRUCTURE

2.1 Introduction

The structure of ghost shrimp burrows varies greatly between species (see review in Dworschak 1983). Previous investigations have reported burrow shapes ranging from simple vertical tunnels with single openings (Atkinson and Nash 1990), multi-layered burrows with many entrances (Nash *et al.* 1984), to very complex burrows with spiral tunnels and chambers (Dworschak and Rodrigues 1997). The diversity of burrow shapes and functional morphology of individual structural elements have been integrated into a formal classification scheme, with the aim to predict trophic mode of a species from its burrow structure (Suchanek 1985, Griffis and Suchanek 1991, Nickell and Atkinson 1995). Specific burrow features are associated with filter/suspension-feeding, deposit/detritus-feeding or seagrass/algae-harvesting (Suchanek 1985), and the burrow shape of a species tends to display features related to a single feeding type. However, some species' burrows possess a broad range of components indicating the potential for variability in feeding behaviour relative to changing environmental conditions (Miller 1984, Dworschak and Pervesler 1988, Nickell and Atkinson 1995, Rowden and Jones 1995). The ability to adopt different feeding behaviours in response to specific environmental conditions would be beneficial for a species, as it would allow it to successfully colonise a range of habitats.

Because there are so many different thalassinidean burrow shapes and burrow structure of a species sometimes varies between sites (as documented for *Callianassa subterranea* by Witbaard and Duineveld 1989, Atkinson and Nash 1990, and Rowden and Jones 1995), it is difficult to predict what the burrow of a

previously unstudied species will be like. Without knowing basic information such as burrow shape and size, and functional morphology of the individual components, it is nearly impossible to make any predictions of the species' impacts on sediment processes.

This section of work aims to describe the structure of *Biffarius arenosus* burrows and gain some understanding of functional morphology as a basis for the larger investigation of burrowing and feeding ecology. This chapter will also attempt to predict what trophic mode *B. arenosus* is most likely to adopt, using published burrow classification schemes as a guide. This prediction will then be tested in more detail in the subsequent chapters.

2.2 Methods

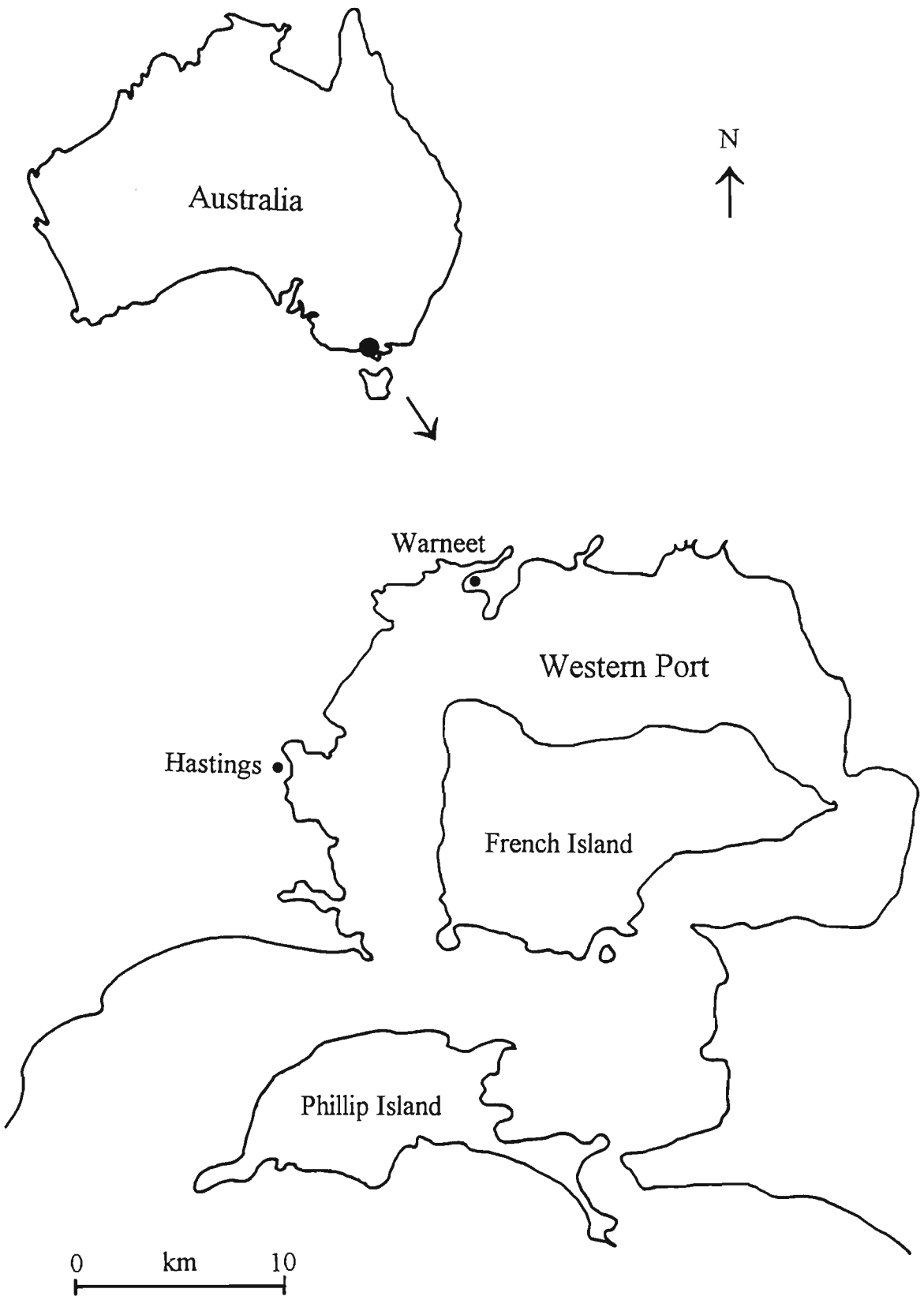
2.2.1 Study site

Western Port is a large, shallow embayment located south-east of Melbourne (37°45'S, 144°58'E) on the temperate southern coast of Australia (Figure 2.1). The bay has an area of approximately 680 km^2, 40% of which is intertidal sand and mudflats (Ministry of Conservation 1975). French Island is situated in the centre of the bay, and Phillip Island narrows the entrance to Bass Strait considerably. The Bay has two deep water channels, North and East Arms, which partially surround French Island and drain the extensive tidal flats situated north of the island. The sediments of these flats vary from fine sand to silt and clay. Seagrass beds (*Heterozostera tasmanica* Martens ex Aschers. and *Zostera muelleri* Irmisch ex Aschers.) are extensive along the edges of the drainage channels and more sparse on the tidal flats. Tidal range at the north end of the Bay is approximately 2.2 m (Ministry of Conservation 1975).

The study site is at the township of Warneet, situated in the Rutherford Inlet, in a north-western arm of Western Port (Figure 2.1). The study population of *Biffarius arenosus* inhabits an intertidal sandflat bordered by saltmarsh (predominantly *Sarcocornia quinqueflora* Bung ex Ungern-Sternberg), with a fringe of mangroves (*Avicennia marina* Forsk Vierh) along the high water mark (Figure

Figure 2.1

A map of Western Port, on the south east coast of Australia. The study site, Warneet, is situated on the northern shore of the bay.


Australia
N
Warneet
Western Port
Hastings
French Island
Phillip Island
0
km
10

2.2). Patchy seagrass beds (predominantly *Heterozostera tasmanica*) extend from the mid-water mark to the channel which drains Rutherford Inlet.

2.2.2 Burrow casting

Biffarius arenosus burrows were cast in November 1994 using a very fluid epoxy resin (CIBA-Geigy Araldite LC3600 resin and LC249 hardener) *in situ*. A $2m^2$ area of sandflat, 5 m below the high water mark, was selected as the casting site and all burrow openings within that area were filled with resin. This sampling design was chosen with the aim to cast complete burrows, by pouring resin into all possible burrow entrances in a given area. The resin was mixed (3:1 ratio by weight, of resin to hardener) on site, and poured into burrow openings at low tide. Plastic cups created a small reservoir at each opening to ensure a continuous flow of resin. After 36 h, the casts were excavated and washed to remove any loose sand.

Burrow dimensions were measured using calipers and a measuring tape. The number of openings per burrow was recorded and burrow volume was estimated by dividing the cast weight by resin (and hardener) density (1.124 g ml^{-1}); the cast weight by resin (and hardener) density (1.124 g ml^{-1}). Burrow surface area was estimated by wrapping a single layer of aluminium foil of known weight per unit area around the cast, as described by Atkinson and Nash (1990). Each cast was wrapped twice and an average calculated. The difference between replicate wrappings was <10%. Least squared linear regressions of the dependant variables (burrow volume and surface area) were performed after data were checked for normality and ln(x+1) transformed. Correlations between the other major independent burrow features were also analysed.

2.3 Results

Twenty-one burrow casts were recovered and analysed. Although each burrow had a distinctive shape, all had consistent features, and the eight examples in figure 2.3 illustrate the intraspecific diversity. Casts were separated into two size

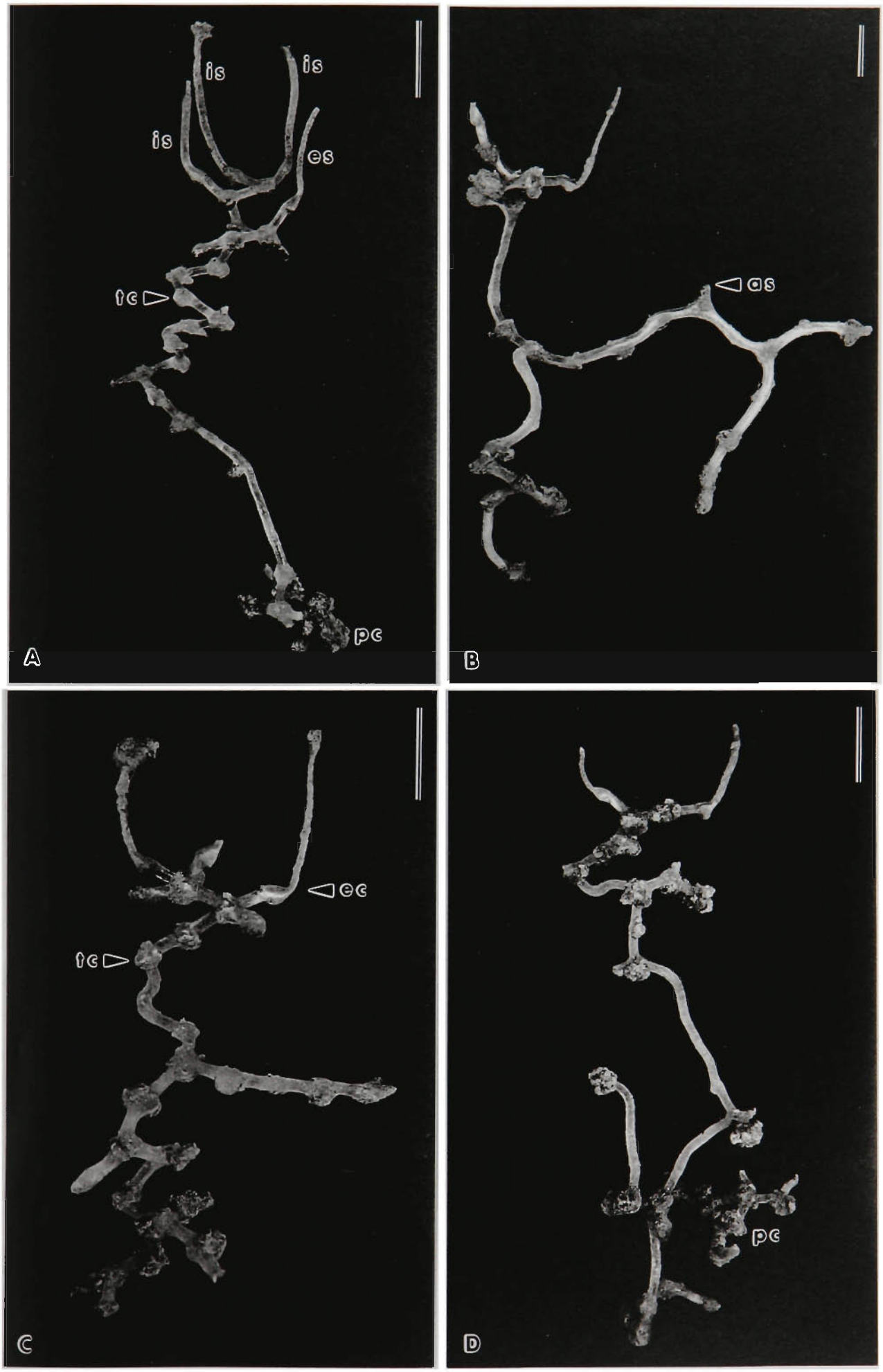
Figure 2.2

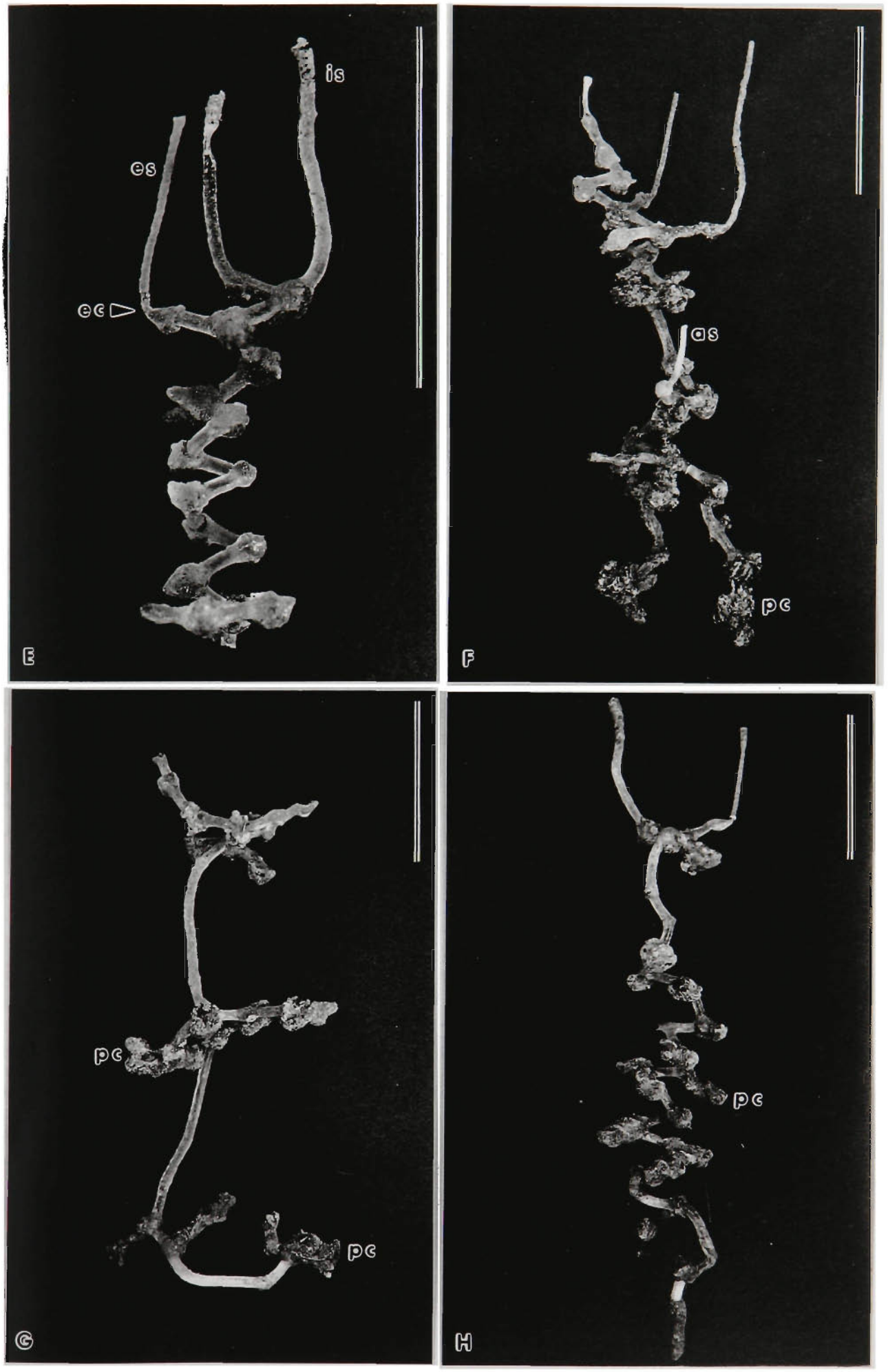
A view of the study site at Warneet, Western Port. This view from the Warneet pier clearly shows the sandflats and patchy seagrass beds exposed at low tide, and the fringing mangroves. All shrimp specimens, burrows and sediment samples were collected from the central sandflat.

Figure 2.3

Resin casts of the burrows of *Biffarius arenosus* showing intraspecific variation. Casts were obtained during field work in November 1994 at Warneet, Western Port. Scale bar = 10 cm.

Abbreviations: is = inhalant shaft, es = exhalant shaft, ec = exhalant constriction, as = additional shaft, tc = turning chamber, pc = peripheral chamber.


is
is
is
es
ic
pc
A
as
B
ec
ic
C
pc
D


is
es
ec
E
as
pc
F
pc
pc
G
pc
H

classes for comparison; 4 small casts presumably inhabited by juveniles, and 17 larger casts presumably inhabited by adults (Table 2.1). Overall, burrow depth ranged from 20 to 58 cm, horizontal burrow extension from 7 to 31 cm, and total tunnel length from 64 to 143 cm (Table 2.1). Grouping around the mean horizontal extension was strong except for two casts with long horizontal tunnels (Figure 2.3b, c). Burrow depth was positively correlated with total tunnel length (Figure 2.4a), but not with horizontal extension (Figure 2.4b). Burrow volume varied between 22 and 174 cm^3, and burrow surface area was between 15 and 638 cm^2 (Table 2.1). Positive linear relationships between surface area and volume (df 1, F 89.61, $P < 0.001$), surface area and total tunnel length (df 1, F 30.89, $P < 0.001$), and volume and total tunnel length (df 1, F 16.71, P 0.001) were identified (Figure 2.5a, b, c).

Burrows were U-shaped at the top with two (13 casts), three (7 casts) or four (2 casts) openings. The mean number of openings was 2.5. Some openings were slightly funnel-shaped (Figure 2.3a, c), but generally the shafts remained linear to the surface. Shafts from the third and fourth openings, when present, connected to the main U at a slightly lower level, creating a multi-layered U connection to the surface (Figure 2.3a, e, f). In some casts, further shafts originated from deeper sections of tunnel (Figure 2.3b, f), providing additional points for surface access.

The first level of the U section of the burrows extended vertically to between 8 and 13 cm depth (Table 2.1). In burrows with more than two openings, the secondary levels of the U section were 1 to 3 cm lower than the first level. Depth of the U represented on average 23% of the burrow depth in adult casts and 38% of burrow depth in juvenile casts.

Diameter of the opening shafts (mean 5.0 mm for large casts) was always less than the diameter of the tunnels (mean 7.1 mm for large casts) (Table 2.1). The difference represents a constriction in the opening shaft cross-sectional area to 13-39% that of the tunnel for adult casts, and 4-36% that of the tunnel for juvenile casts (Table 2.1). Inhalant shafts (indicated by a depression in the surrounding surface) had a larger mean diameter than the exhalant shafts (indicated by surrounding ejecta mound) (Table 2.1), and the exhalant shaft had an additional

Table 2.1 Dimensions of significant features measured from casts of the burrows of *Biffarius arenosus*. Burrows were cast at Warneet, Western Port, at low tide in November 1994.

Cast number	Volume	Surface area	Number of openings	Number of clockwise spirals	Number of anti-clockwise spirals	Burrow depth	Horizontal extension	Tunnel length	Depth of U	Inhalant shaft diameter	Exhalant shaft diameter	Tunnel diameter	Turning chamber diameter	Number of peripheral chambers
	ml	cm^2				cm	cm	cm	cm	mm	mm	mm	mm	
Large casts (adults)														
1	81	423	2	2	0	36	31	115	8	na	3.5	7	16	3
2	150	558	2	2	1	48	8	125	11	6.7	4	6.9	16	18
3	95	412	2	2	1	42	14	98	13	8.2	5	6.7	16	6
4	124	530	2	2	0	46	13	133	9	5.9	3.4	6.3	15	15
5	162	638	2	1	0	55	19	143	11	na	4.4	8.4	19	11
6	174	631	2	0	1	48	16	138	8	na	4.7	7.7	19	15
7	105	418	2	1	1	58	14	99	11	na	5.4	7.6	14	5
8	113	407	na	na	na	51	13	90	13	na	4.7	5.3	16	13
9	114	462	na	0	1	41	16	103	na	na	na	7	15	8
10	130	509	2	2	0	39	12	92	na	na	na	6.6	16	11
11	131	539	na	0	1	42	24	106	10	na	3.9	8.2	18	12
12	160	623	3	2	2	42	11	138	9	na	4	7.5	17	13
13	157	405	3	na	na	32	10	71	9	6.1	4.1	6.5	15	9
14	127	458	3	0	1	40	10	98	10	4.3	na	7.2	17	7
15	75	406	3	1	0	48	13	134	10	6.3	3.7	6.9	16	10
16	110	543	3	1	1	39	20	121	12	6.5	4.8	7.8	17	8
17	72	455	4	1	1	47	16	105	11	5.7	4.2	6.4	13	5
Mean	**122**	**495**	**2.5**	**1**	**1**	**44**	**15**	**112**	**10**	**6.2**	**4.3**	**7.1**	**16**	**10**
Small casts (juveniles)														
18	22	15	3	2	1	20	7	64	9	4.7	3.1	5.2	10	0
20	27	16	3	1	0	30	14	65	11	5.5	2.9	5.8	12	1
21	67	28	4	3	3	36	7	74	8	na	3.9	4.4	12	5
22	25	19	2	1	1	29	7	74	16	4.1	2.9	4.7	12	0
Mean	**35**	**20**	**3**	**2**	**1**	**29**	**9**	**69**	**11**	**5**	**3.2**	**5**	**12**	**2**

Figure 2.4

Correlations between gross dimensions of burrows of *Biffarius arenosus* cast *in situ* on the intertidal beach at Warneet, Western Port in November 1994. (A) tunnel length and burrow depth ($y = 0.24x + 16.22$, $r = 0.68$, $P<0.01$); (B) burrow depth and horizontal extension ($y = 0.15x + 7.69$, $r = 0.24$, $P>0.05$); (C) tunnel diameter and tunnel length ($y = 16.51x - 6.03$, $r = 0.71$, $P<0.01$); (D) tunnel diameter and burrow depth ($y = 4.5x + 11.15$, $r = 0.55$, $P<0.01$); (E) tunnel diameter and number of peripheral chambers ($y = 2.17x - 6.12$, $r = 0.47$, $P<0.05$); (F) chamber width and height ($y = 0.17x + 14.71$, $r = 0.20$, $P>0.05$).

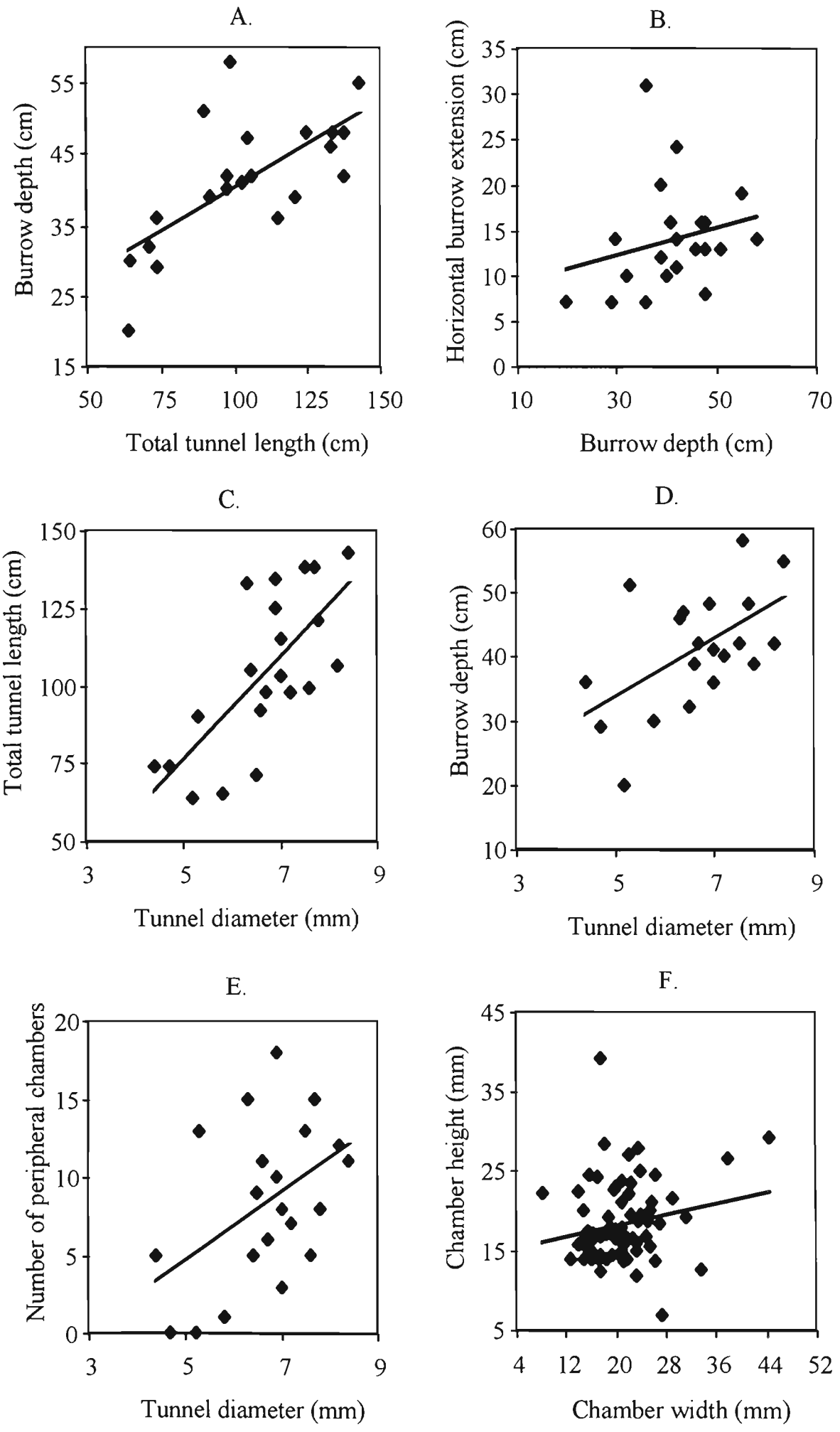
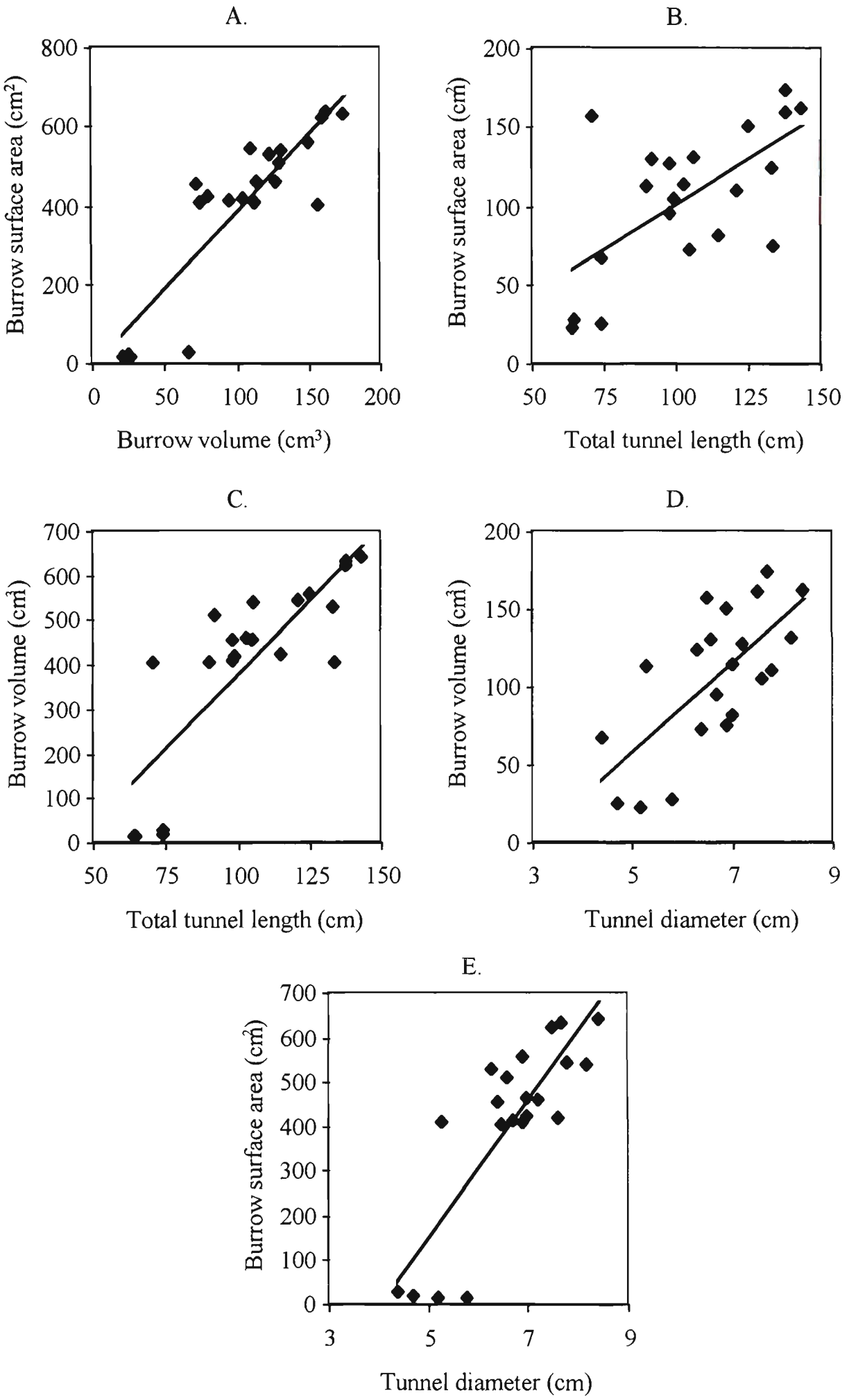

A.
Burrow depth (cm)
55
45
35
25
15
50
75
100
125
150
Total tunnel length (cm)
B.
Horizontal burrow extension (cm)
35
30
25
20
15
10
5
0
10
30
50
70
Burrow depth (cm)
C.
Total tunnel length (cm)
150
125
100
75
50
3
5
7
9
Tunnel diameter (mm)
D.
Burrow depth (cm)
60
50
40
30
20
10
3
5
7
9
Tunnel diameter (mm)
E.
Number of peripheral chambers
20
15
10
5
0
3
5
7
9
Tunnel diameter (mm)
F.
Chamber height (mm)
45
35
25
15
5
4
12
20
28
36
44
52
Chamber width (mm)

Figure 2.5

Positive linear relationships between dependent burrow dimensions measured on burrows of *Biffarius arenosus* cast *in situ* on the intertidal beach at Warneet, Western Port in November 1994.. (A) burrow volume and surface area ($y = 0.42x + 2.16$, $r^2 = 0.83$, $P < 0.001$); (B) tunnel length and burrow surface area ($y = 4.04x - 13.06$, $r^2 = 0.62$, $P < 0.001$); (C) tunnel length and burrow volume ($y = 1.64x - 3.03$, $r^2 = 0.47$, $P = 0.001$); (D) tunnel diameter and burrow volume ($y = 2.42x - 0.03$, $r^2 = 0.47$, $P = 0.001$); (E) tunnel diameter and burrow surface area ($y = 5.98x - 5.69$, $r^2 = 0.62$, $P < 0.001$).


A.
Burrow surface area (cm²)
800
600
400
200
0
0
50
100
150
200
Burrow volume (cm³)
B.
Burrow surface area (cm²)
200
150
100
50
0
50
75
100
125
150
Total tunnel length (cm)
C.
Burrow volume (cm³)
700
600
500
400
300
200
100
0
50
75
100
125
150
Total tunnel length (cm)
D.
Burrow volume (cm³)
200
150
100
50
0
3
5
7
9
Tunnel diameter (cm)
E.
Burrow surface area (cm²)
700
600
500
400
300
200
100
0
3
5
7
9
Tunnel diameter (cm)

constriction followed by an enlargement in the burrow diameter at the base (Figure 2.3).

Unfortunately it was not possible to relate burrow size to ghost shrimp size because no individuals were retained in the casts. However, a positive linear relationship was found between tunnel diameter and burrow volume (df 1, F 16.80, P 0.001), and tunnel diameter and surface area (df 1, F 31.52, P<0.001), and positive correlations were identified between tunnel diameter and total tunnel length, and tunnel diameter and burrow depth (Figures 2.5d, e, and 2.4c, d, respectively), so tunnel diameter is probably representative of shrimp size. This was supported by observations of individual *Biffarius arenosus* burrowing in aquaria, which showed a good animal to burrow fit.

The core of the burrow, extending from the base of the U, usually consisted of a downward spiral, or an irregular spiral combined with some straight sections of tunnel (Figure 2.3b, c, d). This central core branched into further tunnels and chambers. At regular intervals along the tunnels and whenever the tunnel branched bulbous turning chambers were visible (for example, Figure 2.3a, c). Turning points are enlarged regions due to the close match of the shrimp size with tunnel diameter. The diameter of the turning chambers was 120-130% of the burrow tunnel diameter. All tunnels had a circular cross section.

Ninety percent of the casts possessed peripheral chambers. Chamber size and position in the burrow varied between casts, but there was a positive correlation between chamber number and tunnel diameter (shrimp size) (Figure 2.4e). Chambers were found at the edges of horizontal lattices (Figure 2.3d, g, h) or in the deepest section of the burrow (Figure 2.3a, f). A conglomeration of sand grains, shell grit and small pieces of rock always attached to the resin in the chambers (Figure 2.3a, f, g), whereas the tubular burrow walls were always smooth and relatively free of sediment. There was no evidence of decomposing plant material, such as seagrass, in the chambers. Chamber shape exhibited no defined pattern, with no correlation between chamber height and width found (Figure 2.4f).

2.4 Discussion

2.4.1 Burrow features

Despite variations among the different casts, all burrows comprised two dominating features: a U shaped top and a series of tunnels and chambers, both features intrinsically tied to shrimp survival, in terms of burrow irrigation and access to the subsurface food supply. The intraspecific diversity documented here was also remarked upon by Dworschak (1983), who found that *Upogebia pusilla* burrows had a "basic pattern, but great variability in shape".

The fact that individual shrimp were not recovered in any of the casts indicates that the whole burrow may not have been sampled. Dworschak (1983) also rarely found *Upogebia pusilla* individuals in burrow casts and observed individuals blocking themselves into tunnels to avoid the resin. This suggests that *Biffarius arenosus* burrows may be more extensive than the casts suggest.

Thalassinidean burrows can have up to 17 openings (*Calocaris macandreae*, Nash *et al.* 1984). Most species appear to construct between 2 and 4 openings (similar to *B. arenosus*), but this number may vary, depending on physical variables such as sediment porosity (Miller 1984) and organic content (Rowden and Jones 1995). A slight indentation surrounding the incurrent opening was observed in *Biffarius arenosus* burrows, although it was not as pronounced as those described for *Callianassa subterranea* (Atkinson and Nash 1990, Rowden and Jones 1995). The indentation was probably due to continual movement of water into the burrow causing the surrounding sediment to slump into the opening (Rowden and Jones 1995), rather than a distinct structure constructed specifically to trap passing debris, as seen in some other species (Dworschak 1987).

Constricted shaft diameters suggest that *Biffarius arenosus* does not require access to the sediment surface to scavenge or filter food. Narrow apertures are thought to be a response to combat salinity increases in the overlying water (Dworschak 1983), or to help to prevent material and predators from entering burrows (Frey *et al.* 1978). *Callianassa bouvieri* was observed to decrease the diameter of its burrow opening shaft from the surface down using its minor

cheliped (Dworschak and Pervesler 1988). Other reports also suggested that the shaft was narrowed from a wider shaft, after initial construction (Mukai and Koike 1984, Nickell and Atkinson 1995).

Narrow apertures also accelerate the water current, ensuring effective removal of sediment and wastes being ejected from the burrow (Bromley 1990). A constriction followed by an enlargement in burrow diameter was often observed at the base of *Biffarius arenosus* exhalant shafts, a feature documented in other species (Dworschak and Pervesler 1988, Atkinson and Nash 1990, Nickell and Atkinson 1995). This constriction is thought to increase the momentum of moving fluid (described as the Venturi effect) further aiding the successful ejection of sediment from the burrow (Nickell and Atkinson 1995).

Ghost shrimp create water currents by beating their pleopods (Atkinson and Taylor 1988). Passive water movement through the U-shaped section of a burrow also occurs via the Bernoulli effect (Vogel 1981). Flow over the elevated ejecta mound above *Biffarius arenosus* burrows would create different water pressures in the opening shafts, and so cause water to move through the U. Allanson *et al.* (1992) showed that considerable supplementation of burrow water exchange (at least equal to that generated by active pumping) can occur passively via this effect in *Upogebia africana* burrows. The energy cost of pleopod ventilation is probably high (Atkinson and Taylor 1988), so intermittent irrigation and exploitation of the passive current would minimise energy expenditure by the shrimp.

A U shape with 2 openings is essential to maintain water flow through the burrow. A multi-layered U shape, as found for *Biffarius arenosus*, would further enhance efficiency of burrow irrigation, and may be essential for a species constructing complex burrows in anoxic sediments. Ghost shrimp are well adapted to surviving in hypoxic conditions, having relatively low oxygen consumption rates (Anderson *et al.* 1991, Astall *et al.* 1997), a high tolerance to anoxia and low metabolic rates shown in several species (Torres *et al.* 1977, Felder 1979, Thompson and Pritchard 1979, Paterson and Thorne 1993). Some physiological work done on *B. arenosus* shows an adaptive behavioural response to decreasing

oxygen tension in burrow water (Paterson and Thorne 1993). Deposit-feeding species especially are well adapted to cope with hypoxic conditions, as shown by comparative studies of deposit- and suspension-feeding species (Thompson and Pritchard 1979, Mukai and Koike 1984). Deposit-feeding thalassinideans will regularly encounter hypoxic conditions during sediment excavation, and if it is impossible to alleviate anoxia via irrigation, some species can continue to function anaerobically (Torres *et al.* 1977).

High tolerance of sulphide has been reported in several species of thalassinideans (Johns *et al.* 1997), another adaptation especially beneficial for deposit-feeding species. High concentrations of sulphide in the burrow water can be tolerated via a detoxification mechanism which permits aerobic metabolism even under severe hypoxia and toxic sulphide concentrations (Johns *et al.* 1997).

Additional shafts branching from deeper sections of burrow also have been documented previously (Nash *et al.* 1984, Dworschak and Pervesler 1988). It seems likely that these additional connections to the water-sediment interface are vital to ventilate the deeper sections of burrow. Water flow would be most efficient through the U section of burrow, but the blind ended twisting section of tunnel would not be conducive to passive flushing, and would need to be irrigated actively by the inhabitant.

The structure of the secondary U, originating from the deepest point of the primary U, is similar to that reported for *Upogebia pusilla* (Dworschak 1983), but is less complex than the multi-layered U system documented for *Calocaris macandreae* (Nash *et al.* 1984) and *Callianassa subterranea* (Rowden and Jones 1995). Both of these species appear to be functionally adapted to collecting food from the water column rather than enhancing ventilation, although the two processes are intrinsically linked.

Depth of the U in juvenile *Biffarius arenosus* burrows was nearly identical to that in adult burrows, whereas total burrow depth was 33% less. This suggests that the U section of burrow is constructed first and is a relatively permanent structure, compared with the constantly changing deeper tunnels and chambers. Direct

observations of burrowing under laboratory conditions made in this study revealed the dynamic nature of *B. arenosus* burrow shape, a feature observed in other species of thalassinidean (Dworschak 1987, Nickell and Atkinson 1995).

Gross burrow dimensions such as depth, volume and surface area of *Biffarius arenosus* burrows are similar to those of the smaller size range of thalassinidean species. In contrast larger species burrow to depths greater than 2.5 m (Pemberton *et al.* 1976), and have mean burrow surface areas up to an order of magnitude larger than found here for *B. arenosus* (Pervesler and Dworschak 1985). Positive relationships between shrimp size and burrow dimensions have also been found in other species. Tunnel and shaft diameter were positively correlated with shrimp size in *Callianassa subterranea* (Rowden and Jones 1995), supporting my proposal that the tunnel diameter was indicative of shrimp size in *B. arenosus*. A positive correlation between shrimp size and horizontal extension, burrow depth and volume was found for *Neotrypea* (as *Callianassa*) *californiensis* and *Neotrypea* (as *Callianassa*) *gigas* (Griffis and Chavez 1988), whereas no correlation was found between shrimp size and burrow volume in *C. subterranea* (Rowden and Jones 1995). In *B. arenosus*, tunnel diameter (~ shrimp size) appeared to correlate with most dimensions, excluding horizontal extension. Inconsistency of horizontal extension of burrows is probably just the result of an individual responding to local environmental cues and burrowing accordingly, rather than following a specific burrow structure formula.

The long horizontal tunnel present in a few casts was similar to that found occasionally for *Callianassa subterranea* (Atkinson and Nash 1990). The horizontal tunnels may indicate some kind of active searching for food deposits, but most likely just comprise part of the feeding lattice. An alternative hypothesis is that the long horizontal branch is present seasonally and is associated with locating a mate. It remains a mystery as to how these solitary burrowers form unions during the breeding season. Few reports of more than one shrimp per burrow have been documented (Pearse 1945, K. Berkenbusch personal communication). Moreover, when put together, individuals are extremely aggressive (Pearse 1945, F. Bird personal observation 1994). A detailed

investigation of breeding behaviour and seasonal changes in burrow structure is required to test this hypothesis.

Some form of spiral, as described in the burrows of *Biffarius arenosus*, is found in most modern thalassinidean burrows but often is disguised by branching tunnels and nodules (Bromley 1990). Spirals similar to those of burrows of *B. arenosus* have been described for *Callianassa tyrrhena* (Dworschak 1987), *Callianassa bouvieri* (Dworschak and Pervesler 1988) and an undescribed species of *Callianassa* (Braithwaite and Talbot 1972). In all three examples, and *B. arenosus*, the spiral consists of a series of globular chambers connected by tunnel segments with a circular cross section and uniform diameter. The first report of a symmetrical corkscrew spiral in an extant thalassinidean species was recently documented in burrows of *Axianassa australis* from Brazil (Dworschak and Rodrigues 1997). The authors compared *A. australis* burrow shape to paleontological reports of ancestral decapod burrows in the genera *Gyrolithes* (Gernant 1972) and *Ophiomorpha* (Frey *et al.* 1978). In these traces, and burrows documented for *A. australis*, the spiral is an even coil, spiralling in both clockwise and anti-clockwise directions, similar to that shown in Figure 2.3e. but without the globular chambers at the junctions.

The most obvious function of a spiral arrangement is maximal exploitation of a given area of food supply, with area being restricted by spatial competition. Dworschak and Rodrigues (1997) suggested that food content of the sediment containing a spiral is probably evenly distributed throughout. This may explain the inconsistency observed in some *Biffarius arenosus* burrows, with both spiral and non-spiral sections sometimes present in the same cast (for example, Figure 2.3a, c). As the burrower moves through different regions of sediment, it may adapt its burrowing behaviour in response to the available food supply; even distribution - spiral burrowing, uneven distribution - tunnels and chambers. This idea of changing burrowing behaviour relative to food supply will be tested in Chapter 3.

Dworschak and Rodrigues (1997) also discussed the idea of handedness playing a major role shaping spiral burrow sections. However, 40% of the *Biffarius*

arenosus burrows cast contained both clockwise and anticlockwise spirals, indicating that the burrow inhabitants are able to spiral in both directions irrespective of possessing a single enlarged cheliped.

A third explanation for the spiral is that the gently sloping tunnels allow easier movement and sediment processing by the burrow inhabitant (Dworschak and Rodrigues 1997). The gently sloping floors are probably beneficial, but obviously not essential for normal activity by *Biffarius arenosus*, because long stretches of vertical or near vertical tunnels were also present in some casts (Figure 2.3d, g).

Grimm and Föllmi (1994) suggested that the spiral was the result of an optimal strategy for rapid burrowing, after a disturbance event leaves a shrimp exposed on the sediment surface. Once buried in the spiral, the shrimp supposedly resumes 'normal' horizontal branching burrowing. This behaviour seems unlikely for *Biffarius arenosus*, because straight vertical shafts rather than spirals comprise the top of the burrows.

Turning chambers in *Biffarius arenosus* burrows were similar to the 'gallery nodules' (Atkinson and Nash 1990), 'subspherical rooms' (Braithwaite and Talbot 1972), 'bulbous chambers' (Dworschak and Pervesler 1988), 'bulbous turnarounds' (Swinbanks and Murray 1981), 'blind semi-circular bulbs' (Griffis and Chavez 1988), 'nodular chambers' (Nickell and Atkinson 1995) and 'turning chambers' (Ott *et al.* 1976, Dworschak 1983, Rowden and Jones 1995) described by other authors. The chambers act as a point for a directional change, where the shrimp's somersaulting action causes an enlargement of the turning point into an imperfect sphere. The 30% increase in diameter of turning chamber over tunnel diameter found in *B. arenosus* burrows was similar to *Upogebia pusilla* (Dworschak 1983), but smaller than the 70% increase recorded for *Callianassa stebbingi* (Ott *et al.* 1976) and *Callianassa bouvieri* (Dworschak and Pervesler 1988).

In this study the walls of the peripheral chambers adhered to the resin, whereas the walls of the tunnels did not. This was because the chambers were lined with coarser, less firmly compacted sediment than were tunnel walls and the resin

infiltrated the spaces between the coarse particles. Loose sediment or faecal pellets in the chamber also adhered to the resin. Peripheral chambers in other species are thought to be either storage areas for refuse too large to eject from the burrows (Suchanek 1983, Atkinson and Nash 1990, Dworschak and Ott 1993, Rowden and Jones 1995, Nickell and Atkinson 1995) or storage areas of plant material to cultivate fungi and bacteria as a food source (Ott *et al.* 1976, Dworschak 1983, Griffis and Chavez 1988). Refuse storage is common in deposit-feeding species, and plant storage in omnivorous scavenger species. Chapter 3 will investigate if *Biffarius arenosus* actively stores plant material from the sediment surface in its burrows.

2.4.2 Functional morphology of *Biffarius arenosus* burrows - can trophic mode be predicted?

Thalassinidean ghost and mud shrimp appear to fall into three different feeding categories, each with a characteristic burrow shape. Filter/suspension-feeders tend to have simple U-shaped burrows, deposit/detritus-feeding burrows are temporary, deep and a complex series of tunnels and chambers, and seagrass/algae-harvesters have simple holes and deep chambers for storage of plant material (Suchanek 1985). Studies of functional morphology have developed a burrow classification scheme for thalassinideans (Suchanek 1985, Griffis and Suchanek 1991, Nickell and Atkinson 1995). The original model separated burrows into three ecological types based mainly on burrow shape (Suchanek 1985). Griffis and Chavez (1988) found that burrows reflected not only feeding patterns but also environmental constraints, and the classification model was revised to include physical parameters (Griffis and Suchanek 1991). Nickell and Atkinson (1995) noted that some species have plastic feeding behaviour thereby highlighting the deficiencies of the model, and so suggested a less rigid approach, whereby individual features are recognised rather than an overall morphology. The presence of these features, combined with environmental variables allows prediction of the relative importance of the different feeding modes.

Burrows of *Biffarius arenosus* possess seven of the twelve diagnostic features listed in the burrow classification scheme by Nickell and Atkinson (1995). The burrows are deep, and have surface mounds, a tightly layered lattice (spiral), chambers, funnel shaped openings, narrow opening shafts and a circular tunnel cross section. These features are indicative of sediment processing for food and burrow construction, material storage for food or to prevent tunnel blockage by large particles, surface access for feeding or removal of waste and current generation for feeding, irrigation or removal of fine waste (Nickell and Atkinson 1995). Features not shown by *B. arenosus* indicate other properties. A sub-circular tunnel cross-section results from poor burrow wall maintenance and reduces efficient current flow, organic detritus stored in burrows indicates the culturing of food such as bacteria and fungi, oblique tunnels and many tunnel openings enhance surface access for collection of food or removal of waste, and the U or Y shape promotes current generation for feeding and irrigation (Nickell and Atkinson 1995).

The features of *Biffarius arenosus* burrows suggest that the species is predominantly a deposit-feeder, but could also potentially feed on surface sediments and material in the water column. Additionally, the close animal to burrow fit observed for *B. arenosus* also indicates a possible suspension feeding component (Dworschak and Pervesler 1988), although this attribute is also important for adequate ventilation (Dworschak and Rodrigues 1997), an essential requisite for a burrow constructed in anoxic sediments.

CHAPTER 3

FEEDING

3.1 What does *Biffarius arenosus* eat?

3.1.1 Introduction

The structure of *Biffarius arenosus* burrows indicates that the primary feeding mode of the species is deposit-feeding. There has been considerable controversy regarding how deposit feeders gain adequate nutrition from marine sediments. Sediments consist mainly of inorganic grains, and the organic matter buried there is typically refractory humic material (Lopez and Levinton 1987), especially in the deeper zones where the burrows occur. Traditionally, it was thought that deposit-feeders digested organic matter adsorbed to sediment grains, however, particulate organic matter, particularly plant material buried in sediments, is usually of low food quality (Fenchel and Harrison 1976). During digestion, little organic carbon is assimilated, primarily because many consumers are unable to digest the structural plant substances such as cellulose and lignin (Berrie 1976). However, microorganisms can be assimilated much more efficiently (Newell 1965, Hargrave 1970a,b, Fenchel 1970, Yingst 1976, Cheng and Lopez 1991) and they appear to provide a major nitrogen and energy source for deposit feeders (Jumars *et al.* 1990).

Decomposing plant material is colonised rapidly by microorganisms (Cundell *et al.* 1979, Fell and Master 1980). Nutritional quality of the fragmented plant material

(detritus) changes during decomposition, with nitrogen tending to increase and carbon tending to decrease (Tenore 1975, Cundell *et al.* 1979, Fell and Master 1982, Rice 1982, Tenore *et al.* 1984). The detritus is transformed into microbial biomass by bacteria, fungi and other microbes, rendering it less refractory and more attractive to consumers (Cammen 1989). Bacteria utilise nutritional sources unavailable directly to deposit feeders (Jumars *et al.* 1990) and can also assimilate dissolved mineral nutrients from the water column, thereby enriching nutrient poor organic material (Fenchel and Jorgensen 1977).

Even though bacteria provide essential nutrients to consumers, they are probably not the primary source of energy for deposit feeders, simply because not enough are consumed to provide the organisms with adequate carbon, nitrogen and energy (Phillips 1984). Cammen (1980) for example, found that microbial carbon contributed only 25% of that required by the deposit-feeding polychaete *Nereis succinea*. Additional food sources such as plant material, must be consumed to meet energy demands (Lopez and Levinton 1987).

Understanding the source of nutrition for *Biffarius arenosus* will give valuable insight into the speciesí interactions with the biological and physical environment. The food source ingested will ultimately influence the rate of mineralisation, and the return of dissolved nutrients to the water column (Levinton 1989). The most common method employed for a preliminary investigation of diets is gut content analysis. In this section, the diet of *B. arenosus* will be documented using gut content analysis and preference experiments.

3.1.2 Gut content analysis

3.1.2.1 Methods

To investigate what *Biffarius arenosus* ingests, gut contents of 20 intertidal individuals of varying sizes collected from the field were analysed. The shrimps were dissected, and the gut removed intact. The contents were washed from the digestive tract, and mounted on a slide in ultrafiltered water. The whole preparation was viewed under a

Zeiss Axioskop binocular light microscope. Presence/absence data were recorded for any identifiable objects, and abundance noted as rare, moderate or common.

3.1.2.2 Results and discussion

Little variation occurred in gut contents of all individuals sampled. Contents consisted mainly of sand grains and brown unidentifiable organic matter (Figure 3.1.1a), with easily identifiable materials like sheets of plant cells (Figure 3.1.1b) and diatom frustules being present but rare. The presence of some plant cells in gut contents indicated that plant material may have been a food source for the shrimps, so the different plant material sources potentially supplying this population of *Biffarius arenosus* with food were identified and the question posed: Do the shrimps have a preference for one particular food source, or do they feed indiscriminately?

3.1.3 Preference experiments

3.1.3.1 Methods

Seagrass and mangrove material, either fresh or decayed, were offered to the shrimps in the absence of sediment. This experiment was an unnatural setting for the shrimps, since they could not build a burrow, but this was thought necessary to simplify the experiment, and investigate if they would ingest the plant material. Small aquaria (3 replicates of each treatment) were set up containing either fresh or dead seagrass, or fresh, senescent or dropped mangrove leaves, and seawater. Tanks were aerated continuously and a single shrimp allowed to feed in each one. After one month, shrimp were removed, frozen and the gut contents analysed.

3.1.3.2 Results and discussion

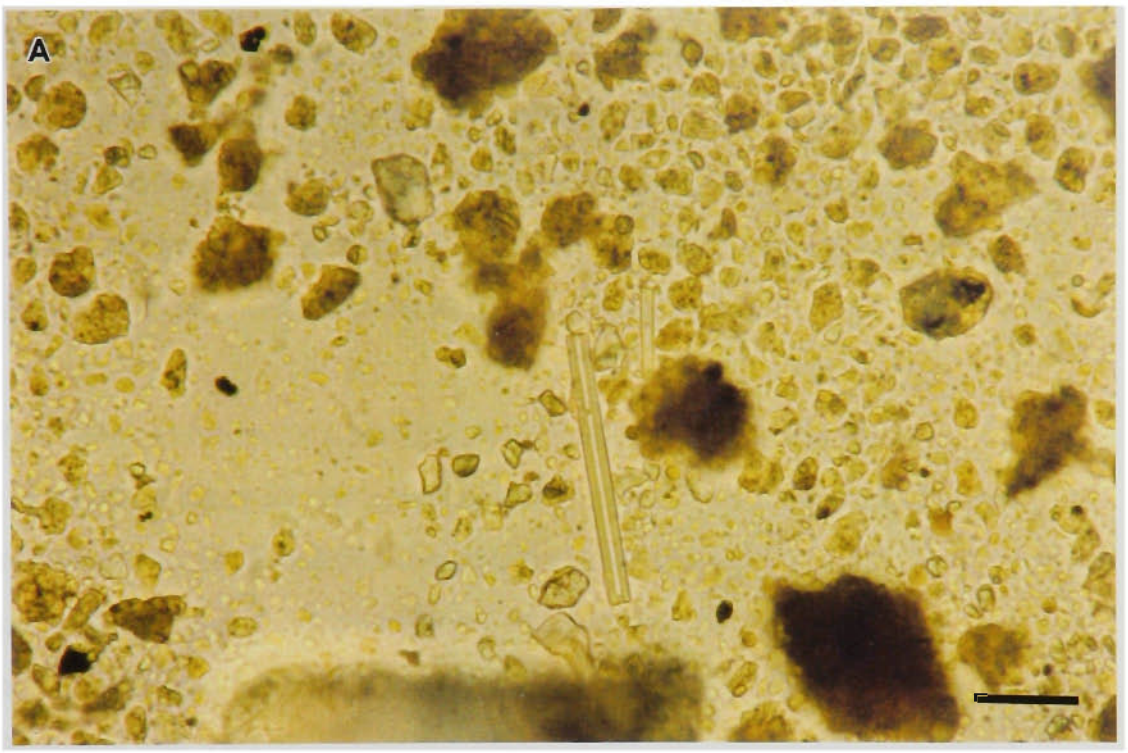
Overall, no conclusive evidence of food source was gained from these feeding observations. Gut contents revealed some sheets of seagrass cells or mangrove trichomes, but again the most abundant material was brown unidentifiable matter. Preference experiments demonstrated that the shrimps would exploit any organic material source available, but choice experiments were made impossible by similarity of gut contents between different diets.

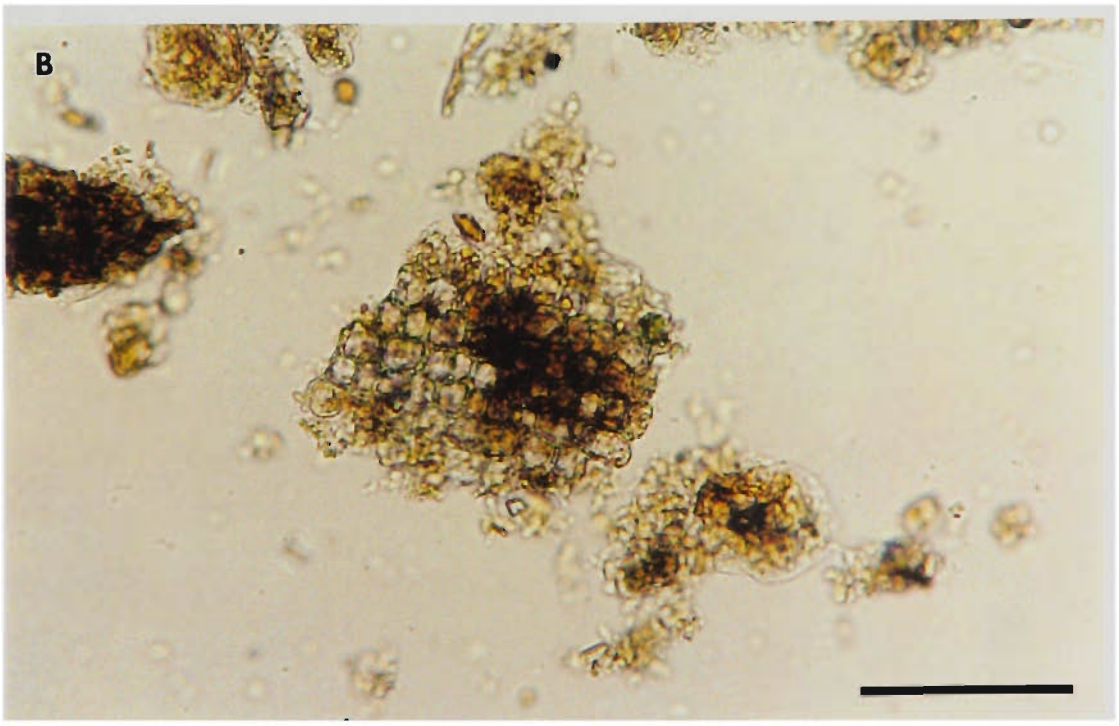
Figure 3.1.1

Contents of the digestive tracts of *Biffarius arenosus* individuals collected from Warneet, Western Port in July 1994.

(A) A typical gut content sample showing sand grains and brown unidentifiable organic matter. Scale bar = 100 μm.

(B) A sheet of vascular plant cells found in the digestive tract of a *B. arenosus* specimen. Scale bar = 100 μm.


A


B

The origins of ingested organic material commonly found in the stomachs of animals are often difficult to trace (Kitting *et al.* 1984). Gut content analysis often only shows food items that have been macerated by mouthparts and/or partly digested, which make them difficult to identify. Some diet components may digest faster than others (usually the preferred food because it is easily digested) giving a biased view of the diet analysed from gut contents (Foale and Day 1992). Additionally, ingestion does not necessarily mean assimilation of those items (Zieman *et al.* 1984).

Gut contents of deposit feeders are particularly difficult to identify due to the complexity of the food ingested. A deposit-feeder's diet consists of a combination of sediment grains and adsorbed material, and detritus comprising plant substrate (cellulose and other structural compounds) and the bacteria, fungi, microalgae, protozoa and small animals attached (Deegan *et al.* 1990), and possibly land-derived organics (Conkright and Sackett 1986). My observations confirmed this idea, and I found that gut contents analysis gave little reliable information regarding exactly what was present. An alternative approach, stable isotope analysis was therefore trialed. Results for this analysis are shown in the next section.

3.2 Stable isotope analysis

3.2.1 Introduction

Stable isotope analysis provides an objective measure of the carbon source assimilated by a consumer (Fry and Sherr 1984, Peterson and Fry 1987, Michener and Schell 1994), and therefore is an excellent way to examine the *Biffarius arenosus* diet. The method is based on the transfer of an isotopic signature (eg. the ratio of $^{13}C/^{12}C$) from food source to the consumer, so that the signature of the consumer indicates feeding patterns of the recent past (Michener and Schell 1994). The success of the stable isotope approach in differentiating possible food sources depends on two major factors: that the animal tissue closely resembles the food source, and that the food source signatures are sufficiently distinct that the diet of the animals can be deduced (Fry *et al.* 1982, Currin *et al.* 1995). Separation of food sources from signatures has been one of the problems associated with the technique throughout its use. The power of interpretation is usually increased by analysing multiple stable isotopes, rather than relying on the information gained from a single isotope (Fry and Sherr 1984, Peterson and Fry 1987, Michener and Schell 1994).

Possible food sources for the *Biffarius arenosus* population in this study originate from both terrestrial and aquatic sources. The sandflat is bordered by saltmarshes and the mangrove *Avicennia marina*, and the seagrass *Heterozostera tasmanica* extends from the lower intertidal zone into the subtidal. Seasonal blooms of the macroalgae *Chordaria cladosiphon* (Phaeophyta) and *Enteromorpha* spp. (Chlorophyta) also may provide dietary carbon, along with microalgae such as seagrass epiphytes, benthic diatoms and sedimented phytoplankton.

Previous use of this method for examining the diet of deposit feeders in coastal habitats has revealed conflicting results (Kikuchi and Wada 1996, Primavera 1996, Riera *et al.* 1996). The major inputs (mangroves, seagrass, seagrass

epiphytes, macroalgae, microphytobenthos and pelagic phytoplankton) are all known to play a role in contributing carbon to the deposit-feeding food chain. Their inclusion in a diet depends on the consumer species, its feeding type and the presence of the potential food item. Previous investigations of other thalassinidean species indicate possible diets of benthic diatoms or phytoplankton, depending on feeding strategies and available food sources (Murphy and Kremer 1992, Rodelli *et al.* 1984, Schlacher and Wooldridge 1996).

This section of work aimed to use multiple stable isotope analysis to identify the primary carbon source of the *Biffarius arenosus* population from Warneet and to clarify the relative importance of the various potential food sources of this deposit feeding species.

3.2.2 Methods

This section of work was supported by VUT Seeding Grant USG 95/02 to P.I. Boon and F.L. Bird, and was done in collaboration with Dr. Stuart Bunn, Centre for Catchment and In-stream Research, Griffith University, Nathan, Australia, and my supervisor P.I. Boon. These results have been published as:
Boon, P.I., Bird, F.L., and Bunn, S. (1997). Organic carbon sources used by intertidal callianassid shrimps in Western Port (southern Australia), determined with multiple stable isotope analyses. *Marine and Freshwater Research* **48**, 503-511.

3.2.2.1 Sample collection

Samples of shrimps and major potential food sources were collected quarterly over a 2-year period. Mid-season sample dates (25 July and 24 October 1994, 9 January , 7 April, 1 August and 10 October 1995, 26 January and 2 April 1996) were chosen to identify any seasonal change in food supply or diet of *Biffarius arenosus*. On each sampling trip, 10 individual shrimp were collected with a bait pump, and at least five replicates of each potential food source. Whole seagrass plants were removed from the sediment, with rhizomes intact, and whole

specimens of macroalgae were collected whenever present on the sandflat. Individual mangrove trees were sampled randomly from the mangrove stand bordering the sandflat, with leaves, branches, and pneumatophores collected from each tree. Similarly, randomly selected specimens of the saltmarsh plant *Sarcocornia quinqueflora* were collected.

3.2.2.2 Laboratory methods

Shrimps were dissected to remove the digestive tract and contents, and abdominal muscle was extracted and frozen. Animals were not acid washed, due to the potential this procedure has for introducing artefacts (Bunn *et al.* 1995). All plant material was washed in filtered seawater to remove any contamination prior to processing. Seagrass was separated into leaves and leaf sheaths, roots and rhizomes, and epiphytes. Epiphytes were removed from the leaves by agitating the plant vigorously in a small volume of filtered seawater, and filtering the suspension onto pre-ashed (500 °C for 4 hours) GF-C filter discs (Bunn and Boon 1993). Pneumatophore epiphytes were scraped from the mangrove pneumatophores and analysed separately. All samples were frozen at -25 °C and freeze-dried at -28 °C for 32 h and then 10 °C for 15 h. The dried epiphytes were then peeled from the filter discs. All plant material was processed the same day it was collected. Individual samples of dried tissue were pulverised with a mortar and pestle, and stored in a desiccator until analysis.

3.2.2.3 Stable isotope analysis

Freeze dried samples were oxidised and the resultant CO_2 and N_2 analysed with a Europa Tracermass (Crew, UK) isotope ratio mass spectrometer. Isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) were compared to conventional standards (PDB carbonate and air N_2, respectively) as follows:

$$\delta\ (‰) = [\text{ratio(sample)/ratio(standard)-1}] \times 1000$$

to produce a delta (δ) value for ^{13}C or ^{15}N. The precision of isotope analyses was determined by analysing sucrose and ammonium sulphate standards and revealed an accuracy better than 0.3 ‰ and 1.0 ‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively.

3.2.2.4 Distinguishing potential food sources for *Biffarius arenosus*

A consumer will take up the signature of its food source, but enrichment often follows a trophic level increase (eg. Fry *et al.* 1982, Gearing *et al.* 1984, Harrigen *et al.* 1989) and must be considered when comparing shrimps and potential food source δ values. Carbon tends to be conservative, with only a minor enrichment (< 1 ‰) if any (Michener and Schell 1994), whereas nitrogen enrichment is usually ~ +3 ‰ (DeNiro and Epstein 1981).

In this study possible food sources were those with a mean $\delta^{13}C$ signature within the range 2 ‰ less than or 1 ‰ greater than the mean shrimp signature, and with a mean $\delta^{15}N$ signature within the range 1-5 ‰ less than the mean shrimp signature (Bunn and Boon 1993). When a mixture of two food sources was a possibility, a simple mixing model for the various foods was applied (Bunn and Boon 1993):

$$P_A = (\delta Consumer - f - \delta B)/ (\delta A - \delta B)$$

where,

P_A = proportion of source A

f = isotopic fractionation (‰) (consumer-diet)

δA = $\delta^{13}C$ or $\delta^{15}N$ of source A (‰)

δB = $\delta^{13}C$ or $\delta^{15}N$ of source B (‰)

In each case, a range of proportions was calculated for the predicted fractionation of between -1 and +2 ‰ for $\delta^{13}C$, and this range was verified using the $\delta^{15}N$ signatures. To ensure that all possible combinations of food sources were considered, a P_A value was calculated for each combination.

Temporal variation was analysed using a one-way ANOVA to compare between sampling dates. Data were checked for homogeneity of variances, using Cochran's C test, and normality, and no transformation was necessary.

3.2.3 Results

3.2.3.1 Isotopic signatures for *Biffarius arenosus* and potential food sources

The mean $\delta^{13}C$ for shrimps collected over the sampling period ranged from -14.4 $\pm$ 0.1 ‰ in April 1996 to -16.3 $\pm$ 0.3 ‰ in October 1994 (Table 3.2.1). A one-way ANOVA indicated significant temporal variation (df 7, MS 0.009, F-ratio 4.214, P 0.002), and a pair-wise Tukey's test revealed that shrimp samples in April 1996 were significantly more depleted in $\delta^{13}C$ than samples in October 94 and January 1996. Similarly, the $\delta^{15}N$ values also showed significant temporal variation (df 7, MS 0.04, F-ratio 2.763, P 0.024), but again the pattern was not consistent over seasons (Table 3.2.2). The range of $\delta^{15}N$ means for *Biffarius arenosus* included 5.3 $\pm$ 0.4 ‰ in April 1996 as a minimum, and 6.6 $\pm$ 0.2 ‰ in July 1994, as a maximum. $\delta^{13}C$ and $\delta^{15}N$ values, averaged over all sampling times, were -15.4 $\pm$ 0.2 ‰ and 6.0 $\pm$ 0.1 ‰, respectively.

Like the shrimps, the seagrasses were relatively enriched in ^{13}C (Table 3.2.1). Seagrass leaves, roots and rhizomes had similar signatures (Table 3.2.1), with a mean $\delta^{13}C$ for all samples being -13.1$\pm$ 1.4 ‰ (n = 50). Delta ^{15}N values of seagrass leaves and rhizomes were also similar, except that rhizome samples in April 1996 were more enriched than at other sampling times (Table 3.2.2). Mean $\delta^{15}N$ value, averaged over all components and sampling dates, was 4.2 $\pm$ 1.4 ‰ (n = 50).

Seagrass epiphytes had a mean $\delta^{13}C$ ranging from -15.8 $\pm$ 0.0 ‰ in August 1995 to 19.6 $\pm$ 0.9 ‰ in April 1996 (Table 3.2.1). Delta ^{15}N values ranged from 2.8 $\pm$ 0.6 ‰ in January 1996 to 8.3 $\pm$ 0.0 ‰ in August 1995 (Table 3.2.2). Averaged

Table 3.2.1. Carbon stable isotope signatures ($\delta^{13}C$) for *Biffarius arenosus* and its potential food sources at Warneet, Western Port. Shrimp and vegetation were collected seasonally between July 1994 and April 1996. Means ± SE are shown. n = 5 unless indicated in parentheses. nd = not determined.

	25 Jul 94	24 Oct 94	9 Jan 95	7 Apr 95	1 Aug 95	10 Oct 95	26 Jan 96	2 Apr 96
Shrimps	-15.3 ± 0.2 (3)	-16.3 ± 0.3 (4)	-15.0 ± 0.1 (4)	-14.9 ± 0.3 (3)	-15.4 ± 0.2(3)	-15.3 ± 0.1 (8)	-16.2 ± 0.6 (7)	-14.4 ± 0.1 (6)
Seagrass								
Leaves	-13.5 ± 0.2	-12.6 ± 0.1	-11.5 ± 0.2	-11.9 ± 0.5	-14.6 ± 0.1	-10.6 ± 0.4	-12.5 ± 0.5	-11.2 ± 0.1
Rhizomes & roots	nd	nd	nd	nd	nd	-10.2 ± 0.2	nd	-10.6 ± 0.1
Mangrove								
Leaves	-25.8 ± 0.3	-26.2 ± 0.1	-27.7 ± 0.4	-26.4 ± 0.2	-27.4 ± 0.3	-26.3 ± 0.1	-25.6 ± 0.3	-25.2 ± 0.5
Branches	nd	nd	nd	nd	nd	-25.4 ± 0.2	-26.1 ± 0.5	-25.2 ± 0.2
Pneumatophores	nd	nd	nd	nd	nd	-28.3 ± 1.0	-25.2 ± 0.5	-26.8 ± 0.8
Saltmarsh	nd	nd	nd	nd	nd	-27.0 ± 0.5 (3)	-27.7 ± 0.2	-26.6 ± 0.3
Algae								
C. cladosiphon	-17.8 ± 0.2	nd	nd	-19.4 ± 0.1	-19.4 ± 0.3	-19.6 ± 0.2	nd	nd
Enteromorpha spp.	nd	nd	nd	nd	-18.8 ± 0.0	-20.6 ± 0.4	nd	nd
Seagrass epiphytes	nd	nd	nd	nd	-15.8 ± 0.0 (1)	-16.2 ± 0.9	-17.5 ± 0.4 (2)	-19.6 ± 0.9
Mangrove epiphytes	nd	nd	nd	nd	nd	-20.1 ± 0.8 (3)	nd	nd

Table 3.2.2. Nitrogen stable isotope signatures ($\delta^{15}N$) for *Biffarius arenosus* and its potential food sources at Warneet, Western Port. Shrimp and vegetation were collected seasonally between July 1994 and April 1996.. Means ± SE are shown. n = 5 unless indicated in parentheses. nd = not determined.

	25 Jul 94	24 Oct 94	9 Jan 95	7 Apr 95	1 Aug 95	10 Oct 95	26 Jan 96	2 Apr 96
Shrimps	6.9 ± 0.6 (3)	6.6 ± 0.2 (4)	5.9 ± 0.2 (4)	5.7 ± 0.2 (3)	5.7 ± 0.2(3)	5.5 ± 0.1 (8)	6.4 ± 0.4 (7)	5.3 ± 0.4 (6)
Seagrass								
Leaves	3.6 ± 0.5	4.2 ± 0.1	2.2 ± 0.5	3.1 ± 0.4	4.6 ± 0.1	3.8 ± 0.4	2.4 ± 0.2 (4)	2.7 ± 0.2
Rhizomes & roots	nd	nd	nd	nd	nd	3.1 ± 0.3(4)	nd	7.1 ± 0.3
Mangrove								
Leaves	2.4 ± 0.3	2.7 ± 0.5	8.8 ± 0.1	3.7 ± 1.3	2.0 ± 0.2	2.6 ± 0.2	2.6 ± 0.3	2.2 ± 0.3
Branches	nd	nd	nd	nd	nd	1.2 ± 0.3	2.2 ± 0.1	3.6 ± 0.2
Pneumatophores	nd	nd	nd	nd	nd	2.3 ± 0.5	2.4 ± 0.5	3.3 ± 0.5
Saltmarsh	nd	nd	nd	nd	nd	2.7 ± 0.4 (3)	6.3 ± 0.4	4.1 ± 1.0
Algae								
C. cladosiphon	7.6 ± 0.5	nd	nd	5.8 ± 0.4 (4)	10.1 ± 0.4	8.9 ± 0.5	nd	nd
Enteromorpha spp.	nd	nd	nd	nd	12.0 ± 0.2	6.4 ± 0.4	nd	nd
Seagrass epiphytes	nd	nd	nd	nd	8.3 ± 0.0 (1)	5.4 ± 0.1 (6)	2.8 ± 0.6 (3)	5.1 ± 0.5
Mangrove epiphytes	nd	nd	nd	nd	nd	nd	nd	nd

over all sampling dates, $\delta^{13}C$ and $\delta^{15}N$ values were -17.7 ± 0.6 ‰ and 4.8 ± 0.4 ‰ respectively.

The two species of macroalgae analysed, *Chordaria cladosiphon* and *Enteromorpha* spp. had similar ^{13}C and ^{15}N signatures (Tables 3.2.1 and 3.2.2), except that *Enteromorpha* spp. was marginally more enriched in $\delta^{15}N$ than was *C. cladosiphon*. Overall mean values for *Enteromorpha* spp. were -19.7 ± 0.4 ‰ and 9.2 ± 1.0 ‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively, and -19.1 ± 0.2 ‰ and 8.2 ± 0.4 ‰ for $\delta^{13}C$ and $\delta^{15}N$ values of *C. cladosiphon*, respectively.

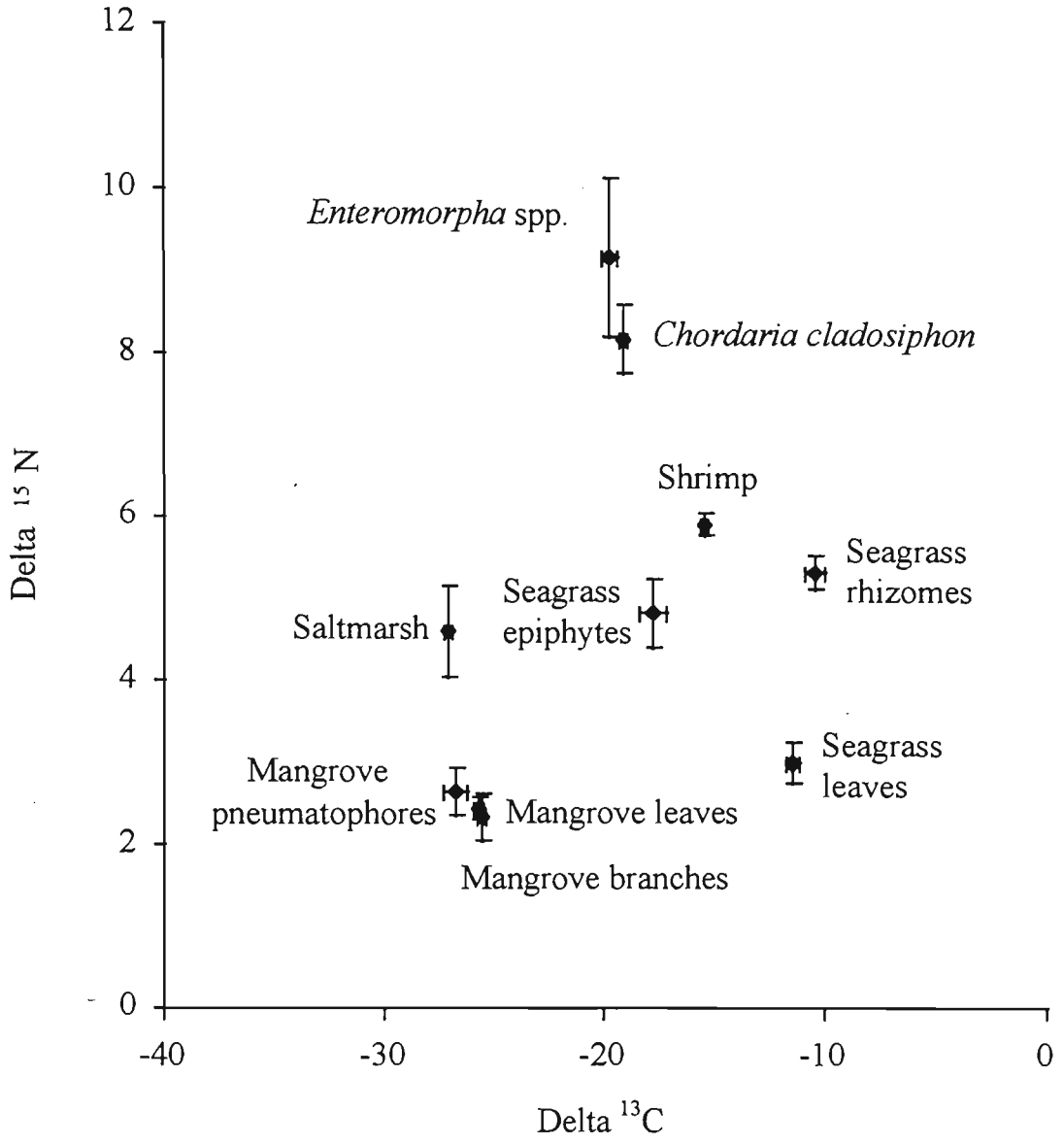
Isotopic ratios of all the terrestrial samples were relatively more depleted in ^{13}C than were aquatic potential food sources and were typical of plants which photosynthesise via the Calvin cycle. The saltmarsh plant, *Sarcocornia quinqueflora*, had a mean $\delta^{13}C$ signature of -27.1 ± 0.2 ‰, with little evidence of temporal variation (Table 3.2.1). The $\delta^{15}N$ value, however, showed greater variation, with values ranging from 2.7 ± 0.4 ‰ in October 1995 to 6.3 ± 0.4 ‰ in January 1996 (Table 3.2.2), and an overall mean of 4.6 ± 0.6 ‰. Mangroves had very similar $\delta^{13}C$ values as *S. quinqueflora* (overall mean -26.2 ± 1.0 ‰), but were relatively depleted in ^{15}N (overall mean 2.8 ± 1.9 ‰). The different mangrove components, leaves, branches and pneumatophores, had similar $\delta^{13}C$ and $\delta^{15}N$ values, and exhibited little temporal variation. The exception was the January 1995 leaf sample which was twice as enriched in ^{15}N as the other samples (Table 3.2.2). Mean $\delta^{13}C$ values of the epiphyte growing on the mangrove pneumatophores, were -20.1 ± 0.8 ‰. Unfortunately the sample was too small to measure $\delta^{15}N$.

3.2.3.2 Delimiting food sources

Both the carbon and nitrogen isotopic signatures of *Biffarius arenosus* were consistent with the utilisation of seagrass and seagrass epiphytes as the major carbon source (Figure 3.2.1). Utilisation of both sources of organic carbon were supported by a 1-2 ‰ fractionation in $\delta^{15}N$ values between the consumer and the food source, a shift well within the proposed limits. Pair-wise calculations

Figure 3.2.1

Delimitation of the potential food sources of organic carbon and nitrogen used by *Biffarius arenosus* in Western Port, southern Australia. Shrimp and vegetation were collected quarterly over a 2 year period between July 1994 and April 1996. Means $\pm$ SE are shown for both the vertical and horizontal axis..


12
10
8
6
4
2
0
-40
-30
-20
-10
0
Delta ^{15}N
Delta ^{13}C
Enteromorpha spp.
Chordaria cladosiphon
Shrimp
Seagrass rhizomes
Seagrass epiphytes
Saltmarsh
Seagrass leaves
Mangrove pneumatophores
Mangrove leaves
Mangrove branches

suggest possible contributions of between 7 and 71% by seagrass epiphytes and between 29 and 93% by seagrass to the shrimp carbon intake. The large ranges were expected because isotopic signatures of the two food sources are reasonably close together, and both were similar to that found for the consumer.

The high $\delta^{15}N$ values of both species of macroalgae preclude them as potential food sources for *Biffarius arenosus* (Figure 3.2.1). However, the terrestrial plants, *Avicennia marina* and *Sarcocornia quinqueflora*, may be potential sources of carbon, albeit minor ones. It is unlikely they would play a major dietary role because the ^{13}C fractionation between consumer and source is > 10 ‰ , a fractionation much higher than the proposed limits. If the terrestrial material did make a contribution, it could only be in combination with seagrass (because algae and seagrass epiphytes were too depleted in ^{13}C) and comprise a relatively minor component (no more than 25%) as calculated from the pair-wise combinations.

These results show the value of analysing multiple stable isotopes, If only $\delta\ ^{13}C$ was measured, results would indicate that the macroalgae was a likely food source of the shrimps. However the combination of both $\delta\ ^{13}C$ and $\delta^{15}N$ indicated that macroalgae were probably not utilised at all.

3.2.4 Discussion

Of all the possible food sources feeding the sandflat communities, the major source of carbon for *Biffarius arenosus* appears to be the seagrass and seagrass epiphytes. Feeding experiments showed that the shrimps are deposit feeders, ingesting sediment and organic material associated with the sediments. Therefore, whatever organic material was buried in the immediate vicinity of the burrow would be the major food supply. Seagrass and its associated epiphytes would probably be buried in sediments adjacent to and immediately surrounding the burrows, during a sedimentation event caused by rough weather, or some other disturbance. Macroalgae too, may be buried on the sandflat by a similar event,

Conversely, the terrestrial plant material would rarely be buried in the immediate vicinity of the burrows, chiefly because mangrove material is not often seen deposited on the sandflat. Van der Valk and Attiwill (1984) estimated that as much as 75% of the fallen leaf litter in Western Port was retained in the mangrove zone, buried rapidly by sediment, whereas the root litter was never exported.

Identifying seagrass and seagrass epiphyte carbon as the primary source for *Biffarius arenosus* is in agreement with recent work demonstrating the importance of seagrass carbon in the benthic food web of Western Port (Nichols *et al.* 1985, Edgar 1996). Similar dietary ties between seagrass and associated invertebrates (Thayer *et al.* 1978, Fry and Parker 1979, McConnaughey and McRoy 1979, Simenstad and Wissmar 1985, Fleming *et al.* 1990), and between seagrass epiphytes and seagrass communities (Fry *et al.* 1983, Fry 1984, Kitting *et al.* 1984) have been reported elsewhere. In some systems, mangroves also contribute dietary carbon to the localised invertebrate population (Rodelli *et al.* 1984, Robertson 1986, Rao *et al.* 1994), but this was not substantially evident for populations of *B. arenosus* in Western Port.

There have been three other stable isotope investigations of thalassinidean diets, and none found results similar to those of this study. The carbon source identified for *Callianassa kraussi* in the Gamtoos Estuary, South Africa, was the suspended particulate organic matter (SPOM) (Schlacher and Wooldridge 1996). The shrimps were strongly depleted in ^{13}C (-32.5 $\pm$ 0.3 ‰), with a similar $\delta^{13}C$ value to the SPOM (-31.2 $\pm$ 0.5 ‰), but clearly different from the other major carbon sources in marginal vascular plants (-25.7 ‰) and detritus (-24.1 ‰). *Callianassa kraussi* typically is a deposit feeder (Branch and Pringle 1987, Griffis and Suchanek 1987), but is also thought to have filter/suspension-feeding tendencies (Day 1981). In the Gamtoos Estuary, microalgae (phytoplankton and benthic) supply 87% of the organic carbon to the system (Schlacher and Wooldridge 1996), and *C. kraussi* is probably exploiting this high water column productivity by feeding off the SPOM brought into its burrows by the irrigation current. The filter-feeding species *Upogebia* sp. from a population in Malaysian

mangrove mudflat, also appears to derive its dietary carbon from phytoplankton (Rodelli *et al.* 1984). *Upogebia* sp. samples recorded a signature of -20.2 ‰, a value more similar to that of phytoplankton (-21.0 ‰) than those of mangroves (-27.1‰) and algae (-18.7 ‰), the other potential food sources (Rodelli *et al.* 1984).

The third thalassinidean study centred on the deposit feeding species *Neocallichrius* (as *Callianassa*) *rathbunae* and *Eucalliax* (as *Calliax*) *jonesi* in the Caribbean (Murphy and Kremer 1987). Possible food sources for this population were identified as seagrass and benthic microflora, and the $\delta^{13}C$ values (-19.2 ‰) indicated that the microalgae (-19.9 ‰) were more likely to be the primary food source than the seagrass (-6.3 ‰).

Additional food sources of *Biffarius arenosus* that were not sampled in this study, but were found to be nutritionally important for other thalassinidean species (Rodelli *et al.* 1984, Murphy and Kremer 1987, Schlacher and Wooldridge 1996), were the benthic diatoms which colonise surface sediments, and the phytoplankton deposited from the water column.

The inability to accurately isolate phytoplankton has represented a major obstacle to resolving the role of microalgae in aquatic food webs (Harrigan *et al.* 1989, Fontugne and Duplessey 1981, Boon *et al.* 1997). Most planktonic marine algae are small, relatively rare and difficult to separate from background particles (Fry 1996). Samples collected from the water column are more typically particulate organic material, comprising live and dead phytoplankton, bacteria and other components (Fry and Sherr 1984). Benthic microalgae, such as diatoms are also difficult to isolate from sediment samples, and only recently have novel techniques been employed to counteract this problem (Currin *et al.* 1995, Riera *et al.* 1996).

Successful sampling of phytoplankton by netting or filtering (eg. Fry and Wainwright 1991), depends on an abundance of phytoplankton, such as during a bloom, and a distinct lack of interfering materials, such as large amounts of

seston. The Western Port water column has a low chlorophyll *a* content, and very high loads of suspended solids (May and Stephens 1996), making sampling of phytoplankton in this study virtually impossible. Western Port suspended matter samples collected using a plankton tow by Nichols *et al.* (1985), were composed mainly of fine sediment and seagrass detritus, and small amounts of zooplankton. Stable isotope analysis of the suspended matter yielded a $\delta^{13}C$ value of - 12.9 ‰ (Nichols *et al.* 1985), a value quite different to the - 22 $\pm$ 5 ‰ average documented for marine phytoplankton (France 1995), and therefore reflects seagrass material not phytoplankton. The mean isotopic signature of phytoplankton was also quite different from the value measured for *Biffarius arenosus*, and France (1995) suggested that benthic invertebrates associated with seagrass beds with a $\delta^{13}C$ value > -16 ‰, probably derived their carbon source from the benthic food web. Conversely, if $\delta^{13}C$ < -18 ‰ , France (1995) suggested that the major carbon source of that consumer was pelagic (ie. phytoplankton). Hence it is reasonable to suggest that phytoplankton would play a minor, if any, role in providing dietary carbon to this population of *B. arenosus*.

Based on the published isotopic signatures for benthic diatoms however, this group may be an important carbon source for *Biffarius arenosus*. On average, benthic diatoms have a $\delta^{13}C$ value of -17.4 $\pm$ 4 ‰, (France 1995, see also Fry and Parker 1979) a value very similar to that gained for the seagrass epiphytes in this study, and therefore well within the possible food source of *B. arenosus*. Bulthuis and Woelkerling (1983) visually inspected the epiphytic material colonising *Heterozostera tasmanica* blades in Western Port, and found the population dominated by pennate diatoms, with smaller proportions of filamentous green and encrusting coralline red algae. This observation would suggest that epiphytic diatoms may contribute significantly to the diet of *B. arenosus*.

There are however, two lines of evidence which do not support the idea that benthic or epiphytic diatoms are a major food source of *Biffarius arenosus*. Firstly, gut contents of *B. arenosus* showed little evidence of a diet of diatoms, with frustules only rarely observed (section 3.1). If diatoms contributed

significantly to the diet, there would be consistent evidence of them in gut contents. For example, another thalassinidean species, *Neotrypea* (as *Callianassa*) *californiensis*, a known suspension feeder, had guts containing fresh plankton (Swinbanks and Murray 1981).

Secondly, for benthic diatoms to make a major contribution to the diet of *Biffarius arenosus*, they must be present, if not common, in the feeding environment. Murphy and Kremer (1992) found evidence of microalgal pigments to a depth of 10 cm in sediment inhabited by *Neocallichirus rathbunae* and *Eucalliax jonesi*, and stable isotope analysis showed that benthic diatoms contributed significantly to the diet of both species. Phospholipid fatty acid profiles of Warneet sediments indicated that benthic diatoms comprised only a minor portion of the sediment microbial community biomass (see section 5.3). A biomarker for benthic diatoms (20:5ω3) was apparent, but rare in surface, burrow wall and surrounding sediments. If *B. arenosus* feeds significantly on diatoms, they would be present in a much greater proportion in the burrow walls, and/or the surrounding subsurface sediment. An elevated biomass of diatoms has been previously documented in burrow walls of benthic fauna, caused by feeding and irrigation activities of the species (Steward *et al.* 1996). However, no such pattern was observed in *B. arenosus* burrow walls, so evidence suggests that the species does not feed principally on benthic diatoms.

When comparing isotopic values of food sources of deposit-feeding consumers, it is assumed that signatures of primary producers do not change during decomposition. Several studies have analysed fresh and decomposing material from saltmarsh plants, mangroves and seagrasses, and have found little, if any fractionation of carbon during decomposition (Haines and Montague 1979, Zieman *et al.* 1984, Stephenson *et al.* 1986, Primavera 1996), but Zieman *et al.* (1984) reported a marked decrease in ^{15}N during decomposition of mangrove leaves. This fact, however, does not alter interpretation of the results of this study.

The isotopic values of plant material measured in this study compare well to previous reports. Mangrove and saltmarsh vegetation had isotopic signatures (approximately -27 ‰) typical of plants fixing carbon via the C_3 pathway (Bender 1971), and were similar to those reported in earlier studies (Rodelli *et al.* 1984, Rao *et al.* 1994, Primavera 1996). Values measured for seagrass however, were not totally consistent with previous reports. Some reports of $\delta^{13}C$ values for *Heterozostera tasmanica* were very similar to the results of this study (McMillan *et al.* 1980, Raven *et al.* 1995), but $\delta^{13}C$ values measured by Nichols *et al.* (1985) were relatively more enriched (-8.7 ‰). The finding that seagrasses were more enriched in ^{13}C than their epiphytes was also consistent with many earlier reports (Fry *et al.* 1983, Fry 1984, Kitting *et al.* 1984, Nichols *et al.* 1985).

3.3 Where is food collected from?

3.3.1 Introduction

It is well known that some species of thalassinidean shrimp collect plant material from the sediment-water interface (Suchanek 1983, Dworschak and Ott 1993, Nickell and Atkinson 1995). Material is usually captured at the burrow entrance and is either directly ingested, worked into the burrow wall or stored in chambers to promote bacterial growth for food (Dworschak 1987, Griffis and Chavez 1988, Dworschak and Ott 1993). Such an activity would ensure an adequate food supply in organic-poor sediments.

The major food sources for *Biffarius arenosus* have been identified on the basis of stable isotope analyses as seagrass and seagrass epiphytes, but the mechanism by which the shrimp obtains this material is unknown. If plant material was collected from the sediment surface by *B. arenosus* and stored in its burrows, the shrimps would be enhancing decomposition processes and bacterial productivity by burying reactive organic material at depth. This behaviour would provide a consistent food supply for the burrow inhabitant, regardless of quality of the local food supply. If this type of activity is prevalent in *B. arenosus*, individuals should display behaviours indicative of feeding plasticity under experimental conditions, such as collection of surface material to enhance subsurface food supply.

The aim of this section of work is to identify where *Biffarius arenosus* collects its food from, and whether this behaviour alters with changes in subsurface food supply.

3.3.2 Methods

To determine if *Biffarius arenosus* leaves its burrow to collect plant material from the surface, a series of 4 treatments was established. Six tanks were filled with

organic 'poor' sediment (washed builder's sand, 0 % organic carbon) and 6 with organic 'rich' sediment (sandflat sediment, 0.34% organic carbon). Three tanks were nominated as 'controls', without shrimps, and 3 as 'experimental' with a single shrimp added. Shrimps were left to burrow for 3 days prior to the experiment start. Four measured lengths of seagrass (8 cm long) were added to the sediment water interface in all replicates, and any changes in length or shape were observed over time (measurements made twice weekly). The experiment was run for 6 weeks.

3.3.3 Results and discussion

Over the 6-week period, there was no evidence of the shrimp leaving their burrows to collect food. The seagrass strips did not alter in length or shape over time, and there was no evidence of shrimp track marks on the sediment surface. Therefore, even individuals inhabiting the organic-poor sediments (0 % organic carbon) did not utilise the surface food source.

Drift-catching thalassinideans have distinctive burrow structures (Griffis and Suchanek 1991). Burrows are characteristically simple, long and relatively straight, and often deep (Suchanek 1985). 'Drift-catchers' burrows also usually have chambers at the base for storage of plant material (Farrow 1971, Pemberton *et al.* 1976, Suchanek 1985, Dworschak and Ott 1993). This burrow structure differs greatly from the complex series of tunnels and chambers described for *Biffarius arenosus*. However, burrow structure alone cannot predict feeding mode of a species.

Individuals of a species of thalassinidean shrimp sometimes display variability in feeding behaviour. *Calocaris macandreae* is primarily a deposit-feeder and rarely seen leaving its burrows. However, few observations indicated that the species may at times utilise food from the sediment surface (Nash *et al.* 1984). Some evidence of surface material collection by *Callianassa subterranea* prompted Nickell and Atkinson (1995) to suggest that the species was also capable of

utilising different food sources depending on the environmental conditions. If this was the case for *Biffarius arenosus*, then shrimps inhabiting organic-poor sediment should display behaviours consistent with exploitation of a alternative food source. Such behaviours were however, not observed.

Overall, the observation that *Biffarius arenosus* rarely, if ever, leaves its burrow, combined with burrow shape and lack of plant material in burrows cast *in situ*, suggest that the species locates all its food below the sediment surface. However, the question still remains: "How do the shrimps obtain their food from the sediment?".

3.4 Do burrows indicate active searching for food?

3.4.1 Introduction

Biffarius arenosus does not appear to leave its burrow to scavenge food, and hence must find adequate nutrition below the water-sediment interface. Stamhuis *et al.* (1996) suggested that the main function of burrowing in the deposit-feeding species *Callianassa subterranea* was mining for food. Sediments, however, are typically nutritionally poor containing < 5% organic matter (Lopez and Levinton 1983), and in Western Port were < 1% (section 4.1). The organic material present is probably of low food quality and often difficult to digest (Berrie 1976). Microbes which colonise and decompose this material, however, improve the nutritional value by combining it with essential nutrients from other sources (Fenchel and Harrison 1976, Fenchel and Jorgensen 1977, Jumars *et al.* 1990). Organic material in marine sediments would typically be in layers due to burial at the sediment surface and subsequent stratification (Reichardt *et al.* 1991), but activity of resident bioturbators often mixes it vertically and horizontally through the sediments (Berner and Westrich 1985, Andersen and Kristensen 1991).

Grimm and Föllmi (1994) suggested that the fossilised burrow structures of thalassinidean ancestors indicated active searching for food. Straight, unbranched vertical tunnels were observed penetrating organic-poor layers, thereby providing access to deeper organic-rich sediments, where burrows branched into a series of horizontal tunnels and chambers. It was suggested that this burrowing behaviour was propelled by olfactory cues, where the organic-rich sediment layer gave off some kind of olfactory signal to the burrowing shrimps (Grimm and Föllmi 1994). Reports of extant species also suggest that the 'mine-like' construction of deposit feeding burrows indicates active searching for organic-rich layers within the sediment matrix (Dworschak and Pervesler 1988, Dworschak 1987, Dworschak and Rodrigues 1997), but few have experimentally tested this idea.

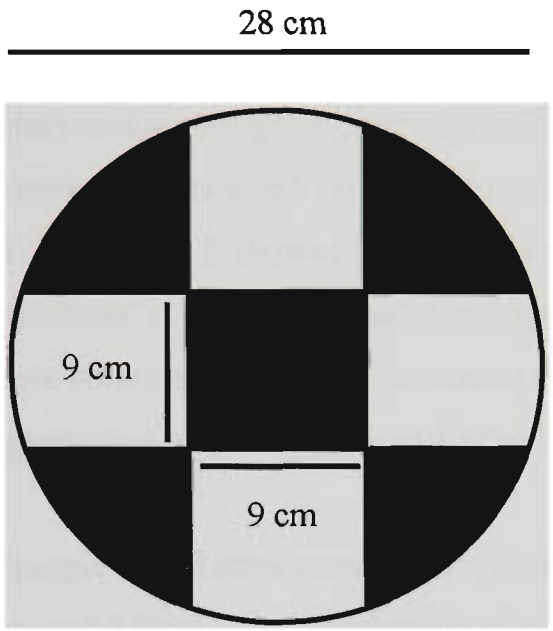
Biffarius arenosus burrows resemble this 'mine-like' construction, so it is reasonable to suggest that the species may actively search for food during burrowing activity. To test this hypothesis, an experiment was designed with alternating columns of organic-rich and poor sediment. It was proposed that if shrimp burrows did indicate active searching for organic-rich deposits, then burrows constructed in organic-poor sediment would be more complex than burrows constructed in organic-rich sediment. Additionally, if a burrow spanned more than one sediment column, the major proportion of it would be concentrated in the organic-rich column.

3.4.2 Methods

A series of vertical columns of alternating organic-rich and poor sediment was created in buckets. This design was chosen to highlight any directional change in burrowing/feeding activity associated with a junction in sediment organic content. Organic 'rich' sediment (sandflat sediment, 0.34% organic carbon) and organic 'poor' sediment (sandflat sediment combusted at 500 °C for 4 hours, 0.02% organic carbon), were added to buckets using a dividing frame, resulting in a 'chequerboard design' of columns with 9 cm sides (Figure 3.4.1). Six buckets were established, 3 with organic-poor sediment in the centre, and 3 with organic-rich sediment in the centre. Seawater was carefully added to the buckets to avoid disturbance of the sediments, and a single shrimp added after 12 hours settling time. The experiment was run for 8 weeks, after which time the water was drained off, and burrows were cast with resin (method described in section 3.2). After 48 hours the casts were removed from the sediment, with careful observations of the position of column junctions on the cast.

Figure 3.4.1

Design of the experiment investigating whether shrimp burrows indicate active searching for food. Solid and open regions represent alternating columns of organic-rich and organic-poor sediment, respectively.



Top view

Side view

3.4.3 Results and discussion

Even though only three of the burrow castings were successful, results do show a clear pattern (Figure 3.4.2). All three casts indicated that the shrimps burrowed in the immediate area below the burrow opening. Burrow A was mainly constructed in an organic-poor column except for two small tunnel extensions into the adjacent organic-rich columns (Figure 3.4.2). Similarly, burrow C was constructed in an organic-poor sediment column except the periphery of two tunnels/chambers which extended into the adjacent organic-rich column (Figure 3.4.2). Burrow B showed the same pattern, except in reverse. The burrow was constructed in organic-rich sediment, and extended to the base of the bucket, where horizontal burrowing intersected two different sediment columns, one organic-poor and one organic-rich (Figure 3.4.2).

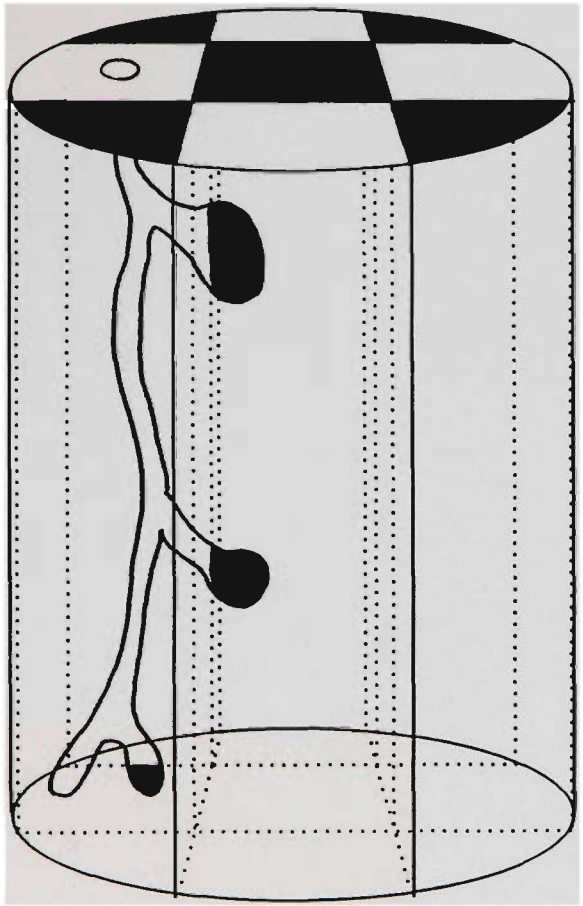
A sample size of three casts was not ideal, and the experiment would have been repeated if there was any indication of active searching in this first experiment. However, in all casts collected, there appears to be no evidence of selection for or against any particular sediment. It was interesting to note that casts did not contain the characteristic U shape and the second opening shaft. It is possible that this opening was not permanently maintained, but was opened whenever sediment needed to be ejected from the burrow, as observed in *Callianassa subterranea* (Nickell and Atkinson 1995).

If the spiral section of burrow combined with the series of tunnels and small chambers does not play some role in searching for food within the sediments, then why are burrows so shaped? This design appears most likely to represent maximal exploitation of a given volume of sediment similar to *Callianassa subterranea* (Nickell and Atkinson 1995). Another consideration is that the regular spirals described in *Axianassa* burrows are thought to indicate that organic matter is evenly distributed throughout the sediment layer the spiral is constructed in(Dworschak and Rodrigues 1997). This idea is supported by the

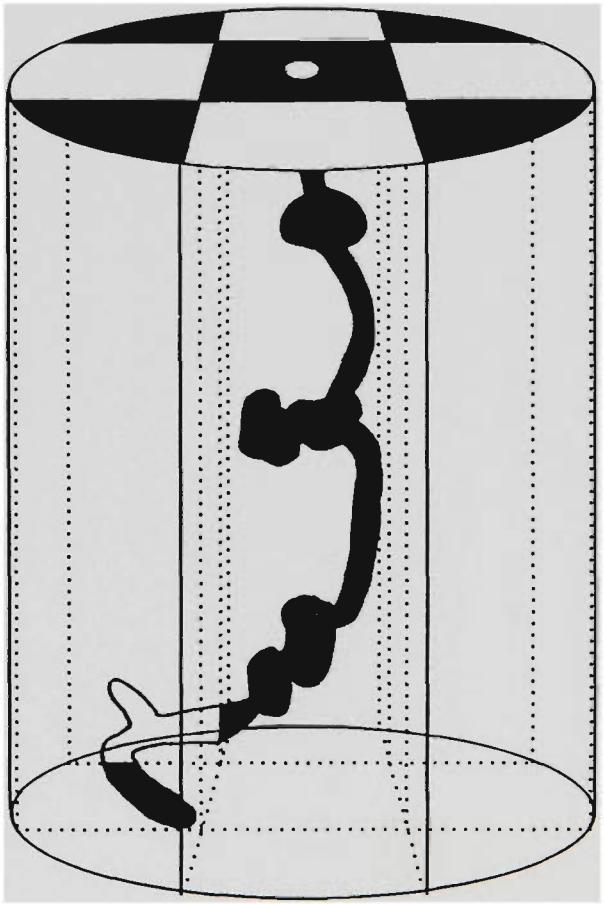
Figure 3.4.2

Results of the experiment investigating whether shrimp burrows indicate active searching for food showing burrow cast shape and position in the three replicate tanks of alternating columns of organic-rich (solid) and organic-poor (open) sediment columns. The casts are coloured relative to the zone of sediment they were constructed in.

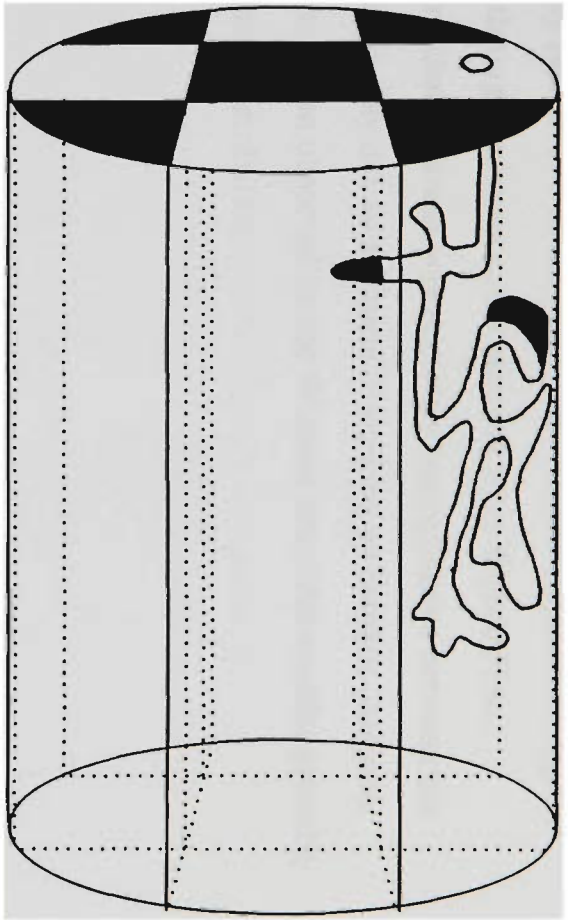
A.



B.



C.



observation that concentrations of buried plant material (such as clumps of seagrass) were rarely seen *in situ* at the study site. An investigation of organic matter distribution through sediment cores sampled from Woods Hole, Massachusetts, found that sediment was relatively homogeneous between the depths 2-20 cm, presumably due to sediment reworking by benthos (Johnson 1977). Intense bioturbation observed on the Warneet intertidal sandflat probably causes a similar organic distribution.

3.5 Sediment sorting

3.5.1 Introduction

Intensive biological reworking of marine sediments has profound effects on a wide range of variables. Impacts are often species specific (Meadows and Tait 1989, Davis 1993, Jones and Jago 1993) depending on burrowing and feeding activities and burrow shape or size.

Sediment particle size distributions can be altered by bioturbating fauna, with finer fractions often being more intensively mixed (Wheatcroft and Jumars 1987). Many chemical properties of sediment are correlated with grain size, so any spatial segregation of particle types would result in a change in chemical composition and reaction rates (Aller 1982). Physical factors such as permeability (Meadows and Tait 1989, Meadows and Hariri 1991, Jones and Jago 1993), shear strength (Meadows and Tait 1989, Meadows and Hariri 1991) and resuspension of sediment (Rhoads *et al.* 1978, Colin *et al.* 1986, Wallin and Håkanson 1992, Davis 1993) all increase in the presence of bioturbating fauna. Effects on sediment stability are not as defined, with evidence for and against stabilisation of sediment, depending on the bioturbating fauna present (Eckman *et al.* 1981, Grant and Dayborn 1994). Sediment reworking may also reintroduce polluted sediment into the water column (Nalepa and Landrum 1988, Officer and Lynch 1989), where contaminants such as radionuclides may be incorporated into the food chain (McMurty *et al.* 1985, Colin *et al.* 1986, Suchanek and Colin 1986, Suchanek *et al.* 1986).

Biogenic sediment reworking also affects the local biological community, and again response of individual species to sediment disturbance depends on the species (Woodin and Marinelli 1991, Dittman 1996). Species of infauna may be inhibited or restricted by biogenic sediment disturbance due to the disruption of feeding apparatus or the changed nature of reworked sediment (Rhoads and Young 1970), whereas the deeper zone of oxygenated and less compacted

sediment may enhance infaunal numbers and diversity (Flint and Kalke 1986). Several studies have addressed the impacts of thalassinidean activity on surrounding fauna, and nearly all have shown negative effects (Tamakai 1988, Riddle *et al.* 1990, Bird 1982, Posey 1986, Posey *et al.* 1991). Bird (1982), however, did find that numbers of commensal organisms inhabiting burrows were enhanced, along with the density of free burrowing amphipods, which obviously benefited from the disturbance.

Of all the bioturbating fauna, thalassinideans, especially, have a large impact on sediment properties, due to their extensive burrow systems and sediment reworking capabilities. However, the impact of the suite of southern Australian species is unknown. Because processing effects are species and site specific, it is difficult, if not impossible, to extrapolate from northern hemisphere studies. Hence, investigating the sediment turnover rates of *Biffarius arenosus* and some effects of the reworking on sediment properties, is an integral part of this larger project studying burrowing and feeding ecology of the species.

3.5.2 Methods

3.5.2.1 Population density

The density of the *Biffarius arenosus* population inhabiting the intertidal sandflat at Warneet, was investigated by counting the number of individuals in a 0.5 m^3 block of sediment. Sediment was excavated from a 0.5 m^2 square marked on the sediment surface, down to a depth of approximately 0.5 m. At this depth, sediment type changed from silty-sand to clay, which was impossible to break-up and sieve. Sampling did not extend into this clay layer, because it existed at the approximate maximum depth reached by the burrows. The sand was sifted through a 1 cm mesh and all individuals removed and counted, and the counts converted to a density m^{-2}. To investigate seasonal variation, ten replicates were sieved at each of 2 dates: 22 December 1994 and 6 July 1995, and seasonal densities were compared with a one-way ANOVA. Data were checked for

homogeneity of variances, using Cochran's C test, and normality and ln(x+1) transformed.

3.5.2.2 Sediment ejection rates

Patterns of sediment ejected from burrows were investigated using sediment traps. Traps were made from small, round plastic food containers, with a 1 cm diameter hole punched in the base. A square was cut in the lid and sealed with plankton netting, to allow the trap to fill with water during high tide, while excluding suspended material. A burrow was randomly chosen, the trap was placed over the opening, and held in place with wire stakes (Figure 3.5.1a). At each high tide change, a new burrow was selected to avoid any possible 'trap effects'. Trapped sediment (Figure 3.5.1b) was emptied after each high tide into plastic bags and the sediment was rinsed, dried at 70 °C and weighed.

Three sets of experiments were undertaken, each treatment sampling from 10 burrow replicates. In February 1995, periodicity in ejecta rates was examined by comparing sediment ejected during low and high tides, treatments (traps over burrow openings) were compared to controls (traps over no opening) and diurnal patterns of activity were investigated by comparing sediment ejected during light (7 am to 8 pm) and dark (8 pm to 7 am) hours. Traps were deployed over a series of 4 days, with a total of 2 high versus low tide experiments, 8 treatment-versus-control experiments, and 4 diurnal experiments. In July 1995, a further 5 treatment versus control experiments were run over a series of 4 days, to examine seasonal variation in amount of sediment ejected from burrows.

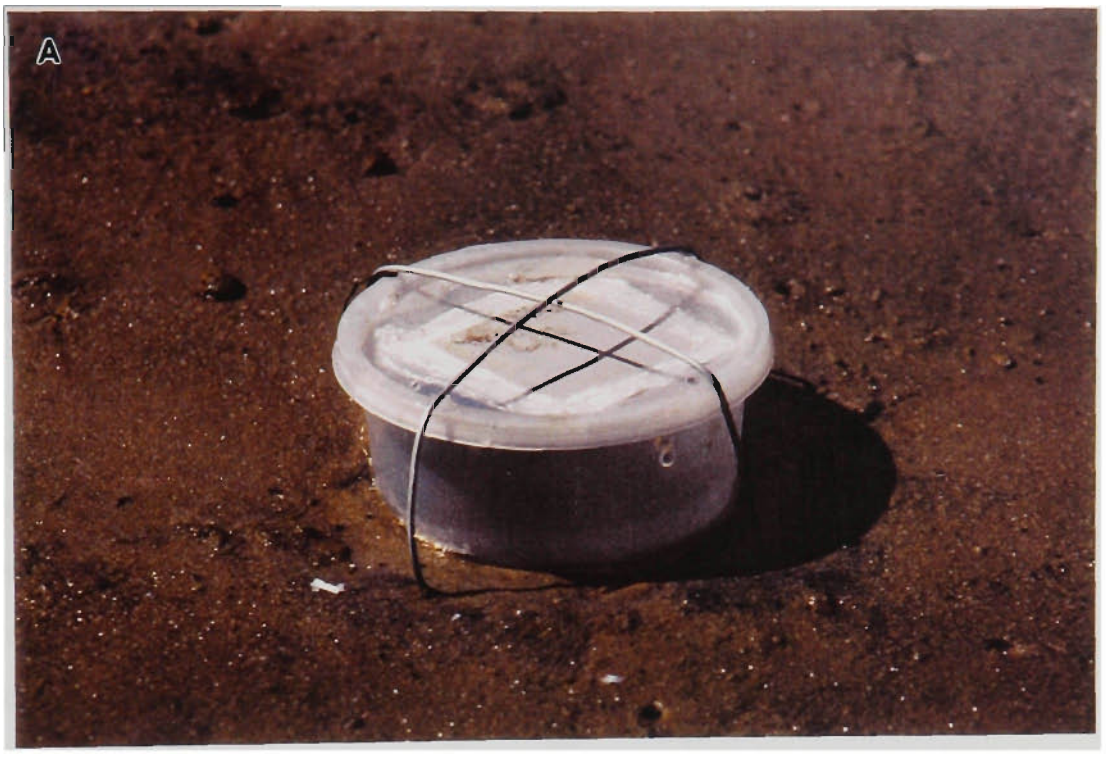
Statistical analysis of the data was performed with the Statistica ® software package. The amount of sediment ejected from burrows during light and dark hours was compared, with a 2 way ANOVA with one random and one fixed factor (run and time, respectively). Seasonal ejecta rates were compared using a 3 way ANOVA with two fixed factors and one random factor (season, treatment and run, respectively). Data were checked for homogeneity of variances and normality, and ln(x+1) transformed.

Figure 3.5.1

The sediment traps used to collect sediment ejected from the burrows of *Biffarius arenosus* on the Warneet sandflat, Western Port, in February and July 1995.

(A)A sediment trap in position on the sandflat. The hole, punched in the centre of the base, was placed over a burrow opening, the lid was attached and the whole trap held in position with wire stacks.

(B) An ejecta mound successfully collected in a sediment trap after 1 tide cycle.


A


B

3.5.2.3 Particle size

Samples of ejecta mound, burrow wall, subsurface and surface sediment were collected from 10 burrow replicates to compare particle size distributions in the different sediment zones. Each sample was oven-dried at 70 °C and weighed, and then washed through a nested sieve series of 1000, 500, 250, 125 and 63 μm mesh sizes. Sediment collected in each sieve was oven dried and weighed, and converted to a proportion of the total sample (by weight).

Statistical analyses were performed with the Primer ® software package (Clarke and Warwick 1994). Results were arcsin transformed to reduce the domination of the very high values, and the particle size distributions of the four sediment types were compared using the Bray-Curtis similarity measure. Representation of the distributions was by non-metric multi-dimensional scaling. To test the null hypothesis (H_o), that there is no significant difference between sediment type distributions, an analysis of similarities was used. This analysis followed 3 steps. Firstly, a statistic (R) was calculated which reflected the observed differences between sediment types, compared with differences among replicates within sediment types. Secondly, R was recalculated after the data labels were randomly redistributed between samples, creating every possible permutation of the data. The recalculation was done 20,000 times. The significance level of differences between sediment types was calculated by comparing R to its permutation distribution. If the observed R appeared unlikely to have come from the permutation distribution, then the H_o was rejected, and vica versa. So, if t of the T simulated values of simulated R are greater than or equal to the observed R, then H_o can be rejected at a significance level of $100(t+1)/(T+1)\%$.

3.5.3 Results

3.5.3.1 Population density

The density of *Biffarius arenosus* did not significantly differ between seasons (df 1, F 0.58, P 0.46). Mean density was calculated at 8.7 individuals m^{-2}.

3.5.3.2 Sediment ejection rates

Even numbers of replicate traps were set out at each run, but some ejecta samples were omitted due to movement or damage of individual traps during the high tide. Unequal sample sizes resulted in an unbalanced data set, thereby decreasing the power of the statistical tests used to analyse the data. The power of a statistical test is the probability of finding a difference when one is present, so a reduction in power increase the chances of a Type II error, where a null hypothesis (stating that there is no difference) is accepted when it is false (Zar 1984). Power of the ANOVA tests appears to be sufficient for all single effects (results are either highly significant or highly non-significant) (Tables 3.5.1 and 3.5.2), but may have been a problem for detecting the interactions between the season and run, and the season, treatment and run effects (Table 3.5.2).

Table 3.5.1. Statistical analysis of sediment ejected from burrows of *Biffarius arenosus* during day and night high tides. Samples were collected from Warneet, Western Port, over a period of 4 days in February 1995.

Effect	df	Mean square	F-ratio	Probability
Day/Night	1	0.122	2.599	0.205
Run	3	0.246	3.329	0.025
Day/Night x Run	3	0.047	0.638	0.593
Error	60	0.074		

Table 3.5.2. Statistical analysis of sediment collected in traps set over burrows of *Biffarius arenosus* (treatment) or bare sediment (control). Samples were collected from Warneet, Western Port, over a period of 5 days in February and July 1995.

Effect	df	Mean square	F-ratio	Probability
Season	1	0.455	1.695	0.263
Treatment	1	5.840	86.532	0.001
Run	4	0.151	1.320	0.265
Season x treatment	1	0.426	1.584	0.277
Season x run	4	0.268	2.333	0.058
Treatment x run	4	0.067	0.587	0.673
Season x treatment x run	4	0.269	2.339	0.058
Error	147	0.115		

Sediment was ejected from the burrows during high tide, regardless of the time of day. Initially, traps were set at low and high tide, but after two runs it was obvious that no activity occurred when burrows were exposed during low tide (Figure 3.5.2). Henceforth, traps were only set during high tide. No significant difference was measured in sediment ejected during day and night high tides (Table 3.5.1), confirming the absence of any diurnal patterns (Figure 3.5.3).

Significantly more sediment was collected in traps set over burrow openings (6.1 $\pm$ 0.6 g dw trap^{-1}), than in traps set over bare sediment (3.0 $\pm$ 0.3 g dw trap^{-1}) (Table 3.5.2). This result demonstrated that the traps were collecting sediment ejected from burrows, and not merely collecting material washed into traps. Sediment ejected did not vary significantly between February and July (Table 3.5.2), suggesting that the shrimps were actively feeding and burrowing year round. Variation around the means (Figure 3.5.4) was quite high, most likely due to a range of shrimp sizes being present within the population. Different sized shrimps would process different amounts of sediment.

Overall, the mean sediment processing rate was 4.26 $\pm$ 1.2 g dw sediment shrimp^{-1} day^{-1}. If this is scaled up to the population level, with 8.7 shrimps m^{-2}, this predicts that 74 g dw sediment m^{-2} day^{-2} is ejected on the sandflat. Over a year, this rate would be approximately 27 kg dw sediment m^{-2} year^{-1}, equating to approximately 4% of the sediment in 1m^2 being returned to the surface, assuming that there is 75 x 10^4 g sediment m^{-2} in 0.5 m^3 and no seasonality in processing rates. This means that the sandflat is turned over to a 50 cm depth (burrow depth) every 25 years by *Biffarius arenosus* alone. The other bioturbating fauna present on the sandflat would probably compound this effect.

3.5.3.3 Particle size

Differences in particle size composition of mound, burrow wall, subsurface and surface sediments are displayed in Figure 3.5.5. The largest dissimilarity was that in ejecta mound samples, 64% of the total weight was composed of particles larger than 250 μm size, whereas the same size range only made up 45-50% in all

Figure 3.5.2

A comparison of sediment collected in traps during high and low tides. Traps were set over openings of the burrows of *Biffarius arenosus* to collect sediment ejected. Collections were made in February 1995 at Warneet, Western Port. Means + SE are shown, and n is labelled above each error bar.

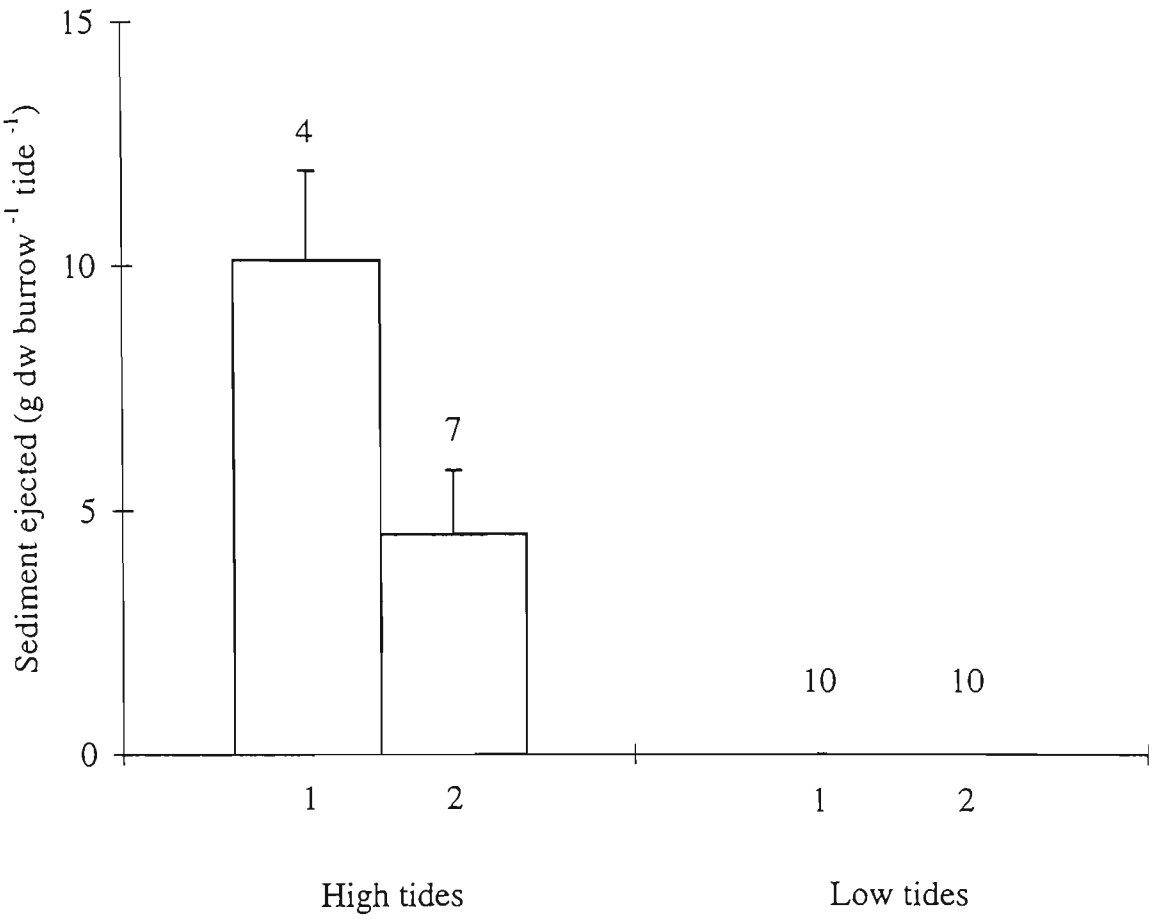

15
10
5
0
Sediment ejected (g dw burrow -1 tide -1)
4
7
10
10
1
2
1
2
High tides
Low tides

Figure 3.5.3

Sediment ejected from burrows of *Biffarius arenosus* during day and night high tides. Sediment collections were made in February 1995 at Warneet, Western Port. Means + SE are shown, and n is labelled above each error bar.

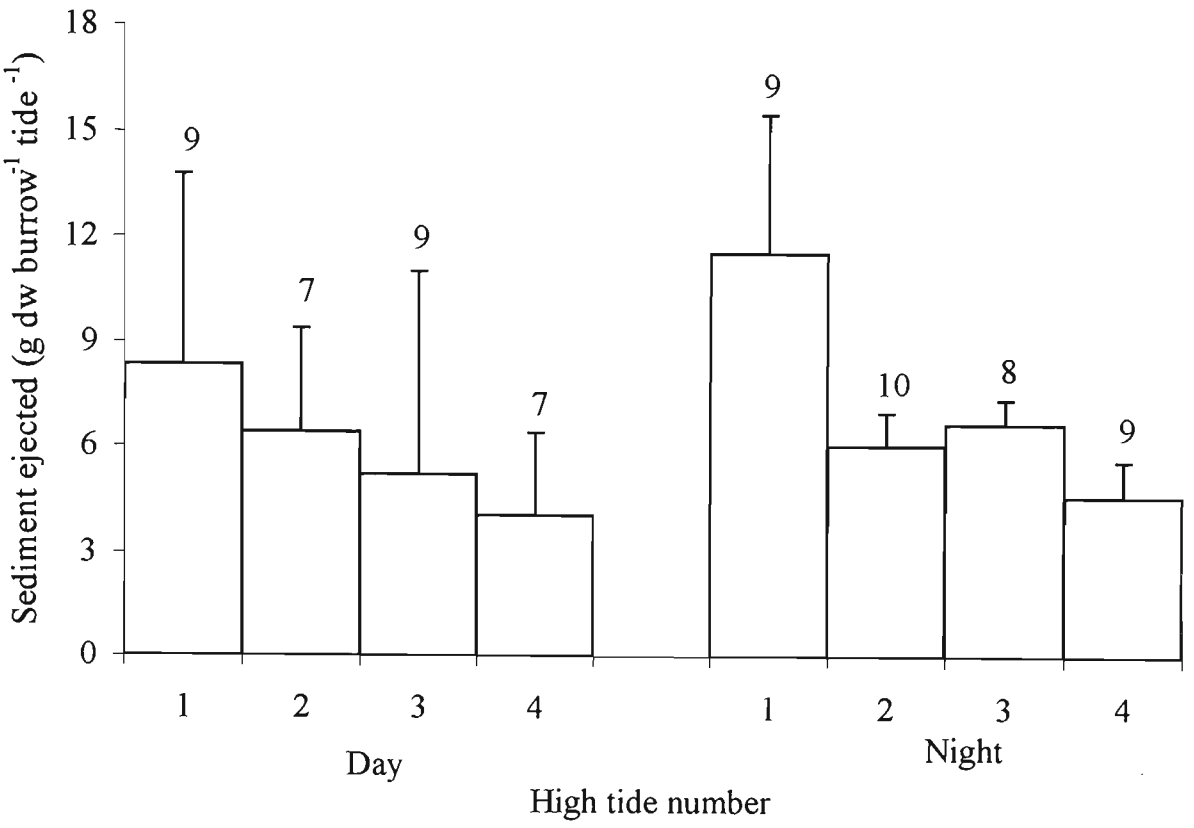

Sediment ejected (g dw burrow^{-1} tide^{-1})
18
15
12
9
6
3
0
9
7
9
7
9
10
8
9
1
2
3
4
1
2
3
4
Day
Night
High tide number

Figure 3.5.4

A comparison of sediment collected in traps over burrows of *Biffarius arenosus* (solid bars), and traps positioned on bare sediment with no burrow opening (open bars), over two seasons: summer (February) and winter (July). Traps were set in February and July 1995 at Warneet, Western Port. Means + SE are shown, and n is labelled above each error bar.

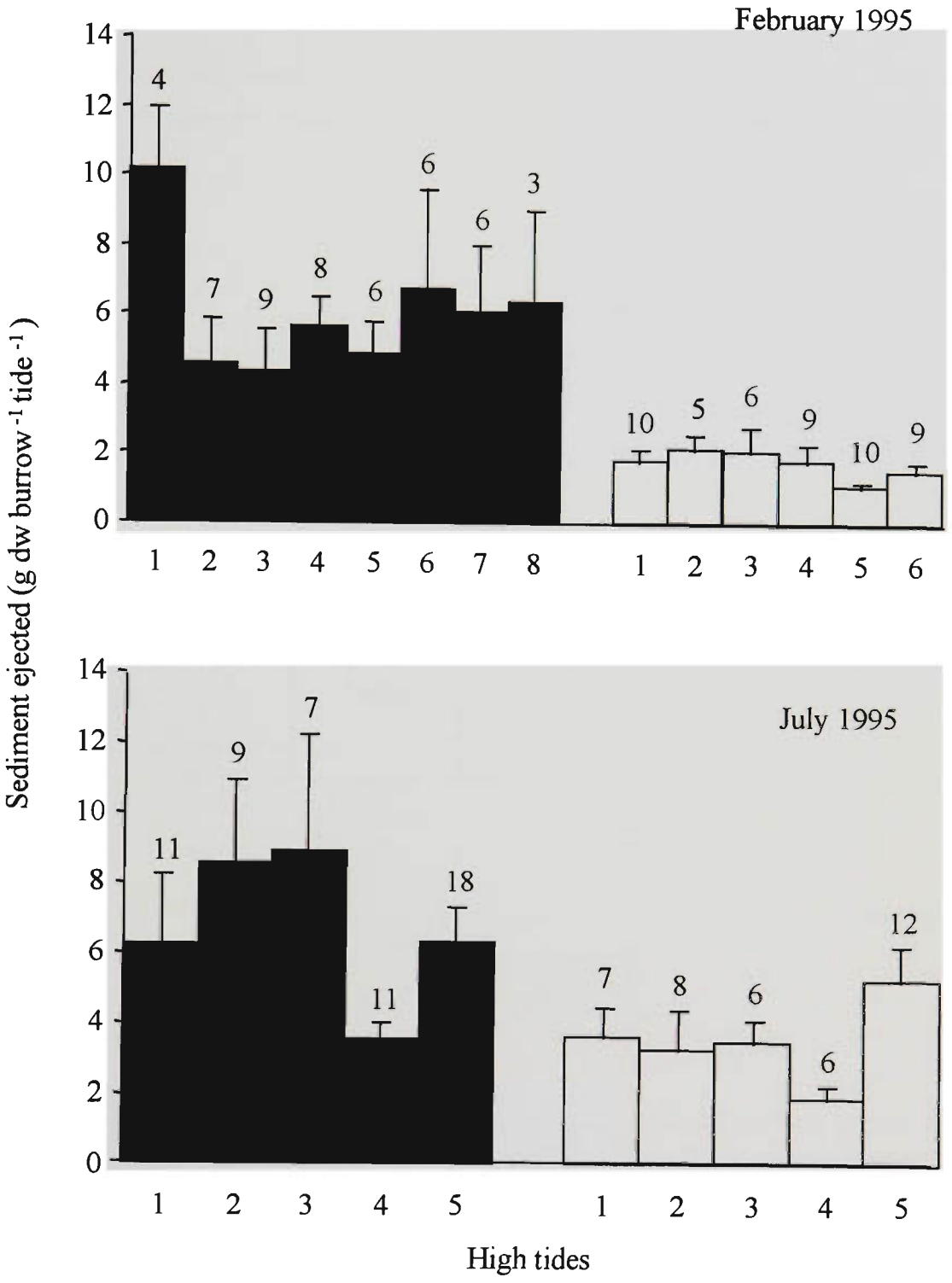
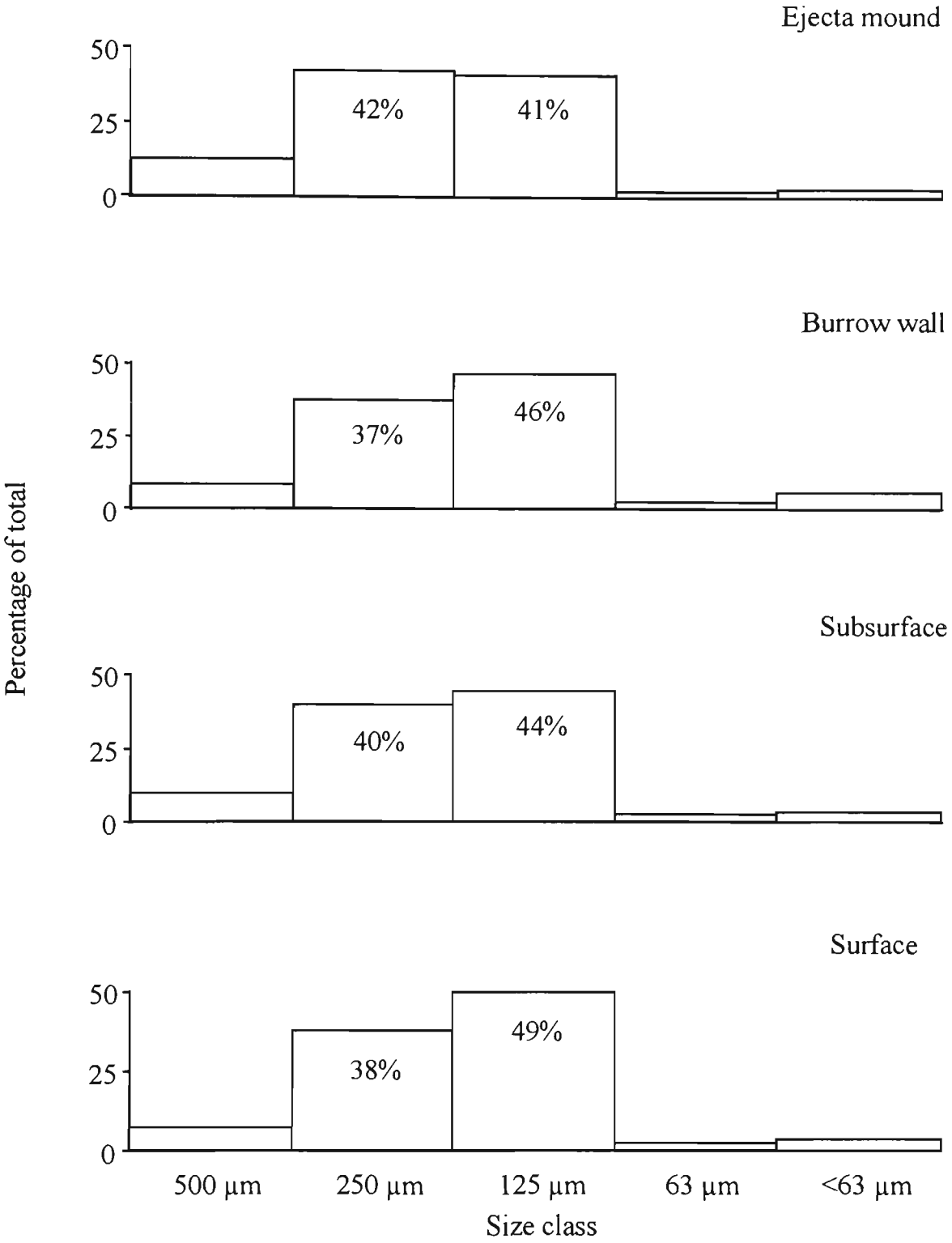

February 1995
July 1995
Sediment ejected (g dw burrow -1 tide -1)
High tides
0
2
4
6
8
10
12
14
1 2 3 4 5 6 7 8
1 2 3 4 5 6
1 2 3 4 5
1 2 3 4 5
4
7
9
8
6
6
6
3
10
5
6
9
10
9
11
9
7
11
18
7
8
6
6
12

Figure 3.5.5

Proportions of sediment size classes present in ejecta mound, burrow wall, subsurface and surface sediments associated with the burrows of *Biffarius arenosus*. Sediment was sampled in January 1997 from Warneet, Western Port.


Ejecta mound
50
25
0
42%
41%
Burrow wall
50
25
0
37%
46%
Subsurface
50
25
0
40%
44%
Surface
50
25
0
38%
49%
Percentage of total
500 µm
250 µm
125 µm
63 µm
<63 µm
Size class

other sediment types (Figure 3.5.5). It appears that the shrimps are selectively ejecting larger particle sizes (>250 μm) from their burrows. Particle size distribution from the ejecta mound was significantly different to distributions found in all of the other sediment types (Table 3.5.3). Burrow wall samples had a marginally higher proportion (6%) of the fine particle sizes (<63 μm) than the other sediment types (3-4%) (Figure 3.5.5), but overall, burrow walls did not differ significantly from subsurface or surface sediments (Table 3.5.3). Subsurface and surface sediments, however differed significantly (Table 3.5.3).

Table 3.5.3. Statistical analysis of similarities between particle size distribution of ejecta mound, burrow wall, subsurface and surface sediments associated with the burrows of *Biffarius arenosus*. Overall and pairwise comparisons of observed R value to its permutation distribution. The overall comparison tests if any differences exist between treatments, and the pairwise tests differences between pairs of sediment types. The probability assigned to reject the H_o is 0.05.

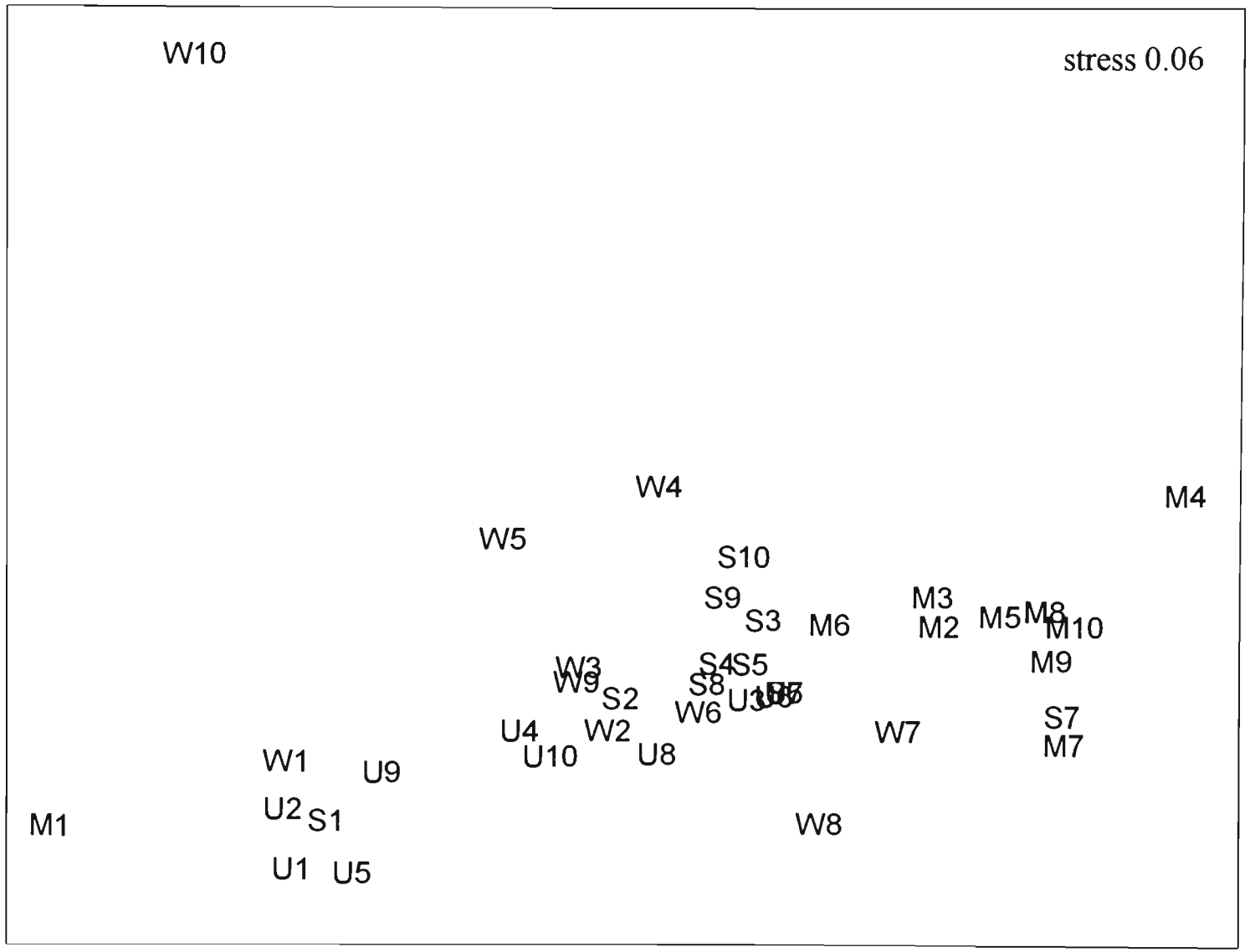
Comparisons	R	Possible permutations	Permutations used (T)	No. of significant statistics (t)	Probability
Overall comparison (Global test)	0.27	1.96E+20	20000	0	< 0.001
Mound/Wall	0.41	92380	20000	3	< 0.001
Mound/Subsurface	0.36	92380	20000	16	< 0.001
Mound/Surface	0.56	92380	20000	6	< 0.001
Wall/Subsurface	0.07	92380	20000	1447	0.072
Wall/Surface	0.06	92380	20000	2934	0.147
Subsurface/Surface	0.18	92380	20000	633	0.032

Representation of the comparisons in 2-dimensional space (Figure 3.5.6), shows that ejecta mound samples are indeed clearly separated from other sediments types. The exception is replicate 1, where all four sediment samples are grouped together in the bottom left corner. The particle size distributions within sediment types in this sample were remarkably similar.

Figure 3.5.6

Similarities between particle size distributions of ejecta mound, burrow wall, subsurface and surface sediments associated with the burrows of *Biffarius arenosus* sampled in January 1997 at Warneet, Western Port.

Abbreviations: M = mound, W = burrow wall, S = subsurface sediment, U = surface sediment. Numbers represent the replicate burrows


W10
stress 0.06
W4
M4
W5
S10
S9
M3
S3
M6
M2
M5
M8
M10
M9
W3
S4
S5
W9
S8
S2
W6
S7
U4
W2
W7
M7
U10
U8
W1
U9
U2
S1
M1
W8
U1
U5

Particle size distribution in burrow wall samples appeared to be intermediate between subsurface and surface samples (Figures 3.5.5 and 3.5.6), a result supported by the statistical comparison (Table 3.5.3). Burrow wall replicate 10 was clearly separated from all other samples (Figure 3.5.6), due to an order of magnitude higher proportion of very fine sediments (<63 μm size class).

3.5.4 Discussion

3.5.4.1 Population density

The *Biffarius arenosus* density measured on this intertidal sandflat of Western Port, was similar to that estimated by Coleman and Poore (1980) from a bay-wide study. A mean density of 12 individuals m^{-2} was estimated for the sandy and muddy sediments shallower than 10 m inhabited by *B. arenosus* in Western Port (Coleman and Poore 1980).

3.5.4.2 Sediment ejection rates

Several studies have investigated sediment processing by thalassinideans, but due to the problems associated with comparing results gained using different methods and units (see review in Rowden and Jones 1993), few studies were directly comparable to these results. Tropical species are known to eject between 71 and 507 g dw sediment $shrimp^{-1}$ day^{-1} (Suchanek 1983, Roberts *et al.* 1981, Vaugelas *et al.* 1986), with some species' rates varying spatially, depending on organic content of sediment or seagrass coverage (Roberts *et al.* 1981, Vaugelas *et al.* 1986). Suchanek *et al.* (1986) suggested an interaction between activity and organic content of sediment, where a shrimp residing in an organic poor environment would need to process a larger volume of sediment to gain adequate nutrition.

The larger temperate species *Neotrypea* (as *Callianassa*) *californiensis* ejects a mean 24 ± 12 g dw sediment $shrimp^{-1}$ day^{-1} from its burrows in British Columbia (Swinbanks and Luternauer 1987), six times the amount processed by *Biffarius*

.arenosus. Sediment ejection rates measured for the small temperate species, *Callianassa subterranea*, however, were much more comparable. Rowden *et al.* (1997) calculated mean ejection rates ranging from 0.16 g dw sediment shrimp^{-1} day^{-1} in winter to 4.8 g dw sediment shrimp^{-1} day^{-1} in summer. The maximal rates were higher than those measured by Witbaard and Duineveld (1989) for the same species. Rowden *et al.* (1997) documented the influence of body size on rate of sediment ejection, with larger individuals ejecting relatively more sediment from burrows. This observation supports the presence of large variation around the mean found in this study.

Activity patterns observed in the present study concurred with previous reports. *Neocallichirus* (as *Callianassa*) *rathbunae* also displayed no diurnal activity patterns (Suchanek 1983), and the lugworm *Arenicola marina* was only actively processing sediment when burrows were submerged (Retraubun *et al.* 1996). No documentation of sediment processing patterns by other intertidal populations of thalassinideans was found, so no comparisons could be made. The only reference to ejection periodicity was for a subtidal population of *Callianassa subterranea*, where shrimp activity was described as a complex pattern of active/inactive periods (Rowden *et al.* 1997).

Seasonal variation in ejection rates was observed in *Callianassa subterranea* (Rowden *et al.* 1997), and *Neotrypea* (as *Callianassa*) *californiensis* (Posey 1987), with increased activity in warmer months. Other reports of seasonality in infaunal activity have also found a positive correlation between activity and temperature in polychaetes (Gordon 1966, Cadee 1976, 1979) and other worms (Retraubun *et al.* 1996). Posey (1987) suggested that a decrease in activity relative to a drop in temperature could be attributed to a simultaneous decrease in salinity of the overlying water.

3.5.4.2 Particle size

Sediment ejected from burrows by *Biffarius arenosus* did not have the same particle size distribution as the surrounding sediment. Particles with diameters of

250 μm, and to a lesser extent 500 μm (these two size classes are the largest), were selectively ejected from burrows by the shrimps. These size classes of sediment particle are similarly ejected from burrows of other thalassinidean species. Most species, such as *Callichirus laurae*, expelled sediments less than 2 mm in diameter (Vaugelas and Buscail 1990) from their burrows. *Neocallichirus* (as *Callianassa*) *rathbunae* and *Eucalliax* (as *Callianassa*) *quadracuta* ejected sediments up to 1.4 mm size, but favoured a smaller particle of 135 μm diameter (Suchanek 1983). An unidentified *Callianassa* sp. studied by Suchanek *et al.* (1986), ejected anything up to 1 mm diameter, but favoured particles sized 250-350 μm a similar size range as *Biffarius arenosus*. *Glypturus armatus* was found to eject particles smaller than 200 μm, and store anything larger (Vaugelas *et al.* 1986). In comparison, *Glypturus acanthochirus* ejected particles larger than 1 mm from its burrows (Dworschak and Ott 1993),

The selective ejection (in terms of particle size) of sediment from burrows, significantly alters properties of surface sediments (Suchanek *et al.* 1986, Dworschak and Ott 1993). A constant supply of fine sediments to the sediment-water interface, along with the burial of coarser particles, would modify sediment stratigraphy (Vaugelas *et al.* 1986). Such a pattern was found in core samples taken in zones of high callianassid density (Suchanek 1983). Particle size distributions of subsurface and surface sediments were significantly different in this study, with surface sediment containing relatively more particles of the size 125 μm. This size class domination did not, however, match with sediments selectively ejected from burrows, so some other factors such as sediment mixing by other burrowing fauna inhabiting the same sandflat must also be affecting surface sediment particle size distributions.

Unlike most other reports of thalassinidean burrow linings, particle size in *Biffarius arenosus* walls was not significantly different to size distributions found in subsurface and surface sediments. The species appears not to selectively incorporate fine particles into the burrow walls. In some replicates a slight increase in very fine particles (<63 μm) was observed, but this result was not

significant overall. Several studies report a fine-grained or silt lining in burrow walls of unidentified *Callianassa* spp. (Shinn 1968, Braithwaite and Talbot 1972, Tudhope and Scoffin 1984, Suchanek *et al.* 1986), and more detailed studies show that burrow walls contain a greater proportion of particles sized ≤ 63 μm (Vaugelas and Buscail 1990, Dworschak and Ott 1993). An elevated proportion of 13-16% of this size range was recorded in *Sergio* (as *Callianassa*) *trilobata* walls compared with 3% in ambient sediments (Dobbs and Guckert 1988). In comparison, *Upogebia pusilla* burrow linings contained a greater proportion of larger particles sized between 500 and 250 μm than surrounding sediments, although this result was thought not to be due to selection by the animal (Dworschak 1983).

A concentration of fine particles in burrow linings would alter the chemical properties of the burrow. It is well known that small particles have a greater surface area for a given volume and therefore higher physiochemical activity (Rashid 1985). Fine sediment particles have an enormous capacity for adsorbing organic material, which in turn associates with reactive materials like metals and radioisotopes. Both materials are known to concentrate on mucus-bound, organic rich thalassinidean burrow linings (Whitehead *et al.* 1988, Over 1990), with concentration often correlating negatively with particle size (Abu-Hilal *et al.* 1988). *Biffarius arenosus* does not consistently concentrate fine particles in its burrow walls, and therefore would have little effect on chemical properties in that regard.

3.6 Chapter summary

Gut contents revealed that *Biffarius arenosus* was a deposit feeder, ingesting sediment grains and organic material collected from the subsurface sediment matrix. The actual source of food was difficult to determine from gut analysis, but multiple stable isotope analysis identified the most likely carbon source for *B. arenosus* as seagrass and seagrass epiphytes.

In the presence of a depleted food supply, *Biffarius arenosus* did not leave its burrow at any time to scavenge food from the sediment surface, indicating that food was collected solely from subsurface deposits. It was thought that burrows may have indicated active searching for organic-rich deposits, as an individual exploited the food supply in its local environment, but no evidence was found to support this.

The ejection of an average 4.26 g dw sediment shrimp^{-1} day^{-1} also indicates that sediment was collected from subsurface deposits and processed for food. The ejection of sediment from burrows occurred only during high tide when the burrows were submerged. Sediments were sorted during processing, with a relatively higher proportion of particles > 250 μm particles being ejected from burrows. This selective ejection did not appear to alter surface sediment particle size distribution significantly.

CHAPTER 4

PHYSICAL CHARACTERISTICS OF THE BURROW ENVIRONMENT

4.1 Organic carbon content

4.1.1 Introduction

Due to the continual rain of organic matter from the water column, surface sediment layers often have the highest concentration of organic matter and the greatest decomposition rates (Jorgensen 1983). Generally only a small proportion of the organic material arriving at the sediment-water interface is buried (Jorgensen 1983, Balzer 1984), but this burial rate is increased by the burrowing and feeding activities of infauna (Aller 1982, Berner and Westrich 1985, Andersen and Kristensen 1991). Sediment reworking by bioturbating fauna mixes organic-rich surface layers with organic-poor subsurface layers, and the fauna may also add new reactive substrates such as mucus and faecal pellets (Kristensen 1988). Burrow walls often contain a relatively higher concentration of organic carbon than surrounding sediments (Aller and Yingst 1978, Kristensen *et al.* 1985, Steward *et al.* 1996). Combined with the oxic environment brought on by the irrigation of burrow water, the presence of reactive organic carbon at depth promotes aerobic decomposition. Additionally the interaction between

aerobic and anaerobic processes leads to a more complete mineralisation of organic material (Fenchel and Jorgensen 1977), suggesting that the redox discontinuity layer at the outer burrow wall is a zone of high metabolic activity.

A concentration of organic carbon has been found associated with thalassinidean burrow walls (Branch and Pringle 1987, Abu-Hilal *et al.* 1988, Vaugelas and Buscail 1990). This section investigates whether *Biffarius arenosus* accumulates organic carbon in its burrow walls by comparing the organic carbon content of wall, surrounding subsurface and surface sediments.

4.1.2 Methods

Sediment samples were collected from the burrow wall, surrounding and surface sediments on the following sample dates; 26 August 1994, 24 November 1994, 8 February 1995, and 19 May 1995. The sampling dates were evenly spaced throughout the calender year to test for any seasonal variation. Burrow walls were sampled by digging up a block of sediment on the sandflat at low tide, and shearing the block so that a burrow was exposed. The burrow wall could then be scraped out. Surrounding sediment samples were collected from the same block of sediment, 5 cm below the surface and 5 cm from the burrow. Surface sediments were scraped to approximately 2 mm deep adjacent to the burrow openings. Approximately 5 g (dry weight) of sediment was collected from each sediment type. Ten replicate burrows were sampled at each date, but only 8 were analysed in November because 2 replicates contained insufficient sample for analysis. Samples were stored in plastic vials and snap frozen in dry ice for transport back to the laboratory, where they were stored at -20 °C. Samples were freeze-dried for 48 h at -28 °C, followed by 20 h at 10 °C, and stored in a desiccator prior to analysis.

A wet digestion technique (dichromate titration) was used to measure organic carbon content of the sediment samples (Allen 1989). Measurement of organic carbon content by this method was checked by analysing 50 mg glucose. The

result indicated a recovery of 93% of the organic carbon present in the glucose sample.

Three dried sediment subsamples were analysed and combined to give a mean result for each sediment sample. Organic carbon content was analysed as follows: 2.5 g of freeze-dried sediment was weighed into 100 ml conical flasks to which was added exactly 20 ml $K_2Cr_2O_7$ (0.5N or 0.083M) and 30 ml of an acid mixture (5:1 v/v of H_2SO_4 and H_3PO_4). The sample was boiled for 30 min at 300 °C, then cooled for 20 min and the sides were rinsed with ultrafiltered water. 0.2 ml of the indicator reagent (*N*-phenylanthranilic acid dissolved in Na_2CO_3 solution) was added, and the sample was titrated with ferrous ammonium sulphate reagent (0.5M). The ferrous ammonium sulphate reacted with the remaining dichromate with an endpoint (colour change) of brown/green to aqua/emerald green. Blanks (no sediment) were run with each analysis. The calculation of percentage organic carbon content is as follows, if 1 ml 0.5N dichromate is equivalent to 1.5 mg organic carbon then:

$$\% \text{ Organic C} = \frac{\text{(blank-sample) ml x 0.15}}{\text{sample weight (g)}}$$

Statistical analysis

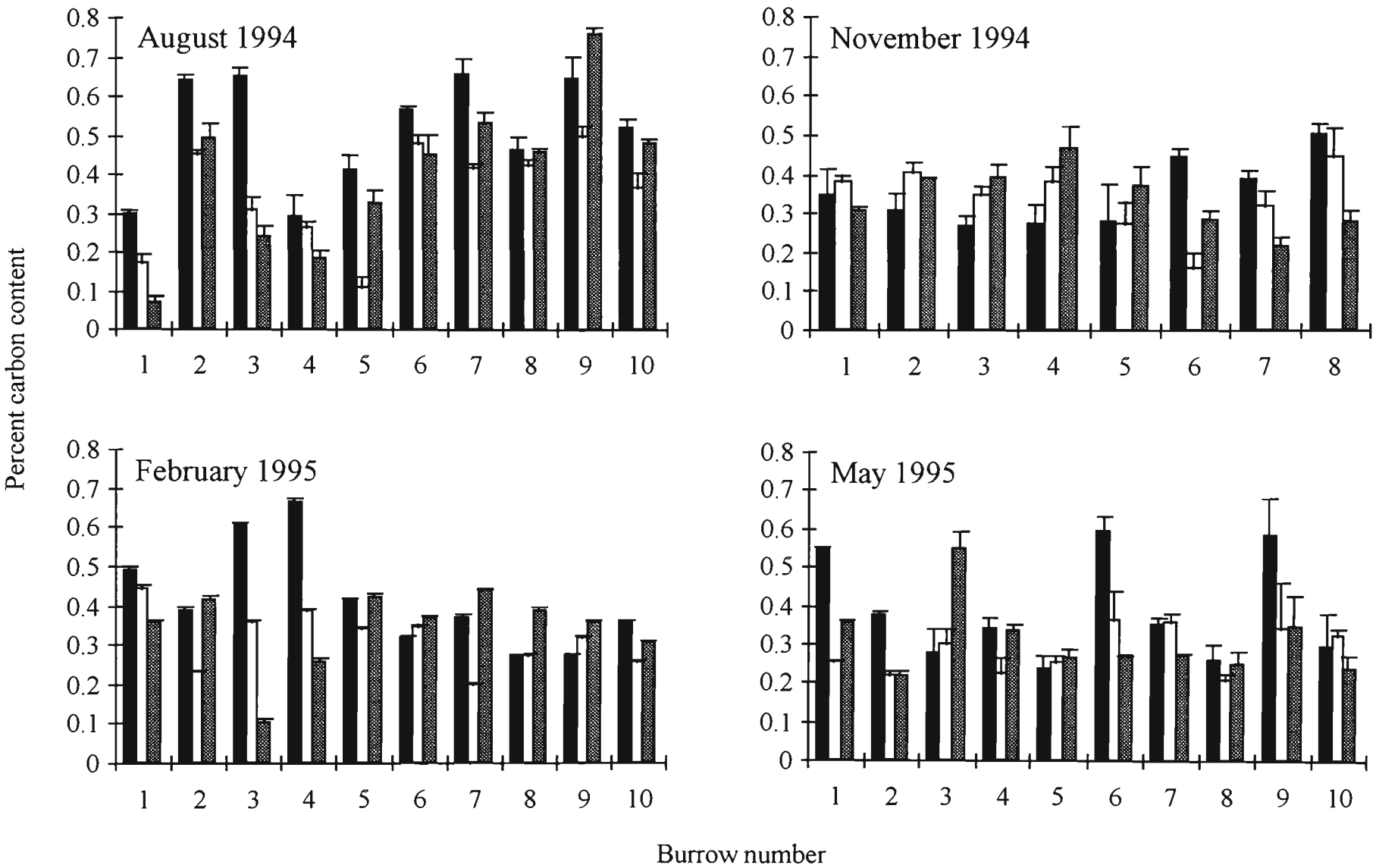
Statistical analysis of the data was performed with the Statistica ® software package. The statistical design was a two-way ANOVA with one random and nested factor. Three sediment types (burrow wall, surrounding subsurface, surface) were each sampled from the 8 randomly chosen replicate burrows, which in turn were nested within the 4 seasons. The data were checked for homogeneity of variances, using Cochran's Test, and normality, and ln(x+1) transformed.

4.1.3 Results

Very low concentrations of organic carbon were measured in all samples. The percentage contents ranged from 0.05 to 0.80% (Figure 4.1.1). No one sediment

Figure 4.1.1

Organic carbon content of burrow wall (solid), surrounding subsurface (open) and surface (shaded) sediments associated with the burrows of *Biffarius arenosus*. Sediments were sampled over 4 seasons between August 1994 and May 1995, from Warneet, Western Port. Means + SE are shown, and n = 3.


August 1994
November 1994
February 1995
May 1995
Percent carbon content
Burrow number
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8

type (burrow wall vs. surrounding subsurface vs. surface sediment) contained a greater concentration of organic carbon than any other. A significant difference between sediment types was found, but this difference varied between burrows, as indicated by the burrow x type interaction (Table 4.1.1). Additionally, no significant seasonal variation was observed (Table 4.1.1).

Table 4.1.1. Statistical results of a two-way ANOVA, with one random and nested factor (burrow), comparing organic carbon content measured in sediment associated with the burrows of *Biffarius arenosus*. Sediment samples were collected over 4 seasons between August 1994 and May 1995, from Warneet, Western Port.

Effect	df	Mean square	F ratio	P value
Season	3	0.348	0.576	0.636
Burrow	28	0.604	4.027	< 0.001
Sediment type	2	2.202	6.690	0.003
Season x type	6	0.222	0.672	0.672
Burrow x type	56	0.330	2.197	< 0.001
Error	184	0.150		

4.1.4 Discussion

Walls of the burrows of *Biffarius arenosus* did not contain a higher organic carbon content than the surface and subsurface sediments. Previous descriptions of thalassinidean burrow walls by other authors discussed features indicative of a high organic content, but rarely quantified it. The few studies that have made measurements found conflicting results. Abu-Hilal *et al.* (1988) documented a 2-10 times greater value of organic content in the mucus-rich wall than in adjacent subsurface sediments of the burrows of *Callichirus laurae* in the Gulf of Aqaba, Red Sea, while Vaugelas and Buscail (1990) found an 11-17 times greater value in the walls than in adjacent subsurface sediments in a nearby population of the same species. Organic carbon content of burrow walls of *Upogebia pusilla*, however, did not differ significantly from that of ambient sediment (Dworschak 1983).

There are several reasons why burrow walls would have a greater organic content than ambient sediment, each reason pertaining to a specific burrowing behaviour. Several species of thalassinidean, such as *Callichirus* (as *Callianassa*) *major*, *Upogebia pugettensis* and *Callichirus laurae*, line and support their burrow structures with mucopolysaccharides (Frey *et al.* 1978, Swinbanks and Murray 1981, Vaugelas and Buscail 1990). The presence of such compounds in burrow walls would increase organic content while providing additional substrate for microbial growth and colonisation (Kristensen 1988). The presence of mucopolysaccharides in *Biffarius arenosus* burrow walls will be investigated in the next section (4.2).

A negative correlation between sediment grain size and organic content, is well established (Hargrave 1972, Meyer-Reil *et al.* 1978, Lohse *et al.* 1996), so the selection of small sediment particles to line burrows would also be expected to enhance organic content of burrow walls. This pattern was prevalent in the burrow matrix of several thalassinidean species, where walls contained a more significant proportion of fine particles than ambient sediment (Shinn 1968, Braithwaite and Talbot 1972, Dworschak 1983, Suchanek *et al.* 1986, Abu-Hilal *et al.* 1988, Dobbs and Guckert 1988, Vaugelas and Buscail 1990, Dworschak and Ott 1993). Sediment particle size in *Biffarius arenosus* burrow walls was not significantly skewed towards organically enriched smaller particles (see section 3.5).

The presence of seagrass or algae detritus in burrows adds organic material to deeper sediment layers. Some thalassinidean species leave the burrow to collect plant material (Nickell and Atkinson 1995), while others position themselves at the burrow entrance and capture material floating past (Suchanek 1983, Dworschak and Ott 1993). Some burrows are specifically constructed with funnel-shaped entrances, to maximise capture of material (Suchanek *et al.* 1986), while all irrigated burrows would benefit from the suspended organic matter transported on the inward current (Witbaard and Duineveld 1989). Seagrass debris or bundles of leaves have been found incorporated directly into the burrow walls (Ott *et al.* 1976, Pemberton *et al.* 1976, Dworschak 1983, Branch and

Pringle 1987, Dworschak 1987, Griffis and Chavez 1988), or filling chambers (Dworschak 1987, Griffis and Chavez 1988, Dworschak and Ott 1993), tunnels (Shinn 1968, Farrow 1971, Braithwaite and Talbot 1972, Vaugelas and Buscail 1990), and deeper sections of the burrow (Ott *et al.* 1976). Storage of plant material within the burrow is often associated with the concept of 'gardening microbes', to enhance the burrow environment for deposit feeding (Ott *et al.* 1976, Dworschak 1987). No plant material was found in *Biffarius arenosus* burrows, either stored in chambers or worked into the burrow walls (see section 2.3).

During feeding, most infauna selectively concentrate richly organic material (Kristensen 1988), and their own mucus-bound faecal pellets are rapidly colonised by microbes (Hargrave 1970a). Hence, the storage of faecal material within burrows would represent another source of organic enrichment. Faecal pellets have been observed on tunnel floors (Dworschak 1983, Dworschak and Pervesler 1988), or stored in backfilled tunnels (Vaugelas and Buscail 1990, Nickell and Atkinson 1995) of some thalassinidean species' burrows. *Callichirus armatus* consolidates burrow walls with faecal pellets in poorly cohesive sands (Vaugelas *et al.* 1986). Occasionally small groups of faecal pellets were observed in turning chambers of *Biffarius arenosus* burrows, but most were expelled from the burrow via the irrigation current.

4.2 Burrow lining

4.2.1 Introduction

Burrows in marine sediments are often lined, presumably to consolidate the burrow structure and protect the inhabitant from a rough surface. Linings include the simple compaction of sediment due to repetitious movement of the inhabitant or animal growth (Dworschak 1983), the selection of specific sediment particle sizes which are consolidated into a lining (Dobbs and Guckert 1988), or the production of mucopolysaccharides which are used to cement the sediment particles together (Vaugelas and Buscail 1990). Presence of a burrow lining also appears to vary between sediment types, with the ghost shrimp *Glypturus armatus* building a thick lining in fine/medium sands, and none in muddy sediments (Vaugelas *et al.* 1986).

A burrow lining is known to affect diffusive permeability of the water-sediment interface (Aller 1983), which is important in predicting the influence of a speciesí burrow on diffusive flux from sediments. Permeability of sediments can be reduced 10-20% (Aller 1988) and 60-90% (Aller 1983) by the presence of burrow linings, depending on the taxon of marine invertebrate studied. Some burrow linings are also known to act as molecular sieves, with negatively charged regions repelling anions and attracting cations, thereby increasing cation diffusion relative to anion diffusion (Aller 1983). This property may be important in, for example, nitrogen-limited coastal environments, where NH_4^+ adsorbs to burrow linings and can be readily flushed into the overlying water via burrow irrigation.

In this larger study of *Biffarius arenosus* burrows, identifying the presence of a discrete burrow lining will assist with understanding overall effects on sediment properties. Both organic content of the wall, and diffusive flux across the burrow wall-water interface will depend on the physical nature of the lining and the presence of mucus bound sediment particles. Additionally the presence of mucopolysaccharides will affect the microbial properties of the sediments,

potentially enhancing biomass and activity of the resident microbial community. This section of work describes burrow wall properties and investigates the presence of mucopolysaccharides.

4.2.2 Methods

4.2.2.1 Physical description of burrow lining

General features of the burrow walls were documented by excavating 5 randomly chosen burrows at low tide on the sandflat at Warneet, Western Port. One centimetre long sections of wall and a similar sized segment of surrounding subsurface sediment were collected carefully to maintain the structure of the sediment matrix and stored in plastic vials. The wall and sediment samples were examined with a Zeiss Stemi 2000C dissecting microscope and a qualitative description made.

4.2.2.2 Presence of mucopolysaccharides

The periodic acid-Schiff (PAS) reaction was used to investigate mucopolysaccharide content of burrow linings. This reaction is based on the oxidative action of periodic acid (HIO_4) on glycol groups (present in glucose residue) which gives rise to aldehyde groups. The aldehyde groups then react with Schiffís reagent, producing a purple compound which indicates the presence of mucopolysaccharides (Junqueira *et al.* 1986).

A detailed description of the procedure is outlined in Whitlatch and Johnson (1974), and recipes for reagents in Humason (1967). Samples of burrow wall, subsurface and surface sediments were collected from 5 randomly selected burrow replicates on 29 April 1997, and preserved in 70% ethanol. For analysis, 0.1 g (wet weight) of sediment was added to a 10 ml plastic vial, and the sample was agitated in 1% aqueous periodic acid for 5 min, 70% ethanol for a further 5 min, and then washed in distilled water for 5 min. Staining occurred in Schiffís reagent for 10 min, after which the sample was agitated in sodium metabisulphite for 3 min, twice. The sample was again washed in distilled water for 5 min, twice, and

finally agitated in 70% ethanol for 30 min and left to stand overnight. The sediment was finally mounted in glycerol and the stained particles viewed under magnification.

4.2.3 Results

4.2.3.1 Physical description of burrow lining

Burrow linings were smooth and shiny, and appeared to be more compacted than were surrounding sediments (Figure 4.2.1a). Turning chambers had a rougher surface, compared with straight sections of tunnel, and sometimes contained loose sand grains or faecal pellets which had settled in the base (Figure 4.2.1b).

4.2.3.2 Presence of mucopolysaccharides

Strong staining for mucopolysaccharides was found in all sediment samples, yet burrow wall sediments appeared to contain no greater concentration of mucus than subsurface and surface sediment samples. This suggests that *Biffarius arenosus* was not contributing any extra mucus during burrow wall construction. Additionally, the presence of mucopolysaccharides throughout the sediment indicates that biological reworking of sediments by organisms other than *B. arenosus* (and the addition of mucus during reworking) was having a significant impact on carbohydrate content of the sediments.

4.2.4 Discussion

The features described for *Biffarius arenosus* burrow walls are similar to previous reports. Burrow walls that are smooth, but not lined with mucus, have been described for several thalassinidean species (eg. *Callianassa* sp. 2 Braithwaite and Talbot 1972, *Upogebia pusilla* Dworschak 1983, *Callianassa bouvieri* Dworschak and Pervesler 1988, *Axianassa australis* Dworschak and Rodrigues 1997). Wall smoothness is probably due to burrow maintenance and animal movement within the burrow (Dworschak 1983).

Figure 4.2.1

Internal characteristics of burrows of *Biffarius arenosus* found at Warneet, Western Port.

(A) A section of the burrow exposed at low tide, showing the compacted nature of the burrow wall.

(B) Burrow lining in a section of tunnel compared with a turning chamber. Faecal pellets (indicated by the arrow) are often present in the chambers.


A


B

The few studies which have used the PAS reaction in a similar way, successfully showed the presence of a mucopolysaccharide lining in the polychaete *Amphitrite ornata* burrows (Aller and Yingst 1978), and the harpacticoid copepod *Pseudostenhelia wellsi* burrows (Chandler and Fleeger 1984). In both studies positive staining of the burrow linings indicated the presence of mucopolysaccharides, but neither study made the comparison to samples of the surrounding sediment, so no indication of the relative contribution of the resident fauna was discussed.

Biffarius arenosus does not appear to contribute mucopolysaccharides locally on burrow walls. The presence of mucus throughout the sediment on the Warneet sandflat, as well as in the wall of *B. arenosus* burrows, indicates a diverse fauna, including microorganisms, meiofauna and macrofauna which secrete mucopolysaccharides during feeding and burrowing activities. Biological reworking ensures that the mucus is well mixed through the sediments.

Mucus production by bacteria, thought to assist with cell attachment (Fletcher and Floodgate 1973, Fenchel *et al.* 1977), binds sediment grains together (Frankel and Mead 1973, Rhoads *et al.* 1978, Uhlinger and White 1983). Benthic diatoms secrete mucus during locomotion and stalk construction (Holland *et al.* 1974, Vos *et al.* 1988), and have been reported to bind sediment grains thereby increasing sediment stability (Neumann *et al.* 1970, Grant 1988, Paterson *et al.* 1990, Underwood and Paterson 1993a,b). Meiofauna burrows, such as those constructed by some harpacticoid copepods, are formed by small particles and detritus being bound with an acid muco-polysaccharide (Chandler and Fleeger 1984), and nematodes are well known for their production of mucus-lined burrows (Cullen 1973). Nematodes also produce a shiny mucus trace associated with feeding (Reimann and Schrage 1978). Macrofauna can add mucus to sediments in two ways: by permanent burrow construction with a mucus lining binding burrow wall sediment particles together (as described for the capitellid polychaete *Heteromastus filiformis* (Rhoads *et al.* 1978)), or by leaving mucus bound traces, as has been described for the polychaete *Paraonis fulgens* (Grant 1983).

In comparison to *Biffarius arenosus*, mucus burrow linings and specialised mucus-producing glands have been documented in some species of thalassinidean shrimp. Vaugelas and Buscail (1990) measured organic content in *Callichirus laurae* burrow walls, as a way of documenting the presence of mucus. They described a 'sticky coating at the inner surface of freshly made or maintained walls', and found a much higher organic content of burrow wall sediments compared with surrounding subsurface sediment. An anatomical description of the genus *Callichirus* describes structures on the third to fifth abdominal somites which may be associated with cement or mucus production (Manning and Felder 1986). Thompson (1972) also related a greater organic content in *Upogebia pugettensis* burrow linings to the presence of mucopolysaccharides, and described mucus producing glands located in the hindgut of the species. *Callichirus* (as *Callianassa*) *major* was observed constructing burrow walls using mucus, whereby the species secrets a milky, gelatinous mucus from a gland in the thoracopod, which it uses to bind sand grains into pellets, and then into the burrow wall (Frey *et al.* 1978). Mucus production has also been described from glands at the bases of mouthparts in *Neotrypea* (as *Callianassa*) *californiensis* (Thompson 1972).

4.3 Redox potential

4.3.1 Introduction

Knowledge of the biogeochemical processes occurring in infaunal burrows is fundamental for understanding sediment-water interactions in marine systems (Aller and Yingst 1978). In non-bioturbated sediments, oxygen penetrates only a few mm into the sediment at the surface (Jorgensen and Revsbech 1985), and electron acceptors (O_2, NO_3^-, SO_4^{2-}) are supplied from above and electron donors (HS^-, NH_4^+, CH_4) from below. Transport of solutes occurs via molecular diffusion and reaction rates are dependant on steep chemical gradients (Fenchel 1996). An irrigated burrow structure, however, supplies an abundance of electron acceptors deep into the sediments and the burrow lining is a source of horizontal redox gradients (Aller and Yingst 1978). The introduction of oxygen into anoxic or reducing sediments changes both the relative dominance and distribution of oxidation-reduction reactions, thereby increasing biogeochemical heterogeneity in the sediments (Aller 1988).

Ghost shrimps, like many types of infauna, irrigate their burrows to renew the oxygen supply and flush out metabolites and other wastes. Despite regular exchange of burrow waters via burrow irrigation, shrimps would still be subject to low oxygen concentrations especially in intertidal environments during low tide. Ghost shrimps appear to be extremely well adapted to surviving hypoxic conditions (Torres *et al.* 1977, Felder 1979, Thompson and Pritchard 1979, Paterson and Thorne 1993, Astall *et al.* 1997), especially deposit feeding species which would also encounter anoxia during sediment excavation for food (Thompson and Pritchard 1979, Mukai and Koike 1984).

The few studies to examine the redox properties of ghost shrimp burrows (Ott *et al.* 1976, Waslenchuk *et al.* 1983, Forster and Graf 1992, Felder and Griffus 1994, Ziebis *et al.* 1996, Nates and Felder 1997) have concentrated on species inhabiting North Sea, Mediterranean and North American sediments. This section

of work intends to describe the electrochemical status of the sediments *B. arenosus* inhabits, and so to provide a physiochemical framework for the further detailed investigation of burrow wall properties.

4.3.2 Methods

Redox potential (Eh) gives a measure of the ratio of oxidised and reduced forms in a contained system (Whitfield 1969). It is a useful means of characterising sediments, but is not a quantitative measurement of redox dynamics (Whitfield 1969, Vershinin and Rozanov 1983). Many of the ion species involved in the important sedimentary redox reactions are not electroactive (do not readily donate or accept electrons at the electrode surfaces), so Eh is not actually measurable in a thermodynamic sense (Berner 1981). However, Eh may be used to describe electrochemical status of sediments, and indicate the likelihood of certain processes occurring. For example, an Eh > 400 mV indicates oxidising conditions where aerobic metabolism predominates and oxygen is the electron acceptor. Similarly, when Eh falls between 100 and 400 mV, conditions are moderately reducing and anaerobic metabolism prevails with nitrate, manganese oxide and ferrous oxide being the electron acceptors. Conditions are considered reducing when the Eh falls between -100 and 100 mV, and sulphidic metabolism predominates with sulphate as the electron acceptor. Finally, an Eh < -100 mV indicates highly reducing conditions where methanogenic metabolism predominates (Bohn 1971, Berner 1981).

4.3.2.1 Equipment

Redox potential (mV) was measured using bare platinum electrodes (with a maximum diameter of 2 mm to minimise sediment disturbance) encased in a glass sheath (5 mm diameter) and a calomel reference electrode connected to a millivolt meter. The inert metal electrode used in conjunction with the reference electrode forms a complete cell (Whitfield 1969). Electrodes were standardised with ZoBell's solution (equal parts of 0.003 M potassium ferricyanide and 0.003 M potassium ferrocyanide, both made up in 0.1 M potassium chloride) prior to use

(ZoBell 1946). All measurements (mV) were converted to Eh by adding 244 mV to correct for the use of the calomel electrode.

4.3.2.2 Redox stability trials

Marine sediments are biochemically active, so electrodes need to be equilibrated to account for any drift in Eh. Extent of the drift in Western Port sediments was investigated by measuring Eh over a 2 h time period with the electrodes constantly in place in the sediments. Results showed that a large decrease (~ 100 mV) was recorded in the first hour, followed by a slight decrease (~ 20 mV) over the next hour of electrode deployment (Figure 4.3.1). A 15 min electrode equilibrium time was chosen, as has been used in previous studies (Howes *et al.* 1981, Vershinin and Rozanov 1983).

4.3.2.3 Laboratory measurements

Tall thin tanks were established to investigate the effect of a burrow on sediment Eh. Sediment was collected from the field site, sieved to removed macrofauna, and mixed thoroughly. Sediment was added to two clear perspex tanks with the following dimensions: 30 cm width, 50 cm depth and 2 cm thickness. Sediments were restricted to a 2 cm thickness to ensure that any burrows constructed would be visible through the perspex. Water was added to the sediment to a depth of 10 cm, and aerated vigorously. One ghost shrimp was added to each tank and left to burrow for a month.

Six platinum electrodes were evenly spaced horizontally across the tanks and fixed in place with foam blocks (Figure 4.3.2). Redox potential measurements were taken at 2 mm, 5, 10, 15, 20, and 25 cm depths, resulting in a composite picture of redox activity in the whole tank. The position of the burrow was traced from the tank surface (identified from sediment coloration and visible tunnel sections) and matched with the redox profiles.

Figure 4.3.1

Measurement of redox potential in intertidal sediments, investigating the drift of Eh over a 2 h period. Recordings were made at Warneet, Western Port, in October 1994. Each data series on this graph represents a different electrode inserted into the same sediment.

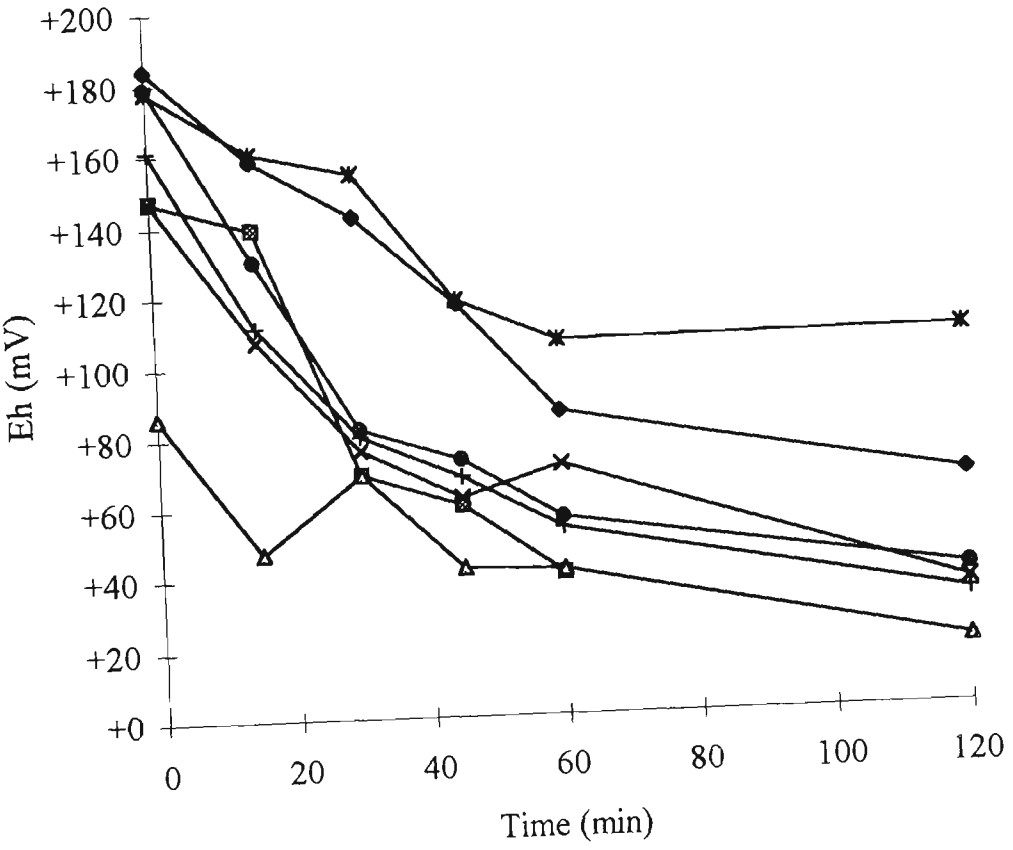

+200
+180
+160
+140
+120
+100
+80
+60
+40
+20
+0
Eh (mV)
0
20
40
60
80
100
120
Time (min)

Figure 4.3.2

Design of the experiment investigating the impact of a single *Biffarius arenosus* specimen on redox potential profiles in tank sediment. The six platinum electrodes (secured with foam blocks) are situated on the right, and the calomel reference electrode is situated on the left. The white dots on the tank indicate the points of measurement. Note evidence of the burrow zigzagging down the left side of the tank.

4.3.2.4 *In situ* measurements

Redox potential, measured from sediments in the laboratory, was possibly not a true representative of *in situ* conditions, because the sediments had been sieved and mixed in air prior to measurement. Therefore, *in situ* measurements were needed for comparison.

Sediments were conceptually divided into burrow, subsurface and surface sediments to investigate the effect of an irrigated burrow on Eh. Sampling occurred at low tide on 19 May 1995. Measurements were made in the burrow environment by gently pushing an electrode into an opening until it came in contact with the burrow wall. Subsurface measurements were made by pushing electrodes into sediments adjacent to the burrow opening. Surface sediment measurements were made by pushing the electrode a few mm into the sediment-water interface. Depth of penetration was marked on the side of the electrodes, which were then left to equilibrate for 15 min after which a reading was taken. Electrodes were then exposed by digging, and the actual position in the sediment was noted. This confirmed, for example, whether an electrode inserted into a burrow was resting in the burrow wall or had penetrated into the surrounding subsurface sediment.

The three different sediment types (burrow, subsurface, surface) were sampled independently, rather than as a set of three associated with a single burrow replicate. This procedure evolved because after measurements were made it was often found that the electrode position was not as intended. This resulted in incomplete sets of measurements from single burrow replicates, so all measurements within each sediment type were pooled. Eleven measurements were recorded from burrows and surface sediments, and 13 from subsurface sediments.

For comparison, adjacent mangrove sediments were sampled on the same day. It was thought that the mangrove sediments would be more reducing than the sandflat sediments, and so the impact of an irrigated burrow on Eh may be more

significant. Nine measurements were recorded from burrows, 14 from subsurface sediments, and 7 from surface sediments.

4.3.2.5 Statistical analysis

Statistical analysis of the data was performed with the Statistica ® software package. The statistical design was a two-way ANOVA. Two sites were sampled (sandflat and mangrove), from which three sediment types (burrow, surrounding subsurface and surface sediments) were compared. The data were checked for homogeneity of variances and normality and ln(x+1) transformed.

4.3.3 Results

4.3.3.1 Laboratory measurements

Burrowing activity of the individual shrimp inhabiting the two replicate tanks varied, thereby creating differing redox patterns (Figures 4.3.3 and 4.3.4). In replicate 1, the sediments are oxidising to moderately reducing to a depth of 5 cm, and reducing below that (Figure 4.3.3). The zone of sediment mixed by the shrimps also extends about 5 cm into the sediment, and the burrow is positioned along the left side of the tank (Figure 4.3.3). Moderately reducing conditions generally occurred in both the zone of mixing and the burrow region, however isopleths did show a localised increase in Eh to 10 cm associated with the burrow (Figure 4.3.3). Redox potentials in replicate tank 2 showed no such localised increase around the burrow structure, with Eh values consistently decreasing from moderately reducing surface sediments to the reducing sediments 5 cm below (Figure 4.3.4). Values were relatively lower in this tank, with the surface sediments less oxidising than in Figure 4.3.3.

4.3.3.2 *In situ* measurements

Redox potentials measured *in situ* showed a more significant impact of the burrow structure on sediment electrochemical conditions. The range of Eh values measured are shown in Table 4.3.1.

Figure 4.3.3

Redox potential profiles measured in tank replicate 1 containing a single *Biffarius arenosus* shrimp and its burrow.

(A) A photograph of the tank showing the zone of bioturbation and the burrow (clearly identified by the lighter sediment extending down the left side of the tank). The white dots represent the points of measurement. Scale bar = 10 cm.

(B) A diagram outlining the position of the burrow and zone of bioturbation (broken lines), points of measurement labelled with Eh values, and the isopleths indicating the regions of redox potential in the sediments (solid lines).


A

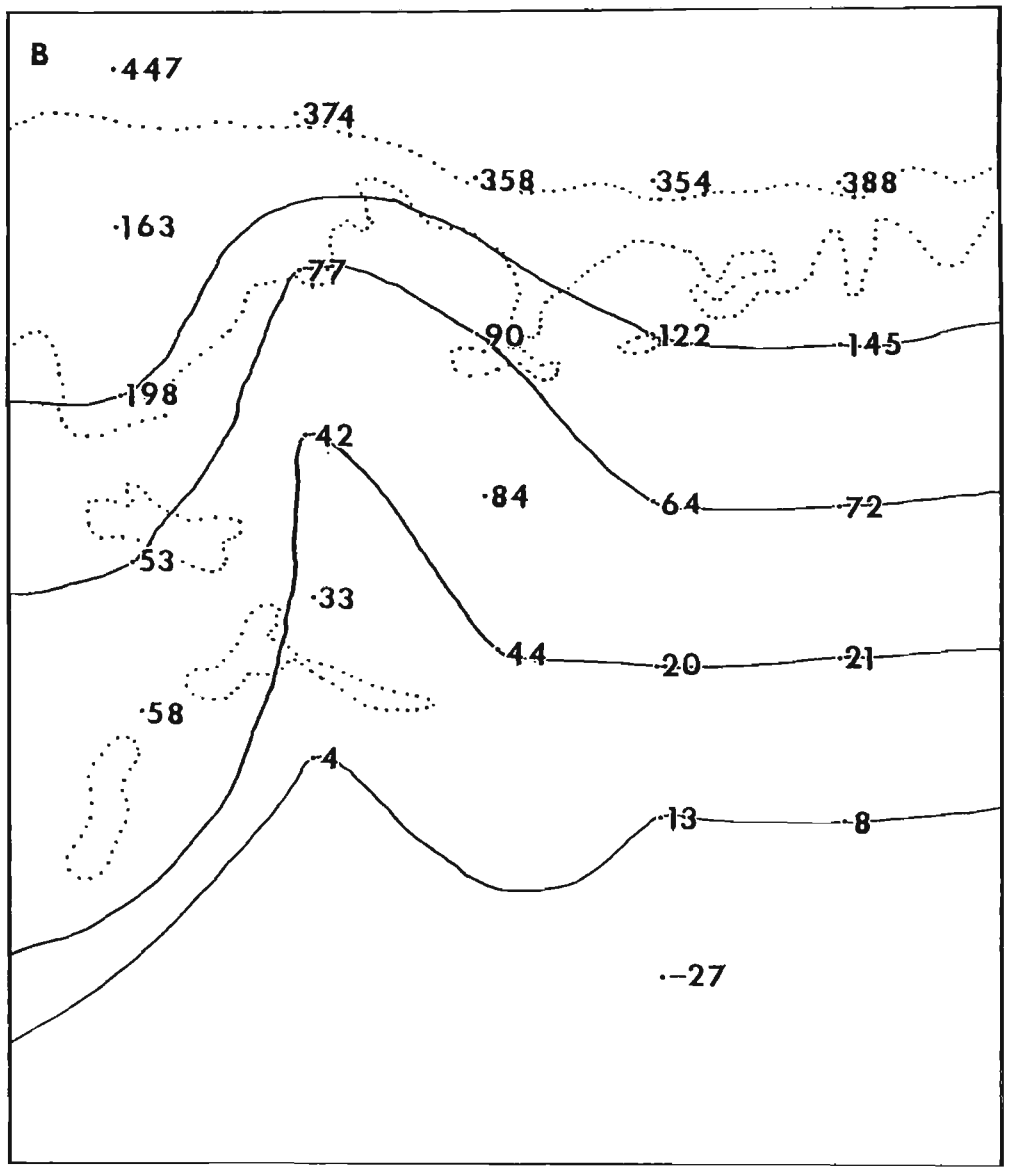
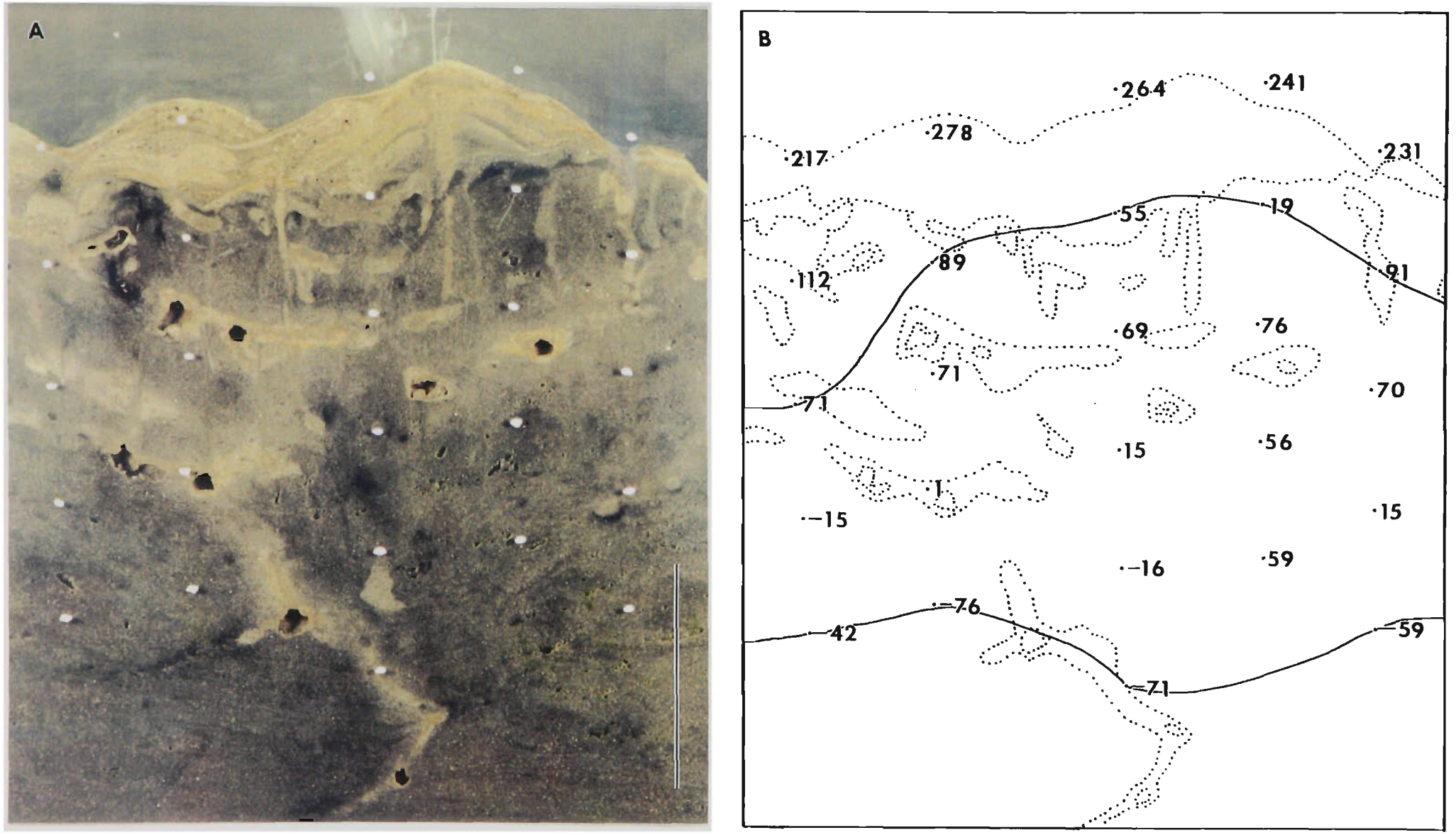

B
447
374
358
354
388
163
77
90
122
145
198
42
84
64
72
53
33
44
20
21
58
4
13
8
27

Figure 4.3.4

Redox potential profiles measured in tank replicate 2 containing a single *Biffarius arenosus* shrimp and its burrow.

(A) A photograph of the tank showing the zone of bioturbation and the burrow (clearly identified by the lighter sediment in the centre of the tank, along with sections of the burrow open to the perspex tank wall). The white dots represent the points of measurement. Scale bar = 10 cm.

(B) A diagram outlining the position of the burrow and zone of bioturbation (broken lines), points of measurement labelled with Eh values, and the isopleths indicating the regions of redox potential in the sediments (solid lines).


A
B
264
241
278
231
217
55
19
89
91
112
69
76
71
70
71
15
56
1
15
−15
−16
59
76
42
59
71

Uneven sample sizes in the data set (Table 4.3.1) would have reduced the power (the ability to detect a difference if one is present) of the statistical analysis. However, power of the ANOVA test comparing redox potential measurements appeared to be sufficient for all effects, as results were either highly significant or highly non-significant (Tables 3.5.1).

The Eh of burrows was not significantly different from that measured in the surface sediments (Table 4.3.2), and both indicated moderately reducing conditions (Table 4.3.1). Redox potential of the subsurface sediments was significantly lower than that measured in the burrow walls and surface sediments (Table 4.3.2), but was also moderately reducing (Table 4.3.1). There was no significant difference between Eh of sediments from the sandflat and the mangroves (Table 4.3.2).

Table 4.3.2. Statistical results of a two-way ANOVA testing difference between Eh values from 2 sites (sand, mangrove) at Warneet, Western Port, and 3 sediment types (burrow, subsurface, surface) associated with burrows of *Biffarius arenosus*.

Source	df	Mean Square	F ratio	P value
Sample site	1	0.012	0.160	0.691
Sediment type	2	3.653	50.707	0.000
Site x type	2	0.537	7.450	0.001
Error	55	0.072		

The site by type interaction (Table 4.3.2) relates to differences between burrow wall and surface sediment redox potentials. Amongst the mangroves, burrow wall conditions were not significantly different than surface sediment redox conditions, whereas on the sandflat surface sediments were significantly more oxidising than burrow walls (Table 4.3.1).

Table 4.3.1. Redox potentials (mV) recorded in the burrows, surrounding subsurface and surface sediments associated with the burrows of Biffarius arenosus. Measurements were made in mangrove and sandflat sediments at Warneet, Western Port, in May 1995.

Site	Eh (mV) Burrow	Subsurface	Surface
Sandflat	251	120	344
	259	164	396
	212	176	262
	218	151	216
	279	90	224
	129	103	278
	177	158	122
	161	128	172
	288	187	191
	256	195	186
	109	99	297
			208
			267
Mean ± SE	**213 ± 6**	**142 ± 11**	**243 ± 21**
Mangrove	337	203	264
	229	188	254
	269	108	348
	357	63	259
	143	102	336
	347	87	235
	168	113	211
	277	117	
	157	120	
		87	
Mean ± SE	**254 ± 28**	**119 ± 14**	**272 ± 19**

4.3.4 Discussion

4.3.4.1 Laboratory measurements

Laboratory redox potential measurements showed a minor impact of bioturbation, with moderately reducing conditions recorded in both the zone of mixing and the vicinity of the burrow. The burrow, however, could be clearly identified by the yellow/brown sediment coloration, caused by the presence of ferric oxyhydroxides (Matisoff *et al.* 1985). This coloration has been observed in burrows of many species (Henriksen *et al.* 1983, Matisoff *et al.* 1985, Hüttel 1990, Davey 1994), and is often used as an indicator of oxidising conditions (Myers 1977, Hylleberg and Henriksen 1980, Kristensen and Blackburn 1987). Oxidising conditions do not, however, necessarily indicate the presence of free oxygen. In fact, Revsbech *et al.* (1980) found no correlation between redox profile and oxygen penetration in surface sediments, and oxygen was only measured in a fraction of the oxidising yellow/brown zone around a burrow. Forster and Graf (1992) also showed that oxygen penetration into sediments from the burrow wall was only one third as far as the elevated Eh.

The laboratory experiment may have been more informative if a comparison between inhabited and uninhabited sediments had been made. Ziebis *et al.* (1996) found that defaunated sediments were reducing at 5 cm depth, compared to an elevated Eh in sediments inhabited by *Callianassa truncata*.

4.3.4.2 *In situ* measurements

More oxidising conditions were found in burrow wall and surface sediments than in subsurface sediments. This result is similar to those of previous reports, which have shown that burrowing fauna increase the penetration depth of an elevated redox potential into sediments (Rhoads 1974, Hylleberg and Henriksen 1980, Flint and Kalke 1986, Ziebis *et al.* 1996, Nates and Felder 1997), caused by both the deepening of surface oxidising layers and creation of oxic microenvironments associated with burrow walls (Myers 1977). Anderson and Meadows (1978) found that the burrow lining in *Nereis diversicolor* (Polychaete) burrows had Eh values more similar to those of surface sediments than those of the subsurface

sediments. In contrast, the burrow walls of *Callianassa stebbingi* were generally more oxidised than were the surface sediments (Ott *et al.* 1976). Ott *et al.* (1976) also found that this result was most pronounced in aquaria, in contradiction to the present study's results. Felder and Griffis (1994) documented 1-2 cm of oxygenated sediment surrounding burrow walls of *Callichirus islagrande*, but did not compare this to conditions in surface sediments. Zones of oxidation around the burrows of *Callianassa subterranea* were similar to if not as high as those measured in surface sediments (Forster and Graf 1992), with Eh dropping from +500 to -100 mV within 1 mm of the sediment-water interface. The oxidised zone around faunal burrows often extends only a few mm into the surrounding sediments (Kristensen and Blackburn 1987, Meyers *et al.* 1988, Forster and Graf 1992, Fenchel 1996).

In contrast, Waslenchuk *et al.* (1983) found that the *Callianassa* spp. burrow environment was enriched in nutrients and sulphides, suggesting that anoxic metabolism prevailed. Mean Eh of burrow waters (-16 mV) was much lower than the mean Eh of overlying waters (213 mV), indicating that irrigation activity was not sufficient to maintain overlying water conditions within the burrow environment (Waslenchuk *et al.* 1983).

4.3.4.3 Interpretation of Eh measurements

One major problem of measuring Eh is that the electrodes disturb the sediments at the point of insertion, possibly disrupting the redox state (Whitfield 1969, Machin and Ott 1972). In the laboratory experiments, burrow walls often collapsed when the electrode was inserted, so the Eh measured was probably not indicative of the actual conditions. In comparison, the field measurements were taken by pushing the electrode gently down the burrow tunnel until the electrode came to a stop in the burrow wall. This method reduced sediment disturbance and possibly provided a better measurement of the upper burrow environment.

The problem of Eh drift after insertion was also observed in this study, with Eh continually decreasing even after a 2 hour equilibration time. In natural systems,

an oxidation-reduction equilibrium is rarely achieved, primarily due to the continual decomposition of organic matter and the associated addition of new electron donors into the environment (Bohn 1971). To account for this, a consistent equilibrium time was used to standardise all the measurements.

Single pinpoint measurements (such as those made in this study) can give a distorted view of the redox status of sediments, compared to a study including variation across time (Machin and Ott 1972). This idea may be particularly relevant for ghost shrimp studies, because short term oscillations in burrow irrigation, due to periodic activity by the inhabitant, or long term changes, due to tidal exposure on intertidal flats, would cause cyclic fluctuations in Eh.

Intermittent exchange of burrow waters promotes variable oxygen conditions, and during periods of rest or low tide, oxygen consumption by the burrow inhabitant and the microbes and meiofauna existing in the burrow wall rapidly exhaust the available oxygen supply (Kristensen 1988). This would also help maintain a low and variable oxygen penetration into the burrow walls (Kristensen 1988). At low tide in *Callianassa japonica* and *Upogebia major* burrows, oxygen saturation dropped by 38%, but within 40 min of the tides return, saturation had recovered to normal levels (Koike and Mukai 1983). The cyclic oxygen presence and associated redox patterns would be accompanied by rapid switching of the dominant metabolic processes in the burrow environment (Aller 1994).

Redox potentials measured in this study showed that sediments were not highly reducing, which is consistent with low organic carbon content of the sediments (< 1%).

4.4 Diffusive flux of a non-reactive tracer

4.4.1 Introduction

Bioturbating fauna are known to increase diffusive flux of solutes between sediments and overlying water (Aller and Aller 1992, Clavero *et al.* 1992, Marinelli 1994). Permanent burrow structures increase the surface area for diffusion along gradients by extending the water-sediment interface deep into the sediments. Burrows can increase the surface area of this interface up to 16 times (Ott *et al.* 1976).

Burrows change the one-dimensional model of diffusion, where solutes can diffuse horizontally into the burrow water as well as vertically from sediments into the overlying water (Aller 1982, 1983). Pressure gradients, active irrigation and animal movement induce convection of water and solutes through the interstitial spaces (Ebenhöh *et al.* 1995). A burrow inhabitant needs to flush the burrow water regularly to remove toxic metabolites (Henriksen *et al.* 1983) and replenish oxygen supply. Regular replacement of burrow water will renew the solute concentration gradients and further enhance diffusive flux. Macrofauna in marine systems are known to enhance flux from sediments by as much as an order of magnitude (Rutgers van der Loeff *et al.* 1984). Such an increase would have far-reaching effects on sediment nutrient dynamics and cycling.

Even though thalassinidean ghost shrimps are among the most common burrowing fauna of estuarine and marine sediments (Dworschak 1983, Griffis and Suchanek 1991), at times building burrows to 5 m depth (Frey *et al.* 1978), no study has yet attempted to quantify their effect on diffusive flux. Most work on this topic has centred on polychaetes (Kristensen *et al.* 1991, Clavero *et al.* 1992, Marinelli 1994), bivalves (Aller and Yingst 1985, Aller and Aller 1992) and freshwater fauna (Matisoff *et al.* 1985, Fukuhara and Sakamoto 1987), with few studies assessing the impact of crustaceans (Henriksen *et al.* 1983). This work aims to investigate the effect of *Biffarius arenosus* burrows and burrowing

activity on diffusive flux of a non-reactive tracer, assisting further with understanding how the species impacts sediment physical properties.

4.4.2 Methods

4.4.2.1 Burrow surface area

An estimate of burrow surface area was gained from the burrow casts collected from the intertidal population of *Biffarius arenosus* in Western Port (see chapter 2 for details). Surface area was measured by wrapping the cast in a single layer of foil. One square centimetre of foil had a known weight and the total foil wrapped around each cast was weighed and converted to an area.

4.4.2.2 Measurement of diffusive flux of a non-reactive tracer

This section of work was done in collaboration with Dr Phillip Ford, from the Centre for Environmental Mechanics, CSIRO, Canberra. The experiment comprised part of a larger study investigating bioturbation by common Port Phillip Bay benthic invertebrates, and was published as Technical Report number 37 of the Port Phillip Bay Environmental Study (Bird *et al.* 1996). The work was funded by the Melbourne Water Corporation.

Because this experiment was associated with the Port Phillip Bay Environmental Study, specimens of *Biffarius arenosus* were collected from a subtidal population in Port Phillip Bay, Victoria. Port Phillip Bay is a shallow, marine embayment of 1950 km^2, adjacent to Western Port on the southern coast of Victoria, Australia. In Port Phillip Bay, *B. arenosus* inhabits a similar sediment type and occurs in similar densities (~ 20 individuals m^{-2}) to populations of the same species studied in Western Port (Poore 1975, Wilson *et al.* 1996).

4.4.2.2.1 Experiment overview

A non-reactive tracer was used in a closed system to examine the effect of burrows and burrowing activity of *Biffarius arenosus* on diffusive flux. Deuterium oxide (D_2O), also known as 'heavy water', is non-reactive and thus diffuses easily across the water-sediment interface. The reduction in

concentration of tracer in the overlying tank water (as it diffused into the sediment) was measured over time. From this reduced concentration, an effective diffusion coefficient was calculated and compared to the diffusion coefficient of D_2O due to purely diffusive flux. This ratio revealed the extent of bioturbatory enhancement on diffusive flux.

4.4.2.2.2 Experimental details

Subtidal specimens of *Biffarius arenosus* and sediments were collected using a Smith-MacIntyre grab from Port Phillip Bay in October 1995. Sediments were sieved to remove fauna and mixed to ensure homogeneity within treatments. Buckets were used as tanks, filled with sediment to a 30 cm depth, and covered with seawater.

Three replicate experimental tanks, each containing two specimens of *Biffarius arenosus* at natural field densities, and three control tanks, containing no animals, were established. Animals were added to the tanks two months prior to the experiment date, to allow the ghost shrimps to build burrows. The experiment was run at 15 °C.

Bubblers were positioned in the centre of each tank and set at a constant aeration rate by timing the displacement of a known volume of water in an inverted measuring cylinder. It was necessary to know how long it would take the tracer to evenly circulate throughout the overlying water to ensure a maximal concentration in the overlying water prior to sampling at time = 0. Fluorescein solution (1 mM) was added to the water above the bubbler and water samples were collected from the centre and four corners at 5, 10 and 15 min. These samples were read immediately (excitation 490 nm, emission 514.5 nm with a Perkin Elmer LS 50B Luminescence Spectrometer). After 15 min, the dye appeared to be well mixed throughout the tank water, so 15 min was chosen as the first sampling time for the experiment.

Five ml of 5% D_2O solution (deuterium oxide purchased from Australian Nuclear Science and Technology Organisation, Lucas Heights) was added to each tank at time = 0. Water samples were then removed from the centre of the

tank at 15 and 30 min, and 1, 2, 4, 8, 18, 24, 30, 42 and 48 h. The samples were transferred to 0.7 ml amber Chromacol vials and crimp sealed immediately. Standards were created by diluting 5 ml D_2O to 1 litre with ultrafiltered water in a standard flask. Samples were taken from the standard flasks at the start and end of the experiment.

Samples were analysed in a VG Optima dual inlet isotope ratio mass spectrometer after reduction of the water to hydrogen gas over zinc at 500 °C. The results are reported as delta (δ) values relative to Vienna standard marine ocean water where

$$\delta = (R \text{ sample} / R \text{ standard} - 1) \times 1000$$

and R is the measured Deuterium/Hydrogen ratio.

Water volumes above the sediment surface were calculated again using fluorescein. Five ml 1 mM fluorescein was added to each tank, and after 15 min a water sample was taken from the middle of the tank and analysed immediately. The concentration of fluorescein in the tank water was compared to that in a standard of known volume, and tank water volumes were calculated.

4.4.2.2.3 Analysis of data

The removal of the non-reactive D_2O tracer added to each tank was treated as happening by a purely diffusive process with the change in the concentration of the added tracer calculated by (Ford *et al.* 1997)

$$c(0,\tau) = e^{\tau} erfc\left(\tau^{1/2}\right),$$

where

$$\tau = \frac{\rho^2 Dt}{L^2}$$

and ρ is the porosity, D is the diffusion coefficient ($cm^2\ sec^{-1}$), t is the time (sec), and L is the height (cm) of the overlying water.

The reduced concentration (actual δ value divided by the δ value extrapolated to t = 0) was plotted against time. On the same axes the reduced concentration,

calculated from the above expression using the measured porosity and water column heights and a range of values of D, was also plotted. This provided an initial estimate of D which was subsequently refined by successive adjustments to achieve greatest concordance (visually estimated) with the experimental data. Figure 4.4.1 shows a representative example.

The measured diffusion coefficients had to be corrected for the tortuosity (θ^2) of the sediment to provide an estimate of the effective diffusion coefficient (D_o) of D_2O in the pore water ie. without the presence of the solid sediment matrix. The tortuosity was calculated from the relationship (Boudreau 1996)

$$\theta^2 = 1 - \ln(\rho^2)$$

and

$$D_o = \theta^2 D$$

An enhancement factor was calculated for all tanks. This factor is the ratio of the measured effective diffusion coefficient to the actual diffusion coefficient for D_2O in water ($1.97 \pm 0.02 \times 10^{-5}$ cm^2 sec^{-1}) (Eisenberg and Kauzmann 1969) at the tank temperature (15 °C). It is a direct measure of the extent that flux of materials to the sediment is increased relative to purely diffusive transfer.

Enhancement factors of the experimental and control tanks were compared using a one-way ANOVA. Data were checked for homogeneity of variances, using Cochran's C test, and normality and ln(x+1) transformed.

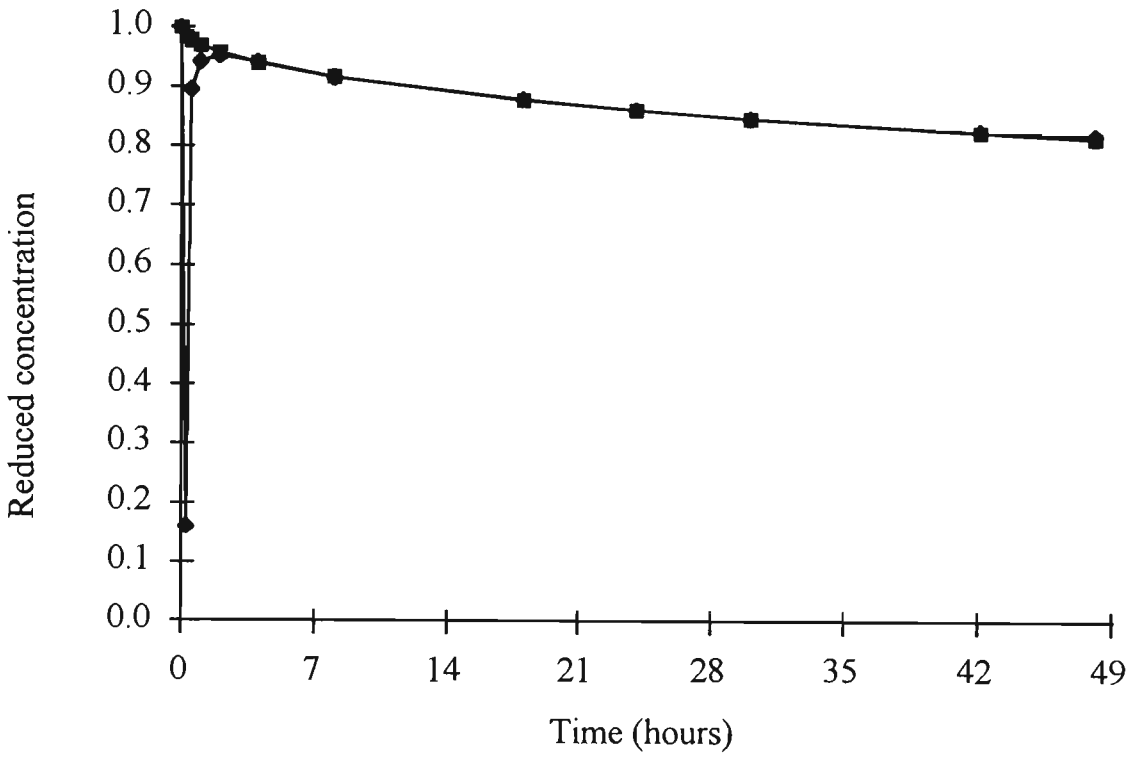
4.4.3 Results

4.4.3.1 Burrow surface area

Mean burrow surface area, measured from 17 casts, was estimated at 495 ± 20 cm^2 (Mean $\pm$ SE). With an average density of 8.7 m^{-2} shrimps inhabiting the intertidal Western Port study site would have a burrow surface area of 0.43 m^2

Figure 4.4.1

Theoretical reduced concentration of the tracer D_2O (square) compared to the actual concentration of D_2O (diamond) in overlying water of Control tank 1. This is the method used to provide an initial estimate of the diffusion coefficient of D_2O in experimental tanks set up to investigate the effect of *Biffarius arenosus* on exchange of dissolved substances in a closed system.


Reduced concentration
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
7
14
21
28
35
42
49
Time (hours)

under 1 m^2 of surface sediment. Therefore, the water-sediment interface would be increased 44% by the shrimps burrows.

4.4.3.2 Diffusive flux of D_2O

The mean effective diffusion coefficient in control replicates was only marginally larger (32%) than the pure diffusive value measured under ideal laboratory conditions (ie. 1.97 $\pm$ 0.02 x 10^{-5} cm^2 sec^{-1} (Eisenberg and Kauzmann 1969)) (Table 4.4.1). In comparison, the diffusion coefficient for the experimental tanks was enhanced by as much as 3.6 times. The mean value was significantly different from that of the controls (df 1, F-ratio 15.44, P 0.02).

Two problems were identified with the experimental data, each potentially affecting the results. Firstly, sediment porosity was only measured in one of the tanks containing fauna (Table 4.4.1). Porosity of the control sediments did not vary much because the sediment was not disturbed throughout the experiment. However, porosity in the bioturbated tanks may well have differed, depending on the activity of the inhabitant. Therefore any comparisons made between experimental tanks will be discussed with caution, because calculations of tortuosity, effective diffusion coefficient and enhancement factor of the tanks containing shrimps, were all based on the same porosity value.

Secondly, in some tanks the relative concentration of D_2O (δ) was still increasing in the water column after t=0, indicating poor mixing (Table 4.4.2). Additionally, this lack of mixing was not consistent over the different tanks. For example, control replicate 1 was well mixed after 15 minutes, whereas control replicate 3 took 120 minutes to achieve this status. Since the samples were not analysed until after the experiment was completed, it was not possible to rectify this problem.

Table 4.4.1. Summary of tank water volume, porosity, tortuosity, measured diffusion coefficients and effective diffusion coefficients and enhancement factors calculated in control (shrimp absent) and experimental (shrimp present) tanks of the experiment investigating the effect of *Biffarius arenosus* on exchange of dissolved substances in a closed system. Estimated porosity values for the experimental tanks are in parentheses.

	Shrimp absent			Shrimp present		
Replicate	1	2	3	1	2	3
Water volume (l)	0.92	0.75	0.97	1.48	1.92	1.62
Porosity	0.61	0.56	0.57	0.50	(0.50)	(0.50)
Measured diffusion coefficient x 10^{-5}	0.71	0.90	0.82	1.83	1.45	2.97
Tortuosity	2.94	3.34	3.28	2.39	2.39	2.39
Effective diffusion coefficient x 10^{-5}	2.09	3.00	2.70	4.37	3.46	7.09
Enhancement factor	1.06	1.52	1.37	2.22	1.76	3.60
Mean enhancement factor ($\pm$ std deviation)		1.32 ± 0.23			2.52 ± 0.96	

Table 4.4.2. Delta (δ) values (concentration of deuterium in samples relative to the concentration in Vienna standard marine ocean water) measured in the overlying water of control (shrimp absent) and experimental (shrimp present) tanks of the experiment investigating the effect of *Biffarius arenosus* on exchange of dissolved substances in a closed system.

Time of sample (hours)	Shrimp absent			Shrimp present		
	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
0.25	25635	29583	4099	15825	13343	14356
0.5	25233	29200	22863	16801	13523	15825
1	24674	28758	24029	16960	13442	15992
2	24080	27890	24314	16768	13240	15732
4	23251	26665	24054	16393	12917	15354
8	22123	25409	23426	15604	12417	14446
18	20780	23382	22541	14613	11737	13234
24	20375	22138	22122	14106	11464	12658
30	20062	22451	21768	13729	11205	12259
42	19390	21718	21258	12771	10752	11499
48	19256	21379	21063	12641	10539	11055

4.4.4 Discussion

4.4.4.1 Burrow surface area

Mean surface area of burrows of *Biffarius arenosus* compared well with that found for similar sized thalassinidean species in the Northern Hemisphere (Koike and Mukai 1983, Dworschak and Pervesler 1988, Atkinson and Nash 1990). Individual burrows of *Callianassa japonica* had a slightly larger surface area than those of *B. arenosus* (~ 625 cm^2) but double the population density (20 m^{-2}), thereby increasing the water-sediment interface surface area by 125% (Koike and Mukai 1983). *Callianassa bouvieri* had burrows with a smaller surface area, ranging between 3.8 and 135.9 cm^2 (Dworschak and Pervesler 1988), but the species was found in much greater densities (454 m^{-2}) and also had a relatively larger impact on the water-sediment interface area. Individual burrow surface area of *Lepidophthalmus sinuensis* and *Callichirus islagrande* was similar to that of *B. arenosus*, but the North American species increased surface area of the

water-sediment interface by 5 and 9 times (respectively) because of much higher burrow densities (100-220 burrows m^{-2}) (Felder and Griffis 1994).

Two studies of *Callianassa subterranea* burrows have documented burrow surface areas ranging from 13 to 2078 cm^2 (Witbaard and Duineveld 1989, Atkinson and Nash 1990). The smaller values (13-68 cm^2) were estimated from burrows constructed in aquaria (Witbaard and Duineveld 1989), which may have influenced burrow size. Atkinson and Nash (1990) reported a mean surface area of 1287 cm^2 from burrows cast *in situ*. When combined with maximum reported densities of the species along Scottish coasts (10 m^{-2}), this corresponds to a 129% increase in water-sediment interface surface area. Witbaard and Duineveld (1989) reported much higher densities (52 individuals m^{-2}) in the Dutch sector of the North Sea, which elevates the potential impact of this species to a 6.7 fold increase of the water-sediment interface. This value is 15 times higher than the 44% increase found for *Biffarius arenosus*, due to both a greater population density and burrow surface area.

Other studies have concentrated on larger species in the genus *Upogebia*, with reports of increases to water-sediment interface of 3-5 times (Dworschak 1981) and 7-15 times in dense populations (Ott *et al.* 1976).

4.4.4.2 Diffusive flux of D_2O

The maximum enhancement of diffusive flux of D_2O by the burrows and burrowing activities of *Biffarius arenosus* (~ 4 times), compared well with previously documented enhancement factors measured for other macrofauna. A mixed community of echinoderms, molluscs, polychaetes and amphipods enhanced the flux of silica between 2 and 10 times (Rutgers van der Loeff *et al.* 1984). Other reports of the combined effect of molluscs and polychaetes showed that this type of community enhanced flux up to 5.2 times (Aller and Yingst 1985, Hopkinson 1987). Single species studies revealed that the polychaete *Nereis* sp. enhanced flux of dissolved phosphate by 1.7- 5.8 times (Clavero *et al.* 1992), and activity by the priapulid worm *Halicryptus spinulosus* resulted in a 10 times increase in the effective diffusion of Br^- (Powilleit *et al.* 1994). Aller and

Aller (1992) found that meiofauna increased diffusive flux rates of the tracers Cl^- and Br^- by 1.7 - 2.3 times, and Green and Chandler (1994) reported similar effects of meiofauna on the diffusive flux rates of cadmium. Matisoff *et al.* (1985) documented enhancement of dissolved silica fluxes (3.1 times the controls) by chironomid larvae in freshwater sediments.

The variation recorded between the 3 experimental replicates could have been due to several factors. Flux enhancement has been found to vary with temperature (Rutgers van der Loeff *et al.* 1984) and possibly faunal composition (Aller and Aller 1992), along with the type and properties of the solute in question (Boudreau and Marinelli 1994). Additionally, density of fauna inhabiting sediments alters the rate of diffusive flux of solutes, with a higher rate correlated with an increased abundance (Clavero *et al.* 1994, Marinelli 1994). These four variables were controlled in this experiment and should have had little impact on the final result.

However, factors such as the size and spacing of burrows are also important (Aller 1980), along with the irrigation behaviour of the burrow inhabitant (Boudreau and Marinelli 1994). Marinelli (1992) showed that fluxes of silicate were positively associated with burrow construction, suggesting that mobile species may have a greater effect than sedentary species. Aller and Aller (1992) suggested that 20 - 40% of the flux increase was due to increased porosity caused by activity of fauna. These factors may well have had some influence on the variation between experimental replicates. Values varied by a factor of 2, and different levels of burrowing activity or irrigation behaviour by individual shrimp may account for these differences. Additionally, the sediments may have had different porosities due to differing activity in the replicate aquaria, which were not accounted for in the calculations of diffusion coefficients. For example, if porosity of the sediment in experimental replicate 1 was increased to 0.40, the enhancement factor would double (Table 4.4.3). Alternatively, if porosity of this sediment was decreased to 0.60, calculated enhancement of diffusive flux would approximately halve. Hence, measuring actual sediment porosity is vital to fully appreciate any differences between replicates and the lack of my doing this limits further interpretation of the results.

Table 4.4.3. Predicted enhancement factors for treatment replicate 1 of the experiment investigating the effect of *Biffarius arenosus* on exchange of dissolved substances in a closed system, with hypothetically higher and lower porosity.

	Measured porosity	Higher porosity	Lower porosity
Porosity	0.50	0.40	0.60
Measured diffusion coefficient x 10^{-5}	1.83	2.86	1.27
Tortuosity	2.93	2.83	2.02
Effective diffusion coefficient x 10^{-5}	4.37	8.10	2.57
Enhancement factor	2.22	4.11	1.30

Despite the obvious limitations with this experiment, some valuable results were gained. Firstly, a novel tracer was used successfully to investigate the effect of bioturbating fauna on diffusive flux. Secondly, it appears that *Biffarius arenosus* does stimulate diffusive flux of D_2O from the sediment into the overlying water, with a possible maximal enhancement factor of ~ 4 times being recorded. Ideally, to gain more conclusive results, this experiment should be repeated with a refined procedure to ensure accurate mixing and porosity measurement, and with more replicate tanks, to account for any variation in burrowing/irrigation behaviour.

4.5 Chapter summary

Burrowing activities of *Biffarius arenosus* clearly impacted the physiochemical burrow environment. No concentration of organic carbon was found in burrow walls of *B. arenosus*, a result which was consistent with the lack of a discrete mucus lining. Burrow walls were, however, lined with compacted and smoothed sediment and were distinctively coloured a light yellow/brown compared to the dark grey of the surrounding subsurface sediments. The contrasting colours inferred differing redox conditions, an observation confirmed by Eh measurements. All three sediment types (wall, subsurface, surface) were moderately reducing, but the burrow wall and surface sediments were significantly more oxidising than the subsurface sediments. Similar redox conditions found in both the burrow wall and surface sediment were indicative of a burrow environment regularly flushed with overlying water to renew the oxygen supply and remove waste products and metabolites. Diffusive flux of D_2O over the water-sediment interface was enhanced by a factor of approximately 4 in the presence of *Biffarius arenosus*. Flux enhancement was probably due primarily to increased surface area of the water-sediment interface caused by the presence of an irrigated burrow but may also have been influenced by steep concentration gradients and convective flow resulting from shrimp activity within the burrow.

CHAPTER 5

MICROBIAL CHARACTERISTICS OF THE BURROW ENVIRONMENT

5.1 Abundance of bacteria

5.1.1 Introduction

Heterotrophic bacteria in coastal environments play a crucial role in decomposition of organic material, cycling of essential nutrients and conversion of dissolved organic carbon into particulate matter available to metazoan consumers (Azam *et al.* 1983, Boynton and Kemp 1985, Moriarty *et al.* 1985, Craven and Jahnke 1992, Alongi 1995). The turnover of carbon, nitrogen and sulphur during bacterial growth, reproduction and death, comprises most of the biochemically mediated cycling of those elements (Vestal and White 1989, Paerl 1993). Quantifying bacterial abundance gives an indication of the microbes' potential to contribute to element cycling, and therefore is a useful measure for studies comparing the properties of different sediment zones.

The supply of organic matter to bacterial populations is a significant factor controlling bacterial abundance and productivity (Moriarty 1989, Moriarty and Hansen 1990, Kjelleberg *et al.* 1993, Danovaro 1996). Bacterial abundances peak at the sediment surface and decrease with depth (Aller and Aller 1986, Moriarty *et al.* 1991), primarily due to the continual sedimentation of organic material from

the overlying water column (Van Duyl *et al.* 1992). Irrigated burrow structures enhance bacterial abundances by providing deeper sediments with reactive organic matter and an oxic environment to promote aerobic decomposition (Anderson and Meadows 1978, Aller and Aller 1986, Aller 1988, Reichardt 1988, Köster *et al.* 1991). Consequently, the irrigated burrow lining has been likened to an extension of the water-sediment interface with similar properties and decomposition rates (Aller and Yingst 1978, Kristensen *et al.* 1985).

Despite the knowledge that thalassinidean shrimps construct extensive burrow systems, very few studies have investigated the potentially significant effect these burrows have on heterotrophic bacteria and therefore on remineralisation of organic matter and nutrient cycling. In one of the few studies undertaken, Branch and Pringle (1987) found that bacterial abundance in Langebaan Lagoon, South Africa, was highest in the burrow linings of *Callianassa kraussi* and decreased rapidly within a few mm of the burrow wall. However, they did not investigate any possible similarities between burrow linings and surface sediments.

The aim of this section of work is to examine the abundance of bacteria in burrow walls, surrounding subsurface and surface sediments, to investigate how the burrow environment of *Biffarius arenosus* affects bacterial abundances.

Direct epifluorescent counting is the favoured method used to enumerate bacteria in complex natural environments (Newell *et al.* 1986, Boulton and Boon 1991), because the classical method of isolating and culturing organisms in media is known to grossly underestimate the population size (Jannasch and Jones 1959, Scholz and Boon 1993). Even though the epifluorescent count technique has its disadvantages, such as incomplete or inconsistent release of bacteria from sediments (Moriarty 1980), unequal fluorescence of different species of bacteria (Ross *et al.* 1996) and the laborious and often subjective nature of sample analysis (Van Es and Meyer-Reil 1982), it is still the most appropriate method for this study.

5.1.2 Methods

Zimmerman and Meyer-Reil (1974) originated the method of epifluorescent staining with acridine orange (3,6-tetramethyl diaminoacridine) (AO), and counting bacteria with epifluorescent illumination on polycarbonate filters. Hobbie *et al.* (1977) refined the technique by staining the polycarbonate filters black prior to adding the stained bacteria, thereby minimising background fluorescence and increasing accuracy of enumeration. Porter and Feig (1980) further modified the method by substituting 4'6-diamidino-2-phenylindole (DAPI) for AO as the bacterial stain, thus reducing non-specific staining of non-microbial DNA components. It is the Hobbie *et al.* (1977) technique which is still the most widely used of the epifluorescence methods, but the DAPI technique is, nevertheless, also used commonly.

5.1.2.1 Methodology trials

5.1.2.1.1 Staining bacteria

Both the epifluorescent stains AO and DAPI were trialed for enumerating bacteria in these sediment samples. Acridine orange has sometimes been found to yield higher bacterial counts than DAPI from the same sediments (Newell *et al.* 1986), so it was initially trialed. The major problem with AO was that it can stain nonspecifically, so bacteria were difficult to distinguish from the background. Conversely, DAPI, which is known for significantly reducing non-specific staining (Porter and Feig 1980), illuminated the bacteria clearly with little background interference. Hence, DAPI was chosen as the appropriate stain to enumerate bacteria in these sediment samples.

Acridine orange

The method outlining AO staining of bacteria was described in Moriarty and Chandrika (1986), and is as follows: 0.5 ml sediment was added to 5 ml tetrasodium pyrophosphate (0.1M) in a centrifuge tube, and the mixture was shaken and sonicated for 5 min. The sample was then centrifuged for 5 min at 3000 rpm. A filter stained in irgalan black (100 mg irgalan black in 100-200 ml 2% acetic acid, filtered) was placed in the filtering apparatus and at least 5 ml

ultrafiltered water was added to the column. 100µl of bacterial suspension and 20µl of AO solution (1 g l^{-1} concentration) were added to the filtering apparatus, stirred and left to stain for 5 minutes. The vacuum pump was turned on, and the solution drawn through the filter. The filter was prepared on a slide for oil-emersion, and bacteria enumerated under 1000x magnification under a Zeiss Axioskop binocular microscope. All glassware was washed and rinsed with ultrafiltered water, in between samples.

Background staining by AO made it difficult to differentiate between bacteria and non-bacteria. A post-filtration wash of the filter, where approximately 10 ml of ultrafiltered water was drawn through the filter following the stained bacterial suspension, aimed to reduce background fluorescence, but had little effect.

DAPI

The same procedure as above was used except DAPI (0.95 mg l^{-1} concentration) was added to the bacterial suspension and the mixture was left to stain for 30 minutes. The filter was washed in the same way highlighting the bacteria on the dark background, making them easy to count.

Bacteria was counted on 8 fields of view (FOV) on each of 3 filters taken from each sample. The values from the FOVs and filters were averaged, and a final value of numbers of bacteria per volume of sediment was calculated using the following formula:

$$\text{No./ml} = \frac{\text{mean x area of filter}}{\text{area of FOV}} \times \frac{1}{\text{vol. filtered (ml)}}$$

where,

Area of filter = 201.06 mm^2

Area of FOV = 0.03 mm^2

Abundance per ml of sediment was also converted to abundance per gram (dry weight) of sediment, for comparison to other values published in those units. Conversion factors were based on Dale (1974).

5.1.2.1.2 Validation trials

Validation trials were performed to adapt the procedure (Moriarty and Chandrika 1986) to these specific sediment samples.

Trial 1. Volume of bacterial suspension

Volume of the bacterial suspension, added to the filtering apparatus, was correlated with bacterial abundance per FOV, so that the abundance fell between 20 and 100 for ease of counting. A range of volumes (5, 10, 20, 40, 80µl) were trialed with 3 replicates each, and the number of bacteria per filter compared. As the suspension volume increased, it became more difficult to discriminate between the bacteria and the background. In replicates 2 and 3, bacterial abundance increased to a peak at 10µl, after which numbers remained similar (Figure 5.1.1). Replicate 1 reached a peak at 20µl, after which a large decrease was recorded. Bacterial numbers dropped or remained constant possibly because individual cells were obscured from the observer's view by the sediment particles and other matter trapped on the filter. Consequently, 10µl of sediment suspension was chosen as the appropriate volume.

Trial 2. Ultrasonication time

The time of ultrasonication, needed to dissociate bacteria from the sediment grains, was investigated via a series of trials. Too little time would only separate a proportion of the cells, while too much time would dissociate all the other matter as well from the particles, and possibly damage cells (Lindahl and Bakken 1995). Sediment samples were initially sonicated for 5, 10, 20, 30, and 60 min, and the bacteria enumerated. Bacterial abundance peaked at 5 min, after which it decreased and remained constant after 20 min (Figure 5.1.2 inset). A second set of samples was sonicated for 1, 2, 3, 4, and 5 min, and bacterial abundance increased over time, reaching a peak at 5 min (Figure 5.1.2). The sonication time

Figure 5.1.1

The effect of bacterial suspension volume on bacterial cell counts. The DAPI staining technique was used to make counts of bacterial cells and variables in the method, such as bacterial suspension volume, were adjusted to suit Warneet, Western Port, sediments. Each data series on the graph represents a replicate. Means $\pm$ SE are shown, and n = 3.

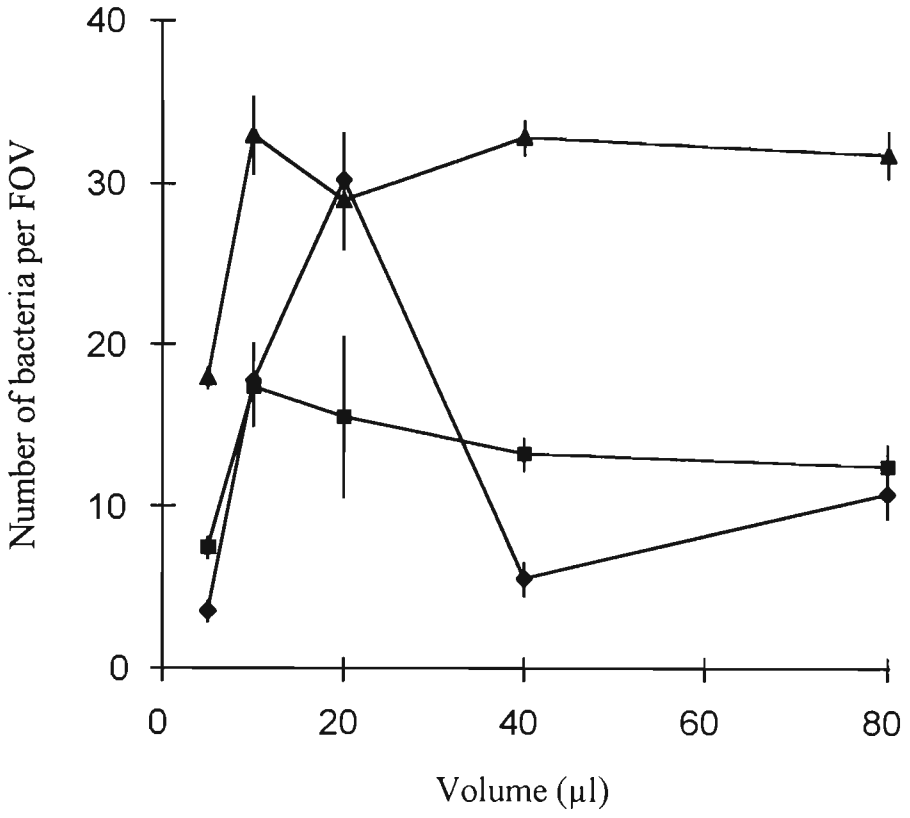
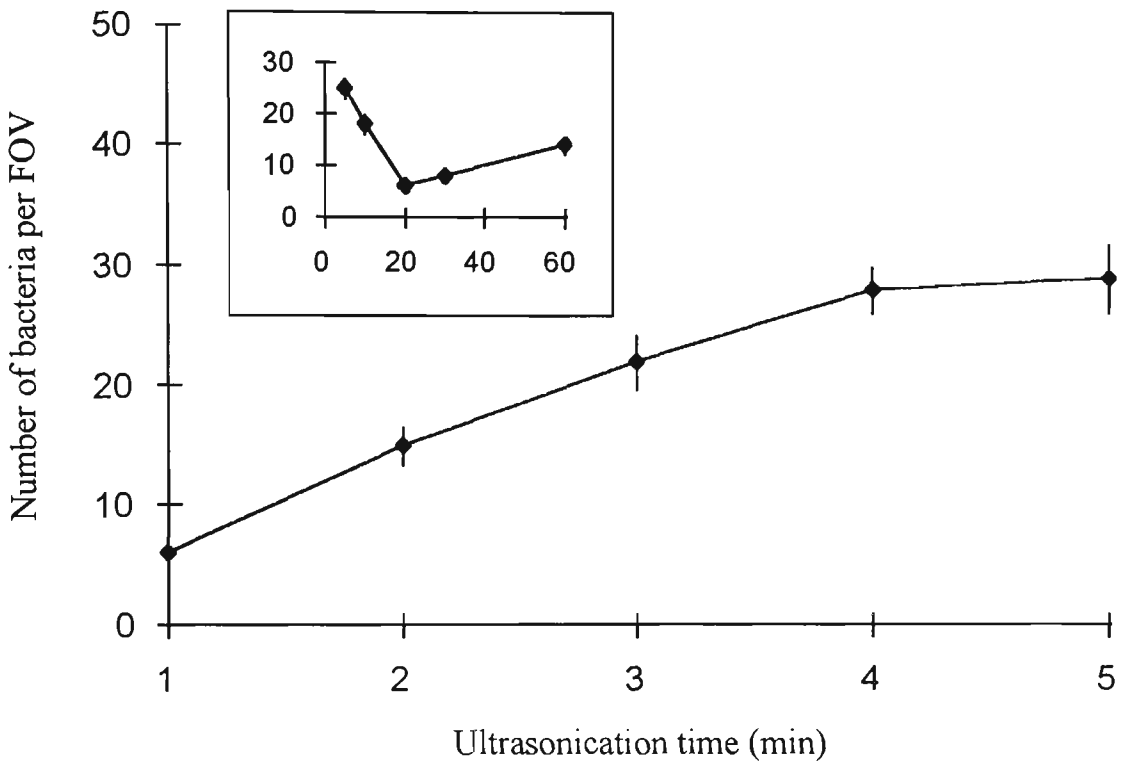

40
30
20
10
0
Number of bacteria per FOV
0
20
40
60
80
Volume (µl)

Figure 5.1.2

Effect of ultrasonication time on bacterial cell counts. The DAPI staining technique was used to make counts of bacterial cells and variables in the method, such as ultrasonication time, were adjusted to suit Warneet, Western Port, sediments.

Inset: Initial trial over time series 5 - 60 min. Means $\pm$ SE are shown, and n = 3.


Number of bacteria per FOV
50
40
30
20
10
0
1
2
3
4
5
Ultrasonication time (min)
30
20
10
0
0
20
40
60

suggested in the published method (5 min) was suitable for these sediment samples.

Trial 3. Source of variation

To improve precision of the direct count method, some studies have reduced the variability by determining the major source of variation and limiting its effect. It appears that more emphasis should be placed on replication at higher levels (the sample), with fewer replicates of filters and fields of view (Kirchman *et al.* 1982, Montagna 1982, Johnson *et al.* 1993). Kirchman *et al.* (1982) found that FOV contributed 60-80% of the total variation, filters contributed 20% and subsamples less than 10%. The optimal method used 6-7 FOV per filter (any less was not recommended due to uneven distributions of bacteria on the filter), approximately 1 filter/subsample, and 2 subsamples from a sample (Kirchman *et al.* 1982). Unfortunately, due to the small size of burrow wall samples collected, this level of subsample replication was not possible in this study.

It was important therefore to identify where the major source of variation was in these sediment samples, and to reduce it if possible. Four independent sediment samples were collected and the procedure followed as above (with the optimal suspension volume and sonicating times). Three filters from each sample were made and bacteria on 8 FOVs were counted for each filter. Statistical analysis of the data was performed with the Statistica ® software package. The statistical design was a one-way ANOVA with a fixed factor (sample), and a random factor (filter) nested within each sample. The data were tested for homogeneity of variances, using Cochran's C Test, and normality, and ln(x+1) transformed.

A very significant difference was found between filters within samples (Table 5.1.1), confirming that numbers of bacteria varied significantly between filters within a sediment sample (Figure 5.1.3). It seemed that the only way to improve variability was to increase the number of subsamples taken from each sample (as suggested by Kirchman *et al.* 1982), but due to the small volume of mud that could be collected (especially for burrow walls) this was impossible.

Figure 5.1.3

Identifying the source of variation within the DAPI staining procedure used to estimate bacterial cell count in Warneet, Western Port, sediments. Bacterial abundances were estimated and compared from each of 3 filters (solid, open and shaded bars) sampled from 4 independent sediment samples. Means + SE are shown, and n = 8.

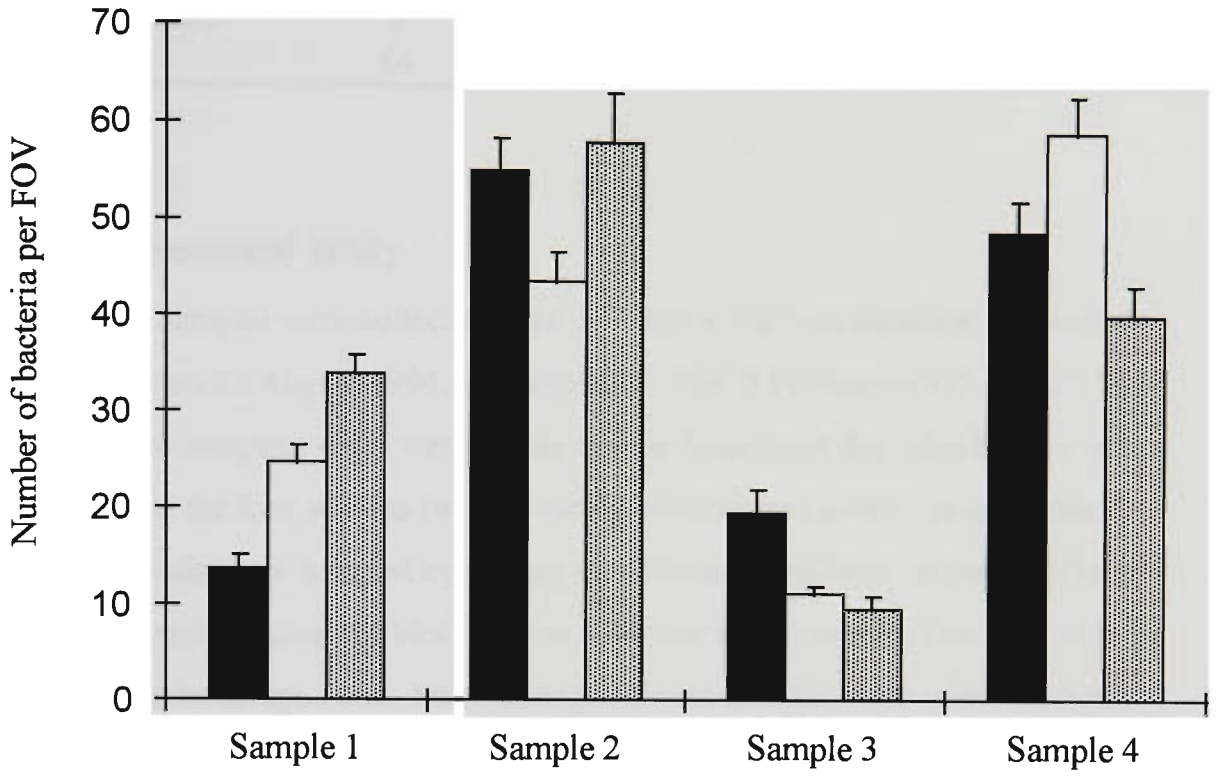

Number of bacteria per FOV
70
60
50
40
30
20
10
0
Sample 1
Sample 2
Sample 3
Sample 4

Table 5.1.1. Statistical analysis of bacteria cell count variation between filters within samples.

Effect	df	Mean square	F-ratio	Probability
Sample	3	11.03	13.13	< 0.003
Filter(sample)	8	0.84	14.42	< 0.001
Error	84	0.058		

5.1.2.2 Seasonal study

Sediment samples were collected from the burrow wall, surrounding and surface sediments on 23 August 1994, 24 November 1994, 3 February 1995, and 19 May 1995. The sampling dates were evenly spaced throughout the calender year to encompass the four seasons (winter, spring, summer and autumn respectively). Burrow walls were sampled by digging up a block of sediment on the sandflat at low tide, and shearing the block so that a burrow was exposed. The burrow wall could then be scraped out. Surrounding sediment samples were collected from the same block of sediment, 5 cm below the surface and 5 cm from the burrow. Surface sediments were scraped to approximately 2 mm deep adjacent to the burrow openings. Approximately 1 cm^3 sediment samples were collected, with three replicate burrows sampled on each date. Sediment was stored in plastic test tubes, and preserved with 1% formalin (filtered through a 0.2 μm mesh size). Samples were refrigerated at 5 °C until analysis.

5.1.2.3 Statistical analysis

Statistical analysis of the data was performed with the Statistica ® software package. The statistical design was a two-way ANOVA with one random and nested factor. Three sediment types (burrow wall, surrounding subsurface, surface) were each sampled from 3 randomly chosen burrow replicates, which in turn were nested within the 4 seasons. The data was checked for homogeneity of variances, using Cochran's C Test, and normality and ln(x+1) transformed. Power of the 2 fixed factors, season and type, was also analysed (Zar 1984).

5.1.3 Results

Surface sediments had the highest bacterial numbers, and subsurface sediments the lowest (Figure 5.1.4). There was no significant difference between bacterial numbers of the surface sediments and burrow walls, and no difference between those of walls and subsurface sediments (Table 5.1.2), but there was a significant difference between abundances in the surface and subsurface sediments. Bacterial numbers tended to increase in the burrow walls relative to the subsurface sediment, because numbers were similar to the surface, but this increase was not large enough to warrant a significant difference. Statistical power of the effect 'sediment type' was only 55%, so an increased number of replicates (ie. greater statistical power) may have shown a greater difference.

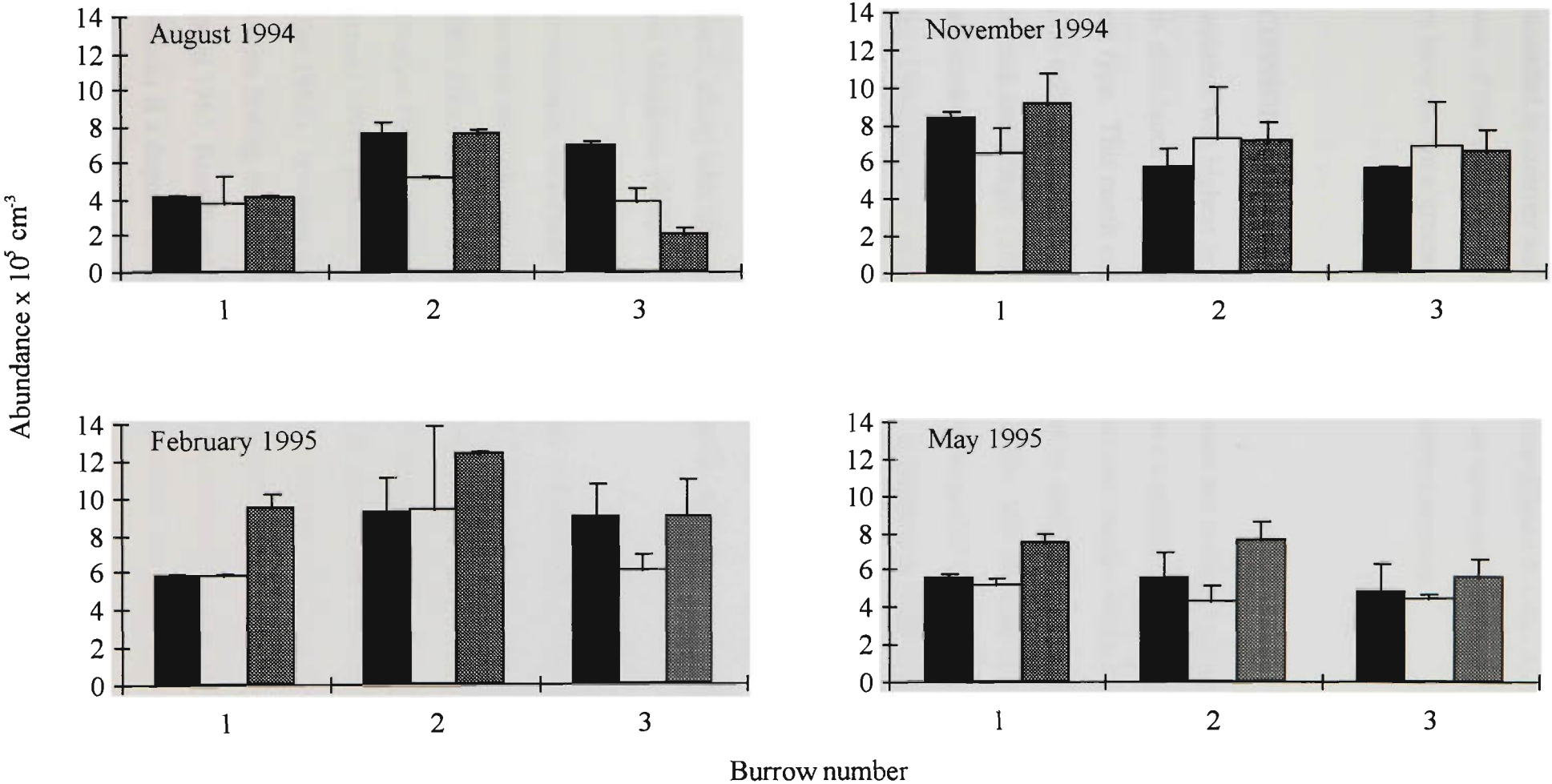
Table 5.1.2. Statistical analysis of bacterial density comparing seasons, burrows (nested within season) and sediment types (sampled from each burrow).

Effect	df	Mean square	F-ratio	Probability
Season	3	0.199	4.269	0.045
Burrow(season)	8	0.047	2.955	0.012
Sediment type	2	0.070	4.166	0.035
Season x type	6	0.032	1.890	0.145
Burrow(season) x type	16	0.017	1.068	0.418
Error	36	0.016		

Burrows showed significant variation, but no burrow by type interaction was observed. The pattern of abundance within burrows (surface abundances generally higher than or similar to the burrow wall, and the wall abundances higher than or similar to the surrounding sediment) remained the same across seasons, with the exception of burrow 3 in winter (Figure 5.1.4). Abundances in this burrow were highest in the burrow wall and lowest in the surface sediments, a quite different pattern from the others. Burrows 2 and 3 in spring were also unusual, with subsurface values resembling both the wall and surface sediment values (Fig. 5.1.4).

Figure 5.1.4

Bacterial abundance in samples collected from burrow walls (solid), surrounding subsurface (open) and surface (shaded) sediments associated with the burrows of *Biffarius arenosus*. Samples were collected over 4 seasons between August 1994 and May 1995 from the sandflat at Warneet, Western Port. Means $\pm$ SE are shown.


August 1994
November 1994
February 1995
May 1995
Abundance x 10^5 cm^-3
0
2
4
6
8
10
12
14
1
2
3
Burrow number

A weakly significant seasonal effect was observed (Table 5.1.2), with highest abundances recorded in summer and lowest in winter (Figure 5.1.4). Again the statistical power of this effect was low (55%), so an increased number of replicates may have shown a greater difference between seasons.

5.1.4 Discussion

Bacterial abundance was highest in surface sediments and lowest in subsurface sediments, but abundance in the burrow wall did not significantly differ from either sediment type. This result conflicts with previous studies which have found that burrows do enhance bacterial densities relative to surrounding anoxic sediments. Branch and Pringle (1987) described a 30-100% increase in bacteria and protozoan numbers in the burrow wall in the presence of *Callianassa kraussi*. Aller and Aller (1986) reported nearly a doubling of bacteria in walls of the polychaete *Amphicteis* sp. burrows, compared to surrounding sediments, as did Köster *et al.* (1991) in deep-sea enteropneust burrows. *Nereis diversicolor* burrow walls were found to resemble surface sediments by having high bacteria numbers, along with high redox, low sulphide and high chlorophyll *a* (Anderson and Meadows 1978).

Grazing by protozoans, meiofauna and macrofauna is important in shaping microbial communities (Johannes 1965, Hargrave 1970a, Gerlach 1978, Morrison and White 1980, Hicks and Coull 1983, Alongi 1985, Moriarty *et al.* 1985a, Gerdol and Hughes 1994). Meiofauna sometimes increase in numbers around burrows (Dittman 1996), possibly in response to increased microbial abundance (Aller and Aller 1986), however this increase is not consistent among species, with some studies finding that meiofaunal numbers actually decrease around burrows (Alongi 1985, Branch and Pringle 1987, Dobbs and Guckert 1988). *Biffarius arenosus* is a deposit feeder, ingesting sediment and plant detritus within its burrow. New diggings and the burrow wall appear to be the main sources of food for this species, so grazing may be important in regulating bacterial numbers in *B. arenosus* burrow walls (Chapter 3). Macrofaunal grazing reduces bacterial

densities in sediments (Hargrave 1976, Moriarty *et al.* 1985a, Gerdol and Hughes 1994), but increases biomass, respiratory activity and biosynthesis of lipids in the surviving microbes (Morrison and White 1980). Hence, we may expect lower numbers in burrow walls, but greater production and activity of the surviving bacteria. One way to investigate further would be to compare bacterial production rates of surface, wall and subsurface sediments. Higher production rates should be positively correlated with increased grazing intensity.

The seasonal change in bacterial abundance observed in these sediments was consistent with the findings of other studies (Meyer-Reil *et al.* 1978, DeFlaun and Mayer 1983, Moriarty *et al.* 1990, Danovaro 1996). DeFlaun and Mayer (1983) described a change in appearance of cells over seasons, with cells appearing shrivelled in winter, and expanding again in higher temperatures. Rublee (1982) also documented a positive relationship between temperature and cell. Growth rate and generation time of marine bacteria are both known to be temperature dependant (Ulitzur 1974).

Variation between burrows was significant, but no burrow by type interaction was observed. The pattern of abundances remained consistent throughout burrows, with surface abundances tending to be the highest, and subsurface sediment abundances tending to be lowest. The significant difference between burrows is probably due to spatial heterogeneity of the sediments, which is often a problem for gaining significant results in this type of study (Montagna 1982, Reichardt 1988). Patchy distributions of organic matter in sediments lead to uneven distributions of microbes associated with the organic material. A burrow near a rich food source such as a buried carcass or concentration of plant material, would have a greater abundance of bacteria than a burrow further away. Additionally, the inhabitant of the burrow close to the source would be subject to greater anoxia because of the increased respiration related to the decomposition of the organic matter, and would therefore need to irrigate its burrow more regularly to maintain livable conditions. Effects of burrows and burrowing activities on biogeochemical qualities of sediment depend on taxon, life habit,

mobility, life history stage and feeding type (Aller and Aller 1992, Steward *et al.* 1996), and may also correlate with a difference in bacteria.

Relative to other studies, the Warneet intertidal sandflat supports a small bacterial population. The bacterial abundance, estimated via direct epifluorescent counts of bacterial cells, ranged from 1.8×10^5 to 1.4×10^6 cells cm^{-3} (corresponding to 4×10^5 to 3×10^6 cells g^{-1} dw) of sediment. Previous reports of abundances in intertidal and shallow subtidal marine/estuarine sediments fall between 10^8 and 10^{10} bacterial cells g^{-1} dw (Hines 1985, Hansen *et al.* 1987, Bianchi 1988, Moriarty *et al.* 1990, Danovaro 1996). This study's results are several orders of magnitude below the previously published range of values, and there are several possible reasons for the discrepancy.

The first is that the Warneet sediments (0.05 to 0.83% organic carbon) are particularly devoid of organic carbon, and hence a food supply for the bacteria. However, Kemp (1987) found 10^8 bacteria cells g^{-1} dw in sediments with 0.5-0.7% organic carbon, and others have documented similar abundances in similarly low % organic carbon (Dale 1974, DeFlaun and Mayer 1983, Aller and Aller 1986), so it is unlikely that low % organic carbon accounts for the result.

Other explanations involve the accuracy of the DAPI direct count method. Direct counts are notorious for underestimating bacterial cell abundance (Newell *et al.* 1986). One major problem is separating the bacteria from sediment grains and detritus into suspension for enumeration. Moriarty (1980) found that detaching microbes from sediment by blending was neither quantitative nor reproducible, whereas ultrasonication, as used in this study, was shown to be more successful (Ramsay 1984). However, ultrasonication also destroys cells, so a sonication intensity which ensures cell survival whilst removing the maximum number of cells is needed (Lindahl and Bakken 1995). Most studies sonicate samples for between 5 s and 2.5 min (Reichardt 1988, Poremba and Hoppe 1995), because cell damage incidence increases with time of ultrasonication (Ramsay 1984). Counts can, however, be adjusted with an appropriate correction factor (Ellery and Schleyer 1984). In the present study, ultrasonication time of 5 min was

chosen because the maximum number of bacterial cells was gained from sediments after this time. Even if a correction factor of 2 (estimated from Ellery and Schleyer 1984) was needed to account for cell damage after 5 min ultrasonication, bacterial counts would still not increase to published levels.

Nearly all other studies used for comparison stained bacteria with acridine orange (AO) rather than DAPI. Newell *et al.* (1986) found that AO yielded higher (up to double) bacterial counts than DAPI from the same sediments. Even if AO was appropriate for these sediments, abundance would still only increase by a factor of 2.

A final consideration is the method used to preserve and store bacterial samples prior to enumeration. In this study, samples were stored in 1% formalin below 5 °C for between 7 and 180 days. Turley and Hughes (1992) documented a loss of only 15 % of the bacterial cells from a seawater sample preserved in 1% formalin and stored a room temperature for a similar number of days. Even if 15% of the bacterial cells were lost during preservation and storage the decrease in cell number would still not account for the unusually small population density recorded.

Hence, no one specific explanation appears to account for the very low population density found in this study, and parallel measurements, estimated from total phospholipid concentrations (section 5.3), should reveal if these abundances are realistic.

5.2 Microbial activity

5.2.1 Introduction

The measurement of the abundance of microorganisms yields valuable information, but it gives no indication of community dynamics or relationships between microbes and environmental factors such as food supply. For instance, many of the bacteria identified with epifluoresence microscopy may be metabolically dormant, and thus not functionally important in organic matter breakdown and nutrient cycling. Measuring microbial activity, in contrast, provides a better idea of these processes, because microbial metabolic activity responds quickly to fluctuating environmental conditions such as alterations to oxygen and dissolved organic carbon supplies (Boulton and Boon 1991).

Microbial activity is closely linked to the decomposition of organic matter. The breakdown of large organic molecules involves both hydrolysis by extracellular enzymes produced by microbes, and the transfer of electrons during remineralisation. The uptake of small organic molecules and inorganic nutrients and oxygen, results in cell maintenance, growth and production. Various techniques are used to quantify different facets of microbial activity (Boulton and Boon 1991). Respiratory activity can be examined by quantifying the rate of respiration (Sinsabaugh and Linkins 1990) or the electron transport system (Zimmerman 1975, Broberg 1985, Stubberfield and Shaw 1990, Songster-Alpin and Klotz 1995, Relexans 1996a,b); extracellular enzyme activity can be measured using colorgenic or fluorogenic substrates (Meyer-Reil 1986, Boulton and Boon 1991, Poremba and Hoppe 1995), and the uptake of radio-labelled organic substrates by bacterial cells can be quantified (Van Es and Meyer-Reil 1982). Bacterial growth and productivity can be determined via adenine incorporation (Craven and Jahnke 1992), thymidine uptake (Moriarty and Pollard 1982, Moriarty *et al.* 1990, 1991), or the incorporation of leucine into protein (Kirchman *et al.* 1985).

Sediment interfaces such as the sediment-water boundary and the redox discontinuity layer are often characterised by high microbial activity (Meyer-Reil 1991). Several studies have documented a peak in activity at the sediment surface and a rapid decrease with sediment depth (Broberg 1985, Meyer-Reil 1986, Novitsky and Karl 1986, Craven and Jahnke 1992, Poremba and Hoppe 1995, Relexans 1996a). Macrofaunal burrows, however, enhance microbial activity below the sediment surface (Köster *et al.* 1991, Boetius 1995), with the result that microbial activity peaks in the burrow walls (Reichardt 1988). Even though it is well known that faunal burrows are highly reactive zones of sediment (Aller 1988), few studies have investigated the impact of burrows on sediment microbial activities. Microbial activity in thalassinidean burrows, especially, has been largely overlooked despite the potential impact these extensive and often deep burrow systems may have on microbial-mediated decomposition processes occurring in marine sediments.

This section of work aims to quantify the activity of microbial populations inhabiting burrow walls of *Biffarius arenosus*, compared with activity in the surrounding subsurface and surface sediments. Two types of activity were chosen to investigate this aim: the electron transport system, quantified through the reduction of the tetrazolium salt INT to formazan; and hydrolytic enzyme activity, measured via the hydrolysis of the substrate fluorescein diacetate.

5.2.2 Methods

5.2.2.1 Methodological trials

The first method used in this study involved the reduction of a formazan salt (INT-tetrazolium), and was unsuccessful because the salt was reduced by both biological and abiological reactions occurring in the sediments. The second method, chosen to circumvent this problem, by measuring enzyme activity rather than bulk reduction, involved the hydrolysis of fluorescein diacetate. Both methods, and their associated problems are described here.

5.2.2.1.1 Tetrazolium salt (INT) reduction

The INT method measures the potential for electron transfers in a biological population via the reduction of a tetrazolium salt ((2-P-iodophenyl)-(3-P-nitrophenyl)-5-phenyl tetrazolium chloride) to formazan (Zimmerman 1975). The salt interacts with the flavoproteins, quinones or cytochromes at the site of electron transfer (Zimmerman 1975), and is widely used for recognising actively respiring bacteria (Newell *et al.* 1986). Because this method interacts with electron transfer it works well in pure cell cultures (Stubberfield and Shaw 1990), but can be subject to abiological interferences in highly reactive sediments (Newell *et al.* 1986, Relexans 1996a). Stubberfield and Shaw (1990) described no background reduction of INT in soil samples, and Songster-Alpin and Klotz (1995) appeared to use the method successfully in freshwater sediments, however there was no mention of validation trials to ensure a linear increase of activity with time.

The procedure described in Zimmerman (1975) was tested first. Two ml phosphate buffer pH 7.7 (KH_2PO_4 and NaOH) and 1 ml INT (1g INT dissolved in 500 ml ultrafiltered water) was added to 1g fresh sediment, and the mixture incubated at 35 °C for 20 minutes. The reaction was stopped with 5 ml organic solvent (2:3 mixture tetrachloroethylene and acetone) and samples were placed on ice and shaken every 15 min for 1 hour, after which they were frozen. The extracted formazan was separated from the sediments by centrifugation (3000 rpm for 5 min), and the optical absorbance read at a wavelength of 496 nm. This procedure was unworkable because the solvent was insoluble in water, so inaccurate readings were gained if the cuvette was not totally dry after rinsing between samples.

A more workable solvent, ethanol, was suggested by Stubberfield and Shaw (1990). One ml phosphate buffer and 0.5 ml 8 mM INT and 0.5 ml 0.2M sodium succinate (pH 7.5) was added to 1g sediment and the sample incubated at 25 °C for 20 min. The reaction was halted using 5 ml ethanol and the samples were shaken and placed on ice for 1 h. The sample was removed and placed in an

ultrasonic bath for 5 min to ensure all formazan was dissolved in the ethanol, centrifuged (3000 rpm for 5 min) and the optical absorbance read at 461 nm.

Documented wavelengths varied (Zimmerman 1975, Stubberfield and Shaw 1990), so the wavelengths of a range of dilutions of the formazan in ethanol were measured. The measured wavelengths were then related back to the dilutions suggested in the method, and the appropriate wavelength was found to be 461 nm.

The Stubberfield and Shaw (1990) version of the INT method was further examined to see whether responses were linear with time. Various treatments were:

1. method as described with fresh sediment;
2. method as described with autoclaved sediment, to test if the activity measured originated from a biological source;
3. method as described without sediment, as the first control;
4. method as described with sediment but without INT, as the second control;
5. method as described with sediment but without 0.2M sodium succinate as an additional electron donor.

Four replicate sediment samples were used per treatment. Treatments 3 and 4 gave very low and relatively constant absorbance readings, as expected (Figure 5.2.1). This demonstrated that INT alone and sediment alone did not give rise to significant optical absorption at a wavelength of 461 nm. The treatment containing autoclaved sediment gave absorbance readings much lower than normal sediment, suggesting that the INT method was measuring biological activity. The presence of sodium succinate had little influence on the result. However, the result from treatment 1, showed that the fresh sediment contained a compound(s) that immediately reduced the INT to its formazan salt. If purely

Figure 5.2.1

Validation trials of the INT-tetrazolium assay. This assay relies on the electron transfer system to give a measurement of microbial activity in a sample and was trialed as a technique for possible use with Warneet, Western Port sediments. Means ± SE are shown, and n = 3.

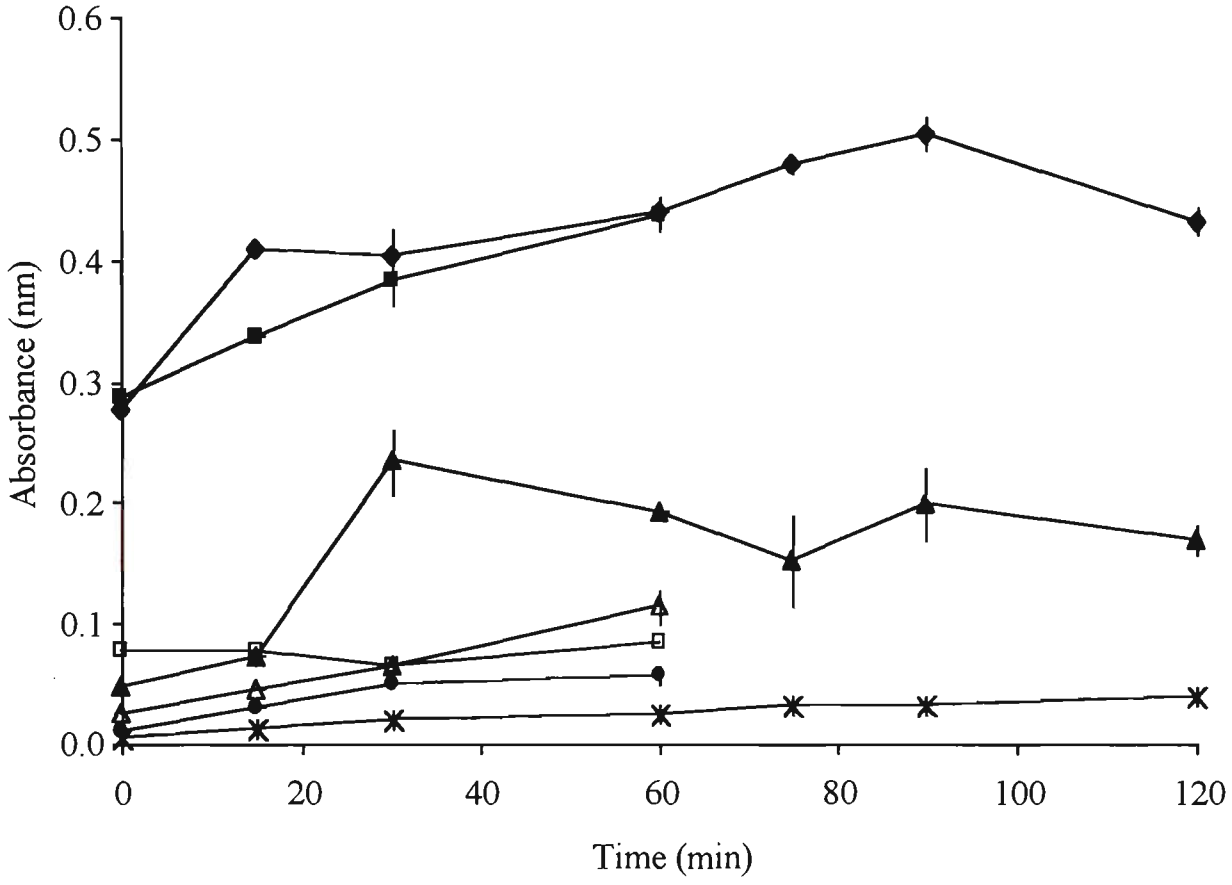
Key:

Symbol	Fresh sediment	Autoclaved sediment	INT	Sodium succinate
◆	+	−	+	+
■	+	−	+	−
▲	−	+	+	+
△	−	+	+	−
×	−	−	+	+
●	−	−	+	−
□	+	−	−	+

where:

+ indicates presence, and

- indicates absence.


0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance (nm)
0
20
40
60
80
100
120
Time (min)

biological activity was being measured, time courses would have been linear under treatment 1. Autoclaving apparently removed these interfering compounds, as well as stopping all biological activity. Relexans (1996a) also found that the chemical reduction of INT was a major problem when using this technique to examine electron transfer in reduced coastal sediments. Due to the difficulty of separating biological and abiological reduction of INT, this method was deemed not suitable for measuring microbial activity in these sediments.

5.2.2.1.2 Fluorescein diacetate

The fluorescein diacetate (FDA) assay is a better measure of microbial activity than the INT assay in a range of environmental materials (Stubberfield and Shaw 1990). FDA is hydrolysed by non specific esterases, such as phosphatase, lipase and carbohydrate and protein-degrading enzymes (Meyer-Reil 1991), causing a release of highly fluorescent fluorescein, which permits very sensitive determinations (Meyer-Reil and Köster 1992). Measuring extracellular enzymatic hydrolysis gives insight into a key process of decomposition, because organic matter must first be decomposed extracellularly before it can be incorporated into bacterial cells (Meyer-Reil 1991). Enzymes which may hydrolyse the FDA are either excreted from living cells (mainly microorganisms) or are liberated during cell lysis (Meyer-Reil 1981). Dead cells can be either plant, animal or microbial (Gumprecht *et al.* 1995), and are known to persist and be active for some time after lysis (Burns 1980).

It is well known that disturbance alters metabolic rates and biomass of microbes (Findlay and White 1984) by disrupting fine structure of sediments, destroying microhabitats and bacterial aggregates, isolating and optimally suppling cells with substrate in a competition-free zone (Meyer-Reil 1986). In this technique, rates are measured from sediment slurries under FDA saturation, measuring potential activity, not actual activity (Poremba and Jeskulke 1995).

The method was originally described by Swisher and Carroll (1980) for microbial activity on coniferous needle surfaces, but the basic technique described by

Stubberfield and Shaw (1990) was used here. A known weight of sample was added to 19 ml 0.1M phosphate buffer (pH 7.6) and 0.1 ml FDA solution (4.8 mM in acetone), and the mixture shaken for 2-4 hours at a constant temperature. After incubation the reaction was slowed by keeping the tubes on ice. The sample was filtered and the fluorescence read at excitation wavelength of 490 nm. This method needed to be validated for the sediment samples to be analysed. Five validation trials were designed, each testing some part of the methodology:

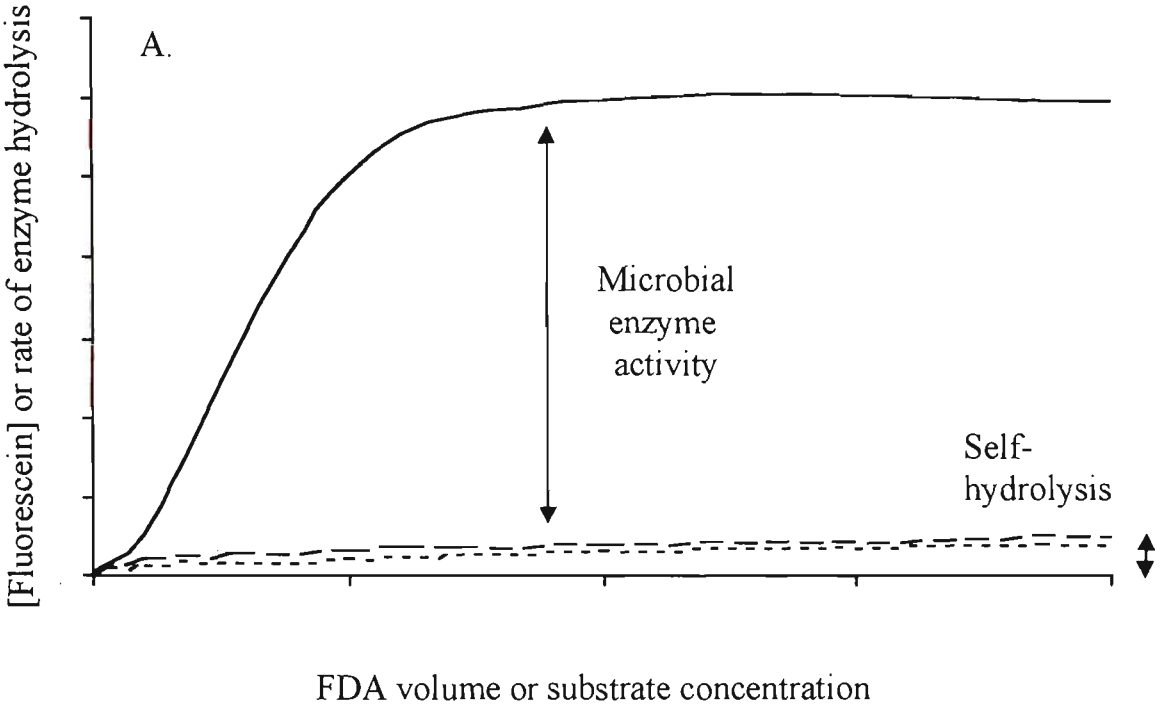
1. method as described, except the volume of FDA was varied to ensure saturation;
2. method as described without sediment, to test if the buffer hydrolysed the FDA substrate to any extent;
3. method as described with autoclaved sediment, to ensure that the assay was measuring purely biological activity;
4. method as described, except the amount of sediment was varied to ensure that measurement was taken on the linear part of the curve;
5. method as described, except incubation time was varied to test for linearity.

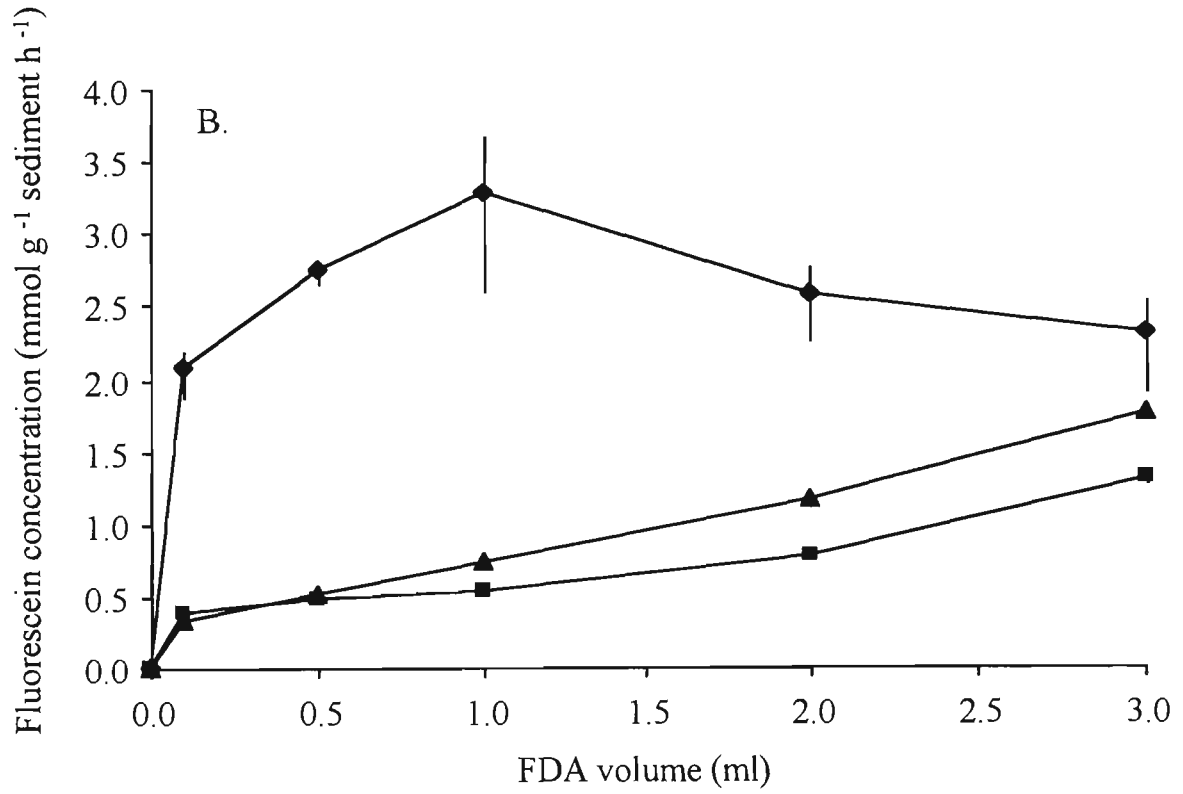
One gram of sediment was added to 19 ml buffer, and the volume of FDA varied within the range of 0 - 3 ml. The ideal volume of FDA would saturate the sample, so maximum activity level for 1 g of mud could be measured. Figure 5.2.2a shows the predicted increase in activity with FDA concentration, compared to predicted activity in autoclaved sediment, and the control assay containing no sediment. The predicted increase with FDA concentration should start at zero and increase rapidly until maximum activity is reached. This curve resembles a rectangular hyperbola. Of course this relationship will not apply infinitum, because there will be compounding factors. For example in this assay, precipitation was present at high concentrations of FDA, probably contributing to the drop in measured activity after 1.0 ml FDA (Figure 5.2.2b). One ml of FDA was chosen as the ideal volume because it produced the highest concentration of fluorescein (Figure 5.2.2b).

Figure 5.2.2

(A) Theoretical response of microbial activity to increasing FDA volume in different sediment treatments: fresh sediment (solid line), autoclaved sediment (broken line) and no sediment (dashed line).

(B) Effect of different volumes of FDA on enzyme activity in fresh (diamond) and autoclaved (square) sediment, and a control assay containing no sediment. Means $\pm$ SE are shown, and n = 3.


A.
[Fluorescein] or rate of enzyme hydrolysis
Microbial enzyme activity
Self-hydrolysis
FDA volume or substrate concentration


B.
Fluorescein concentration (mmol g -1 sediment h -1)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
FDA volume (ml)

Sediment was autoclaved for 35 min at 120 °C to kill the bacteria and to observe if the assay was affected by any abiological activity. Activity measured in the autoclaved sediment treatment and the assay without sediment was considerably lower than that measured in the fresh sediment assay (Figure 5.2.2b) but was higher than expected (Figure 5.2.2a), indicating that the buffer was hydrolysing the FDA to a small extent. The apparent hydrolysis appeared to increase with increasing FDA volume, suggesting that the volume of FDA used in the assays should be kept at a minimum.

Activity appeared to double with each linear increase in sediment weight (Figure 5.2.3). This doubling theoretically should continue until the FDA supply is depleted. The wet weight of sediment chosen for the final method, was relative to the volume of mud held in a small spoon. Because the assay would be run in situ, it was important to choose a sampling technique which would be reproducible. The final validation involved running a time series (with 1 g sediment and 1 ml FDA), to ensure that the enzymatic response was linear over time, therefore justifying extrapolation of activity rates. Microbial activity increased linearly until 3 h (Figure 5.2.4), after which the curve began to flatten. Two hours was chosen as the incubation time.

5.2.2.2 Seasonal study

Samples were collected on 26 March 1996, 23 July 1996, 23 October 1996 and 14 January 1997, each date evenly spaced throughout the year to encompass seasonal variation. In the field, 3 samples of 1.2 g (wet weight) of sediment were collected from each sediment type (burrow wall, subsurface and surface sediments) from each of 5 replicate burrows. Nineteen ml 0.1M phosphate buffer (pH 7.6) and 1 ml FDA solution were added, and the mixture was agitated and incubated, buried in the sediment (at *in situ* temperatures) and agitated at half hourly intervals for 2 h. Samples were snap frozen in dry ice pellets and kept frozen for at least 2 h to halt activity, during transport to laboratory. After defrosting, the samples were filtered, diluted 1/100 and read immediately

Figure 5.2.3

The effect of mud weight on enzyme hydrolysis of FDA. The FDA hydrolysis technique was used to quantify microbial activity and variables such as mud weight were adjusted to suit Warneet, Western Port, sediments. Means $\pm$ SE are shown, and n = 3.

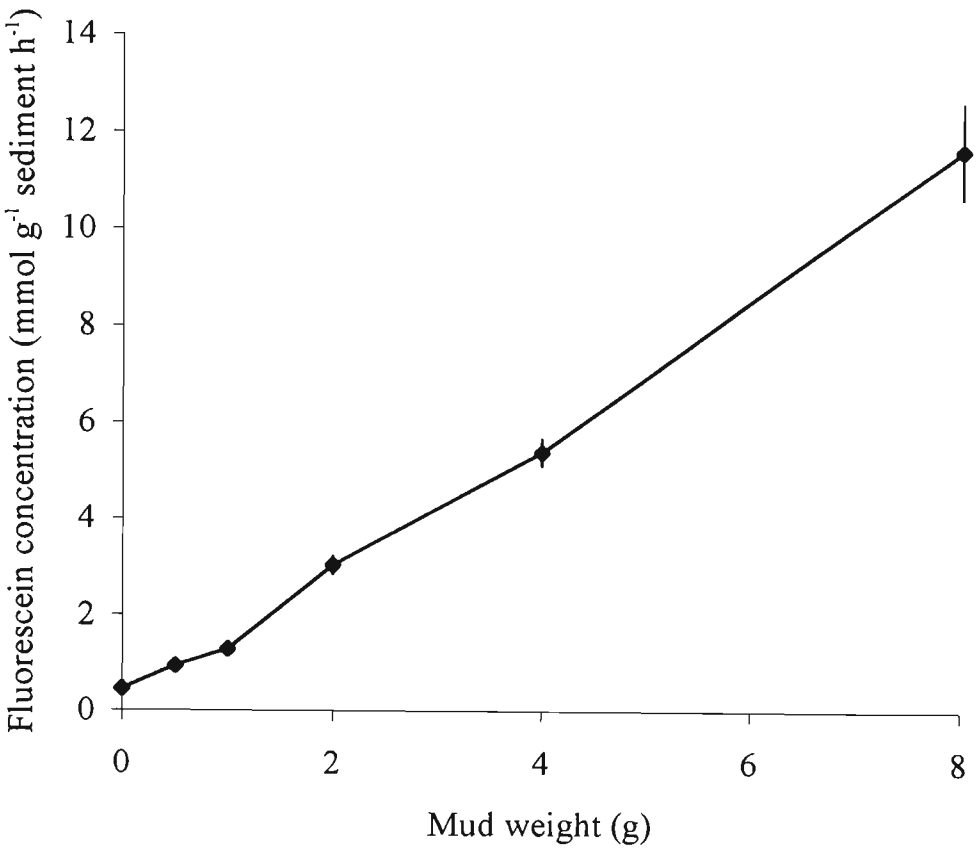
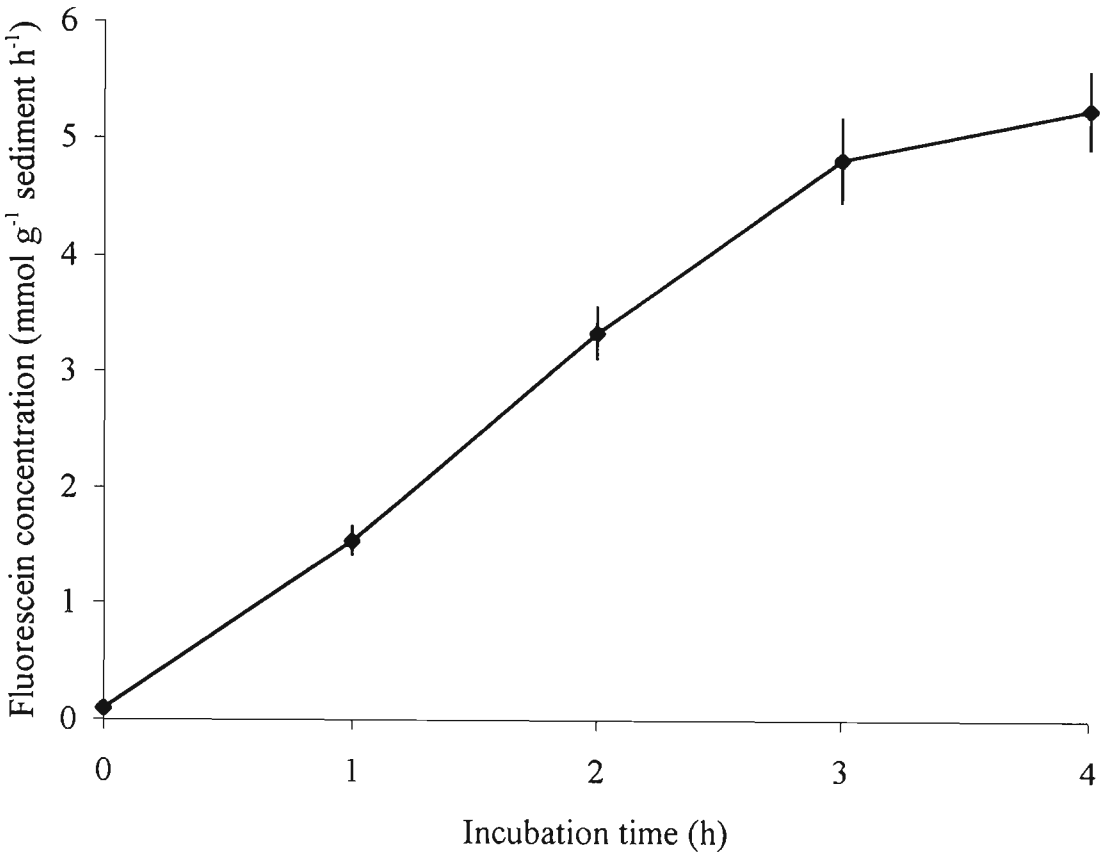

Fluorescein concentration (mmol g-1 sediment h-1)
14
12
10
8
6
4
2
0
0
2
4
6
8
Mud weight (g)

Figure 5.2.4

The effect of incubation time on enzyme hydrolysis of FDA. The FDA hydrolysis technique was used to quantify microbial activity and variables such as incubation time were adjusted to suit Warneet, Western Port, sediments. Means $\pm$ SE are shown, and $n = 3$.


Fluorescein concentration (mmol g-1 sediment h-1)
0
1
2
3
4
5
6
0
1
2
3
4
Incubation time (h)

(excitation 490 nm, emission 513 nm with a Perkin Elmer LS 50B Luminescence Spectrometer).

Frozen samples (post-incubation) could be stored overnight with negligible loss of fluorescein from the sample extract (Table 5.2.1). Blanks (no sediment) were assayed along with sediment samples at each sampling date to quantify the amount of FDA hydrolysis caused by the phosphate buffer. This value was usually very low, and was subtracted from the measurements to compensate. A further 5 burrow replicates were sampled, and the sediment kept at *in situ* temperatures for transport to the laboratory. Upon arrival, the assay was initiated with these samples, with the incubation temperature being a consistent 20 $\pm$ 1 °C (water bath) over all seasons. The purpose of this assay was to investigate if temperature had a major influence on microbial enzyme activity.

Table 5.2.1. Rate of fluorescein release (mmol g^{-1} h^{-1}) from two 1g sediment samples incubated for 2 h, and then frozen for either 2 or 24 h, to investigate the loss of fluorescein due to prolonged freezing.

Time left frozen (hr)	Range	Mean
2	3.82 - 4.44	4.03
24	3.52 - 3.85	3.65

The microbial activity was measured as arbitrary fluorescence units (AFU) g^{-1} ww sediment h^{-1}, and then converted to concentration of fluorescein (mmol g^{-1} h^{-1}) by dividing by 1.23 (slope of linear standard curve) and 1000 (nmol to mmol).

5.2.2.3 Statistical analysis

Statistical analysis of the data was performed with the Statistica ® software package. The statistical design was a two-way ANOVA with one random and nested factor. Three sediment types (burrow wall, surrounding subsurface, surface) were each sampled from 5 randomly chosen burrow replicates, which in turn were nested within the 4 seasons. The data were checked for homogeneity

of variances, using Cochran's C Test, and normality and ln(x+1) transformed. Power of the statistical comparisons was tested by post-hoc power analyses (Zar 1984), and results showed >99% power for each fixed effect: season and sediment type.

5.2.3 Results

5.2.3.1 Problems with homogeneity of variances

One central assumption of an ANOVA is that within-treatment variances are homogeneous. After transforming data to improve normality, the variances of the measurements taken at in situ temperatures were still heterogeneous (Cochran's C = 0.118, P = 0.025). Box plots of each variable were viewed and since variation around the means was satisfactory, the significance level of the ANOVA was reduced from 0.05 to 0.01 to increase robustness. Heterogeneous variances increase the risk of a type one error (finding there is an effect when there is not) but because results were very significant (Table 5.2.2), this was thought not to be a problem.

Table 5.2.2. Statistical analysis of microbial activity measured in and around burrows of *Biffarius arenosus* at in situ temperatures, comparing seasons, burrows (nested within seasons) and sediment types (sampled from each burrow). Measurements were made over 4 seasons between March 1996 and January 1997 at Warneet, Western Port.

Effect	df	Mean square	F ratio	P value
Season	3	27.230	317.34	< 0.001
Burrow (season)	16	0.086	2.37	0.004
Sediment type	2	15.798	224.40	< 0.001
Season x type	6	0.251	3.56	0.008
Burrow (season) x type	32	0.070	1.94	0.005
Error	120	0.036		

5.2.3.2 Microbial activity at in situ temperatures

Microbial activity in burrow walls was not significantly different from that measured in surface sediments, and both were significantly greater than subsurface sediments (Table 5.2.2). This pattern was consistent over all burrows and seasons (Figure 5.2.5). Microbial activity also showed significant seasonal variation (Table 5.2.2), with summer recording the highest activity, and winter recording the lowest (Figure 5.2.5).

There was a significant seasonal interaction with sediment type, indicating that the relative importance of type changed with seasons (Table 5.2.2). For example, in summer, activity in the surface sediments was higher than in the burrow walls, whereas in spring, burrow walls recorded a higher activity than surface sediments (Figure 5.2.5). There was also a significant difference between burrows, and a burrow by type interaction, indicating that differences between sediment types changed between burrows (Table 5.2.2). For example, in February 1995 microbial activity was highest in the surface sediments of burrow 4, whereas burrow wall sediments recorded the highest activity in burrow 5 (Figure 5.2.5). Activity measurements in wall and surface sediments, were however, always significantly higher than subsurface values.

5.2.3.3 The effect of temperature on microbial activity

With all seasonsí assays incubated at the same temperature (20 ± 1 °C), the seasonal variation was not as marked as for the assays incubated at in situ temperatures (Figure 5.2.6). Summer, again, had the highest microbial activity, but there was little difference between the three other seasons.

Other patterns were consistent with the assays incubated at in situ temperatures. Sediment type varied in the same way, with microbial activity being similar in the wall and surface sediments, but both significantly greater than the subsurface sediments (Table 5.2.3). There was also a significant difference between burrows, and a burrow by type interaction indicating that the relative importance of the wall and surface sediments differed between burrows.

Figure 5.2.5

Microbial enzyme activity of burrow wall (solid), surrounding subsurface (open) and surface (shaded) sediment incubated at *in situ* seasonal temperatures. Sediment samples were collected in and around burrows of *Biffarius arenosus* at Warneet, Western Port over 4 seasons between March 1996 and January 1997. Means $\pm$ SE are shown, and n = 3.

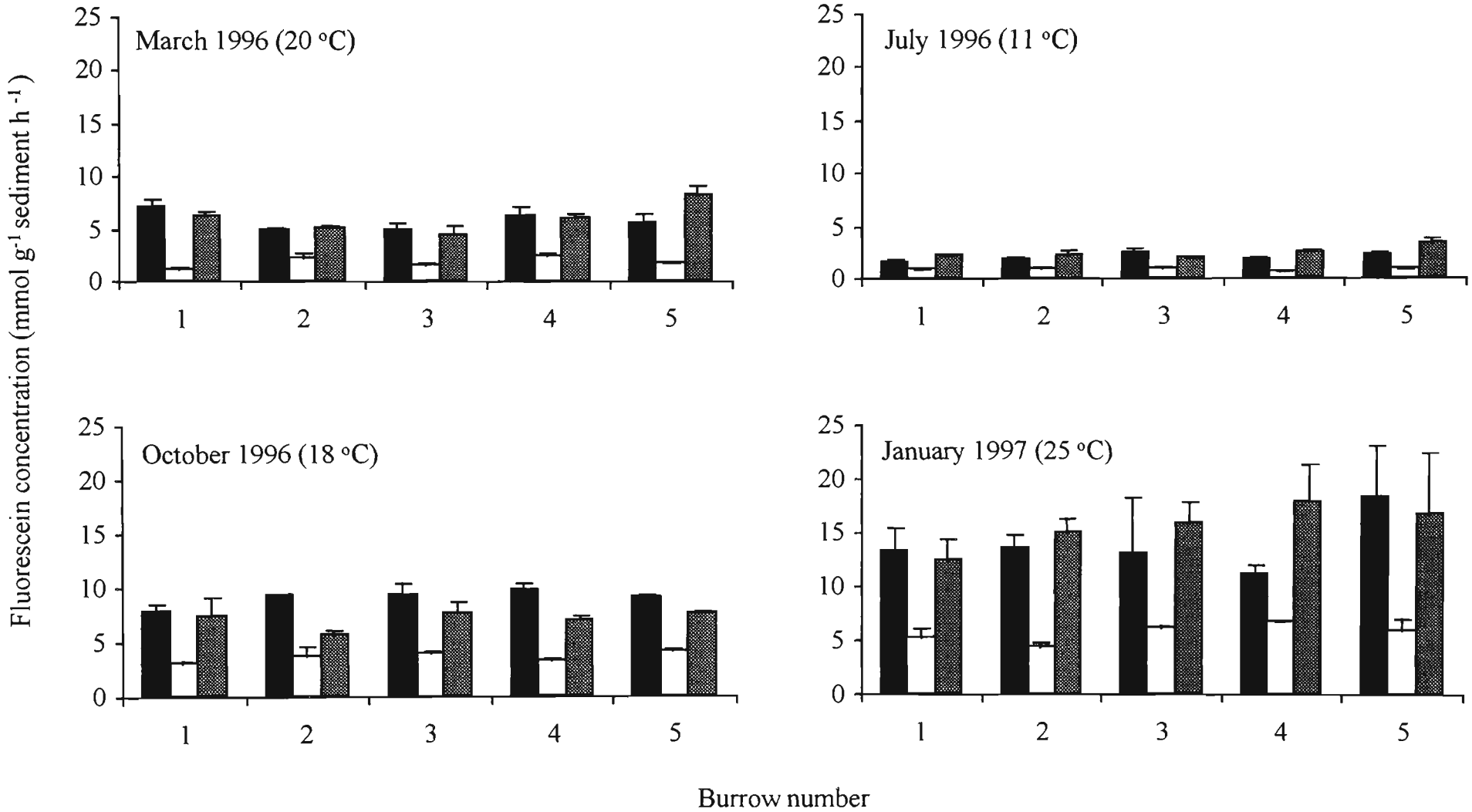

March 1996 (20 °C)
July 1996 (11 °C)
October 1996 (18 °C)
January 1997 (25 °C)
Fluorescein concentration (mmol g-1 sediment h-1)
Burrow number
0
5
10
15
20
25
1
2
3
4
5

Figure 5.2.6

Microbial enzyme activity of burrow wall (solid), surrounding subsurface (open) and surface (shaded) sediment incubated at a standard 20 °C ± 1 °C each season. Sediment samples were collected in and around burrows of *Biffarius arenosus* at Warneet, Western Port over 4 seasons between March 1996 and January 1997. Means ± SE are shown, and n = 3.

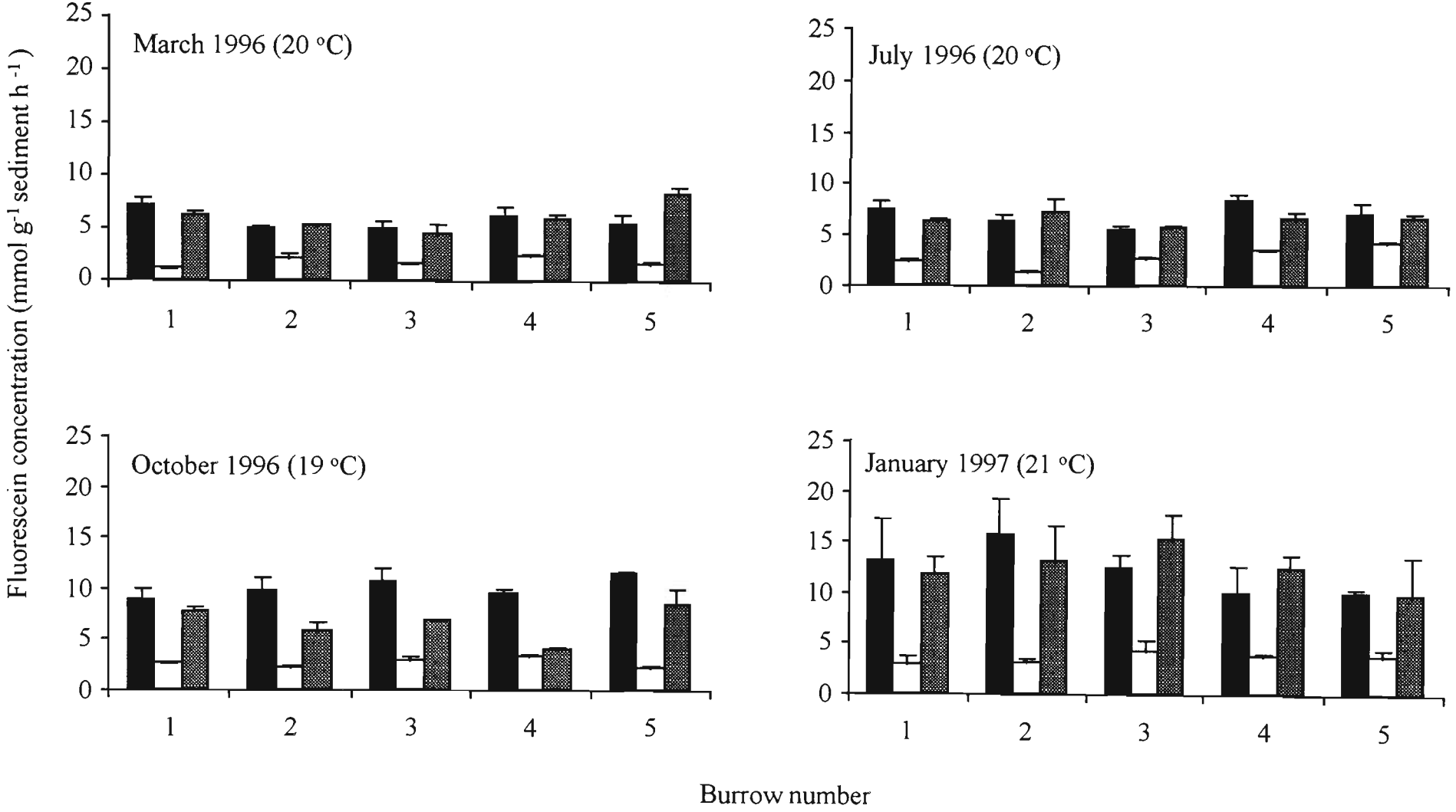

March 1996 (20 °C)
July 1996 (20 °C)
October 1996 (19 °C)
January 1997 (21 °C)
Fluorescein concentration (mmol g-1 sediment h-1)
Burrow number
0
5
10
15
20
25
1
2
3
4
5

Table 5.2.3. Statistical analysis of microbial activity measured in sediment samples collected in and around burrows of *Biffarius arenosus* and incubated at a consistent 20 ± 1 °C. Sediment samples were collected from Warneet, Western Port over 4 seasons between March 1996 and January 1997. Analysis compared seasons, burrows (nested within seasons) and sediment types (sampled from each burrow).

Effect	df	Mean square	F ratio	P value
Season	3	3.541	29.72	< 0.001
Burrow (season)	16	0.119	3.62	< 0.001
Sediment type	2	21.528	169.08	< 0.001
Season x type	6	0.282	2.21	0.068
Burrow (season) x type	32	0.127	3.87	< 0.001
Error	120	0.033		

5.2.4 Discussion

Microbial enzyme activity in the wall and surface sediments was consistently 3 times greater than activity measured in the subsurface sediments. Köster *et al.* (1991) also found a 3 fold elevation of FDA hydrolysis in deep sea enteropneust burrow walls compared to surrounding subsurface sediments. In comparison, the localised enhancement of microbial activity was not as pronounced in echiuran burrow walls (Köster *et al.* 1991). Using different methods, other studies have documented that bacterial growth and metabolism is elevated in burrow walls (Aller and Yingst 1985, Reichardt 1988), especially in the inner most surface (Aller and Yingst 1978, 1985, Aller and Aller 1986).

Three major factors are known to control enzyme activity: availability of substrate (ie., ultimately organic matter as a carbon source), availability of enzymes, and temperature. Several studies show that organic matter primarily controls microbial activity (Meyer-Reil 1987, Meyer-Reil and Köster 1992, Gumprecht *et al.* 1995). Aller and Yingst (1985) suggested a similar explanation for increased activity in polychaete (*Heteromastus* sp.) burrow walls, a result consistent with the idea of microbial gardening, where a deposit feeder stimulates bacterial growth and production through grazing.

In this study, percentage organic carbon content did not differ between sediment types (section 4.1), so it provides little explanation of the activity differences. Reichardt (1988) found that heterotrophic activity (measured via carbon uptake) was maximal in the burrow wall, compared to surface and subsurface sediments, but these differences also did not correspond to total organic matter or protein content of sediments. Even though Boetius (1995) suggested that an increase in activity at depth was due to burial of organic material rather than changes in microbial biomass, it is possible that bacterial numbers would be positively correlated with enzyme release and therefore hydrolytic activity if the cells were active. If cells were dormant, however, there would be no such correlation. In this study, bacterial abundances were significantly greater in the surface than the subsurface sediments, and burrow wall abundances tended to increase relative to subsurface abundances (section 5.1). This may account for the significantly higher enzyme activity recorded in these zones of sediment.

Temperature played a major role in controlling enzyme activity in these sediments, but obviously was not the major variable accounting for the differences between sediment types. Seasonal measurements varied greatly with temperature, with the highest activity recorded in the highest temperatures (January) and the lowest when the sediments were coldest (July). From this data it appeared that temperature was an important factor. This observation was supported when temperature was controlled at a constant 20 °C over the seasons, and activity levelled out in autumn (March), spring (October) and winter (July). Measurements of activity in summer (January), however, still showed much higher activity, indicating that some other factor was important. Was there more food available in summer? Bulk organic carbon measurements showed no seasonal changes. Were there more bacteria and enzymes present in summer? DAPI counts were highest in summer and lowest in winter, so this may have had some influence. However, if temperature was the controlling factor, then activity recorded at 20 °C in winter should have been lower than that recorded in spring and autumn at the same temperature, because bacterial numbers were lowest in winter. Results suggest seasonal fluctuations in per-cell activity.

Overall it appears that temperature has the largest influence of the factors controlling microbial activity, but bacterial abundances (availability of enzymes) and organic matter concentration may also be important. Similarly, other factors such as pH, pressure, salinity, and ionic composition, all known to have an influence (Van Es and Meyer-Reil 1982) but not measured here, may also be important.

Significant differences between burrows within seasons displayed evidence of heterogeneity of sediments, a result also found with bacterial abundances. The fact that activity in surface sediments was not consistently greater or less than activity in burrow walls, indicates that samples with higher activity may have been taken from sites of greater microbial significance. Faecal pellets are significant sites for microbial growth and production. It is well known that faecal pellets are rapidly colonised by microorganisms (Hargrave 1970a), and that the creation of new aerobic environments encourages active bacterial growth (Aller and Yingst 1985) and microbial activity (Hargrave 1976). *Biffarius arenosus* ejects faecal pellets from its burrows, and the pellets collect in groups on the sediment surface. Physical action of the tide receding often breaks the pellets and mixes patches of the material into the surface sediments, creating a heterogeneous microbial distribution. Burrow walls can also have patchy microbial distributions. Factors such as sediment particle size and topography (Hargrave 1972, Anderson and Meadows 1978), aerobic/anaerobic microhabitats (Dobbs and Guckert 1988) and the presence of mucopolysaccharides (Aller and Yingst 1978), can all contribute to creating a heterogeneous environment.

5.3 Microbial biomass and community structure

5.3.1 Introduction

Direct counts of bacteria provide an estimate of bacterial abundance, but no indication of abundance of the other microorganisms, such as micro-eukaryotes (eg. protozoans) and fungi which may also inhabit the sediments. Microbial biomass is a measure of the whole microbial community, and can be quantified by measuring total phospholipid (PL) concentration of a sample. Phospholipids are found in all cellular membranes and are denatured rapidly on cell death (White 1988), so measurable amounts in the environment are correlated with viable cell biomass. Total phospholipid concentration can also be converted to an estimate of cell number using conversion factors calculated for an average sized *Escherichia coli* bacterial cell (White *et al.* 1979, Balkwill *et al.* 1988). Because the DAPI microscopic counts in this study measured unusually lower bacterial numbers than other studies, another estimate of cell number was needed to test the validity of these microscopy results.

Abundance, biomass and activity measurements yield valuable information, but are integrative approaches. They give little indication of the types of bacteria and other microorganisms present (Boulton and Boon 1991). In other words, they give no information on community structure. In a highly oxidising environment of the surface sediments we would expect a large proportion of aerobic microbes such as diatoms and aerobic bacteria, whereas in highly reducing sediments of deeper zones we would expect low numbers of aerobic microbes and a dominance of anaerobes such as sulphur-reducing and methanogenic bacteria. In terms of understanding microbially-mediated decomposition and nutrient cycling in sediments, it is important to know which are the most dominant functional groups of bacteria. Analysis of phospholipid fatty acid methyl ester (PLFAME) profiles of sediment microbial assemblages reveals community structure and relative

proportions of each functional group of microorganism present. Microbes which contain distinctive fatty acids, rare in other microbial profiles, can be isolated as unique biomarkers. If a distinctive biomarker is present in a communityís profile, then the microbe it codes for is also present in that community. For example, a sediment sample containing any of the fatty acids 16:1ω3t, 20:5ω5 or 20:5ω3 would contain diatoms (Vestal and White 1989). Thus, analysis of phospholipids can generate data not only on total microbial abundance (to test the validity of earlier DAPI counts) but also on the types of bacteria present in the sediments.

The ability to discriminate among phospholipids of different bacterial species is due to the fatty acids esterified to the glycerol phosphate backbone (Bobbie and White 1980). During analysis, the ester-linked fatty acids are cleaved from the backbone, producing a profile of fatty acids within that phospholipid sample. Gas-chromatography is then used to separate the various fatty acids, where peak size correlates with concentration of fatty acid. GC/mass spectrophotometry can then be used to verify the structure and identify the peaks (Guckert and White 1986).

Analyses of PLFAME profiles has shown that microbial communities in marine sediments are often dominated by prokaryotes (Federle *et al.* 1983, Parkes and Taylor 1983, Parkes 1987), but some eukaryotes, protozoa and fungi are also present (Bobbie and White 1980, Federle *et al.* 1983, Dobbs and Guckert 1988). The presence of the different microbial functional groups depends on the physical environment and the dominating metabolic processes, and bioturbating activities may alter these conditions and hence the resident assemblage. For example, Branch and Pringle (1987) found that viable diatoms can be found at unusual depths due to sediment mixing by benthic fauna.

Despite its potential, there are few cases of this PLFAME biomarker approach being used to analyse community structure of macrofaunal burrows. Two studies only have applied the PLFAME technique to investigating the effect of an irrigated burrow on localised sediment microbial assemblages, and found that

burrow walls supported a distinctive community structure (Dobbs and Guckert 1988) which is intermediate between surface and subsurface microbial communities (Steward *et al.* 1996).

The final aim of this section of work on the microbial aspects of shrimp burrows is to examine the microbial assemblage of the environment around the burrows of *Biffarius arenosus*, to investigate if burrowing activity alters the microbial community structure.

5.3.2 Methods

5.3.2.1. Seasonal sampling

Sediment samples were collected from the burrow wall, subsurface and surface sediments on the following sample dates: 26 August 1994, 24 November 1994, 8 February 1995, and 19 May 1995. The sampling dates were evenly spaced throughout the calender year to represent seasonal variation. Burrow walls were sampled by digging up a block of sediment on the sandflat at low tide, and shearing the block to expose a burrow. Samples from the burrow wall were collected between 1 and 14 cm depth, as were the subsurface sediment samples, except they were sampled from sediment parallel but 5 cm from the burrow. Surface sediments were scraped to approximately 2 mm deep, adjacent to the burrow openings. At least 4 cm^3 of sediment was collected from each sediment type, and 10 replicate burrows were sampled at each date. Samples were stored in plastic vials, snap frozen in dry ice in the field, stored frozen at -20 °C, and then freeze-dried for 48 h at -28 °C and then 20 h at +10 °C.

5.3.2.2 Total phospholipid content via extraction and digestion

The method, modified from White *et al.* (1979), involved the following steps. All glassware was acid-washed in 1M HCl and rinsed in ultrafiltered water.

5.3.2.2.1 Extraction

One gram of freeze-dried sediment was measured into a 250 ml flask, and 5 ml of phosphate buffer (8.7 g K_2HPO_4 dissolved in 1 litre of ultrafiltered water, and neutralised to pH 7.4), 15 ml 0.05% anhydrous methanol and 7.5 ml of chloroform were added. The mixture was shaken vigorously and allowed to extract for 3.5 h. An additional 7.5 ml of chloroform and 7.5 ml of ultrafiltered water was then added, and the mixture was again shaken vigorously and allowed to separate for 24 h. The chloroform phase was removed and a total volume was measured. One ml of chloroform was added to each of 3 test tubes, and the solvent evaporated under a stream of nitrogen gas. At this stage, it was appropriate to include a blank with 1 ml of new chloroform to which the rest of the procedure was applied. The blank would reveal any additional phospholipid or other contaminants added during the extraction.

5.3.2.2.2 Digestion

The dried lipid was digested in 1.5 ml of 35% perchloric acid, which was heated to 180 °C for 2 h in a heating block. Samples were cooled before 2.4 ml molybdate reagent (4.4 g ammonium molybdate $(NH_4)_2MoO_4$ and 14 ml concentrated H_2SO_4 dissolved in 1 litre of ultrafiltered water) and 2.4 ml diluted ANSA reagent (30 g sodium bisulfite $Na_2S_2O_5$, 2 g sodium sulfite Na_2SO_3 and 0.5 g 1-amino-2naphthol-4-sulfonic acid dissolved in 200 ml of ultrafiltered water, and diluted before use 1:12) were added. The mixture was heated in a boiling water bath for 7-10 min and then cooled. The absorbance was measured at 830 nm on a Varian Cary UV/visible spectrophotometer.

A standard curve was plotted from serial dilutions of a stock solution (4.58 g l^{-1} solution of Na_2HPO_4 diluted 1 in 250 times) containing a known concentration of phosphorous (4 mg l^{-1}). The linear curve (equation: $y = 0.067x + 0.017$) was used to convert absorbance (nm) readings (from digested sediment samples) to concentration of phosphorous (µg) per test tube.

When some sediment samples were run, results showed that very little phospholipid was present in the sediments. This technique of measuring total phospholipid was obviously not sensitive enough for these sediments. An alternative method, phospholipid fatty acid methyl ester (PLFAME) profiles was used to provide a measure of total phospholipid, an estimate of cell number, and an indication of microbial community structure.

5.3.2.3 Total phospholipid, cell number and community structure via fatty acid methyl ester profiles

This section of work was performed at the CSIRO Division of Marine Research, Hobart, under the guidance of Dr Peter Nichols. Due to time and financial constraints, this analysis was limited to a maximum of 20 samples. The main aim was to compare phospholipid content of sediments collected from burrow walls, subsurface and surface sediments, and also to investigate any seasonal variation. With a limit of 20 samples, one burrow replicate (containing 3 sediment types) was randomly chosen from 26 August 1994, 24 November 1994, and 19 May 1995, and three burrow replicates from 8 February 1995. It was expected that this range of samples would show any seasonal variation, and any variation between burrows within a season.

5.3.2.3.1 Extraction and Elution

The lipid was extracted from sediment samples using the procedure outlined above, dried under a stream of N_2 gas, and stored frozen at -20 °C. Lipids were dissolved in chloroform and separated with silicic acid column chromatography into neutral lipids, glycolipids and phospholipids using the solvents chloroform, acetone and methanol respectively. The lipid fractions were reduced under N_2 gas and stored frozen at -20 °C.

5.3.2.3.2 Methylation

The following series of extractions, separated the phospholipids into fatty acid methyl esters (FAME). Three ml of methylation solution (10:1:1, MeOH, HCl, $CHCl_3$) was added to the phospholipid, and the solution heated for 2 h at 80 °C in

a heating block. After cooling, 1 ml of ultrafiltered water was added, and the methyl esters extracted 3 times with C_6 / $CHCl_3$.

5.3.2.3.3 Silylation

The FAME fraction was reduced under a stream of N_2, and 50 µl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and two internal standards, C_{23} FAME and DEC_{16} were added. It was thought that the sediment samples may contain evidence of methanogenic (Archaea) bacteria, so BSTFA was added to convert the diether lipids (PLEL), present in Archaea, to their corresponding *O*-trimethylsilylethers (Virtue *et al.* 1996).

The purpose of adding the internal standards was to create reference peaks of known concentration that could be compared to the individual FA and EL peaks for calculation of the concentrations of individual FA and ELs. C_{23} FAME was the reference for ester-linked phospholipid fatty acids (PLFA), such as those derived from eubacteria. In contrast DEC_{16} was the reference for ether-linked PLEL, such as those derived from archaea. If internal standards were not present, FAME peaks could only be interpreted qualitatively.

Initially 50 µl of C_{23} FAME internal standard was added to the samples, but this volume was reduced to 25 µl to increase sensitivity. Two samples (8S2 and 5S2) were more concentrated than the others, so these samples were diluted by reducing the sample under a stream of N_2 and adding 25 µl of $CHCl_3$ and 475 µl of C_{23} FAME.

5.3.2.3.4 Gas Chromatography and GC-Mass Spectrometry

FAMEs and PLELs were separated and detected with gas chromatography (GC) on a Hewlett Packard 5890 GC equipped with a 50 m x 0.32 mm i.d. cross-linked methyl silicone fused-silica HP1 capillary column and a flame ionization detector. Hydrogen was used as the carrier gas. Samples were injected with an HP 7673 autoinjector at 50 °C in the splitless mode with venting time of 2 min. After 1 min, the oven temperature was programmed to increase from 50 °C to 150 °C at

30 °C min^{-1}, then to 250 °C at 2 °C min^{-1} and finally at 4 °C min^{-1} to 300 °C. The temperature was maintained at 300 °C for 15 min. The detector and injector were maintained at 290 °C and 310 °C respectively.

Chromatography software from DAPA Scientific Software (Kalanumdra, WA) assisted with quantification of peak areas, and individual FAMEs were identified by comparing GC retention time and mass spectral data, with the peaks obtained for the internal standard. GC-MS was performed with a Fisons MD800, using Fisons Masslab software.

5.3.2.3.5 Nomenclature

The fatty acid nomenclature used, designates the total number of carbon atoms: number of double bonds, with the position of the double bond closest to-the aliphatic (ω) end of the molecule indicated with the geometry 'c' for *cis* and 't' for *trans*. The prefixes 'i', 'a' and 'br' refer to iso, anteiso and methyl branching respectively. Other methyl branching is indicated by the position of the extra methyl group from the carboxyl end, for example, 10Me16:0. Cyclopropyl fatty acids are designated as 'cy'.

5.3.2.3.6 Calculations

Total phospholipid concentration was calculated using the following equation:

Concentration (μg) = Area of peak/Area IS x A/B x C x 100/D x 1/E x 1/1000

where,

A = volume of sample in vial

B = volume of sample injected into GC

C = concentration of internal standard (ng)

D = percentage of total solvent extract taken

E = volume or mass of sample

1/1000 = conversion ng to μg

Concentration of total PL was used to estimate bacterial density of the sediment samples. The calculations involved the following factors: average bacteria contain 100 μmol PLFA/g dw, average molecular weight of a polar lipid derived FA is 270, and 1 g of bacteria is equivalent to 5.9×10^{12} cells (White *et al.* 1979, Balkwill *et al.* 1988). This calculation assumes that the PL content of bacteria does not fluctuate under natural conditions (White *et al.* 1979).

5.3.3 Results

5.3.3.1 Phospholipid concentration and cell number

Phospholipid concentrations varied between 1 and 105 μg g dw^{-1} but most of the values were below 5 μg g dw^{-1} (median value 4.54 μg g dw^{-1}) (Table 5.3.1). There was no consistent pattern between sediment type, and no one type was consistently higher or lower than the others. The two extraordinarily high values (May subsurface and especially August subsurface) were evidence of the heterogeneity of sediments.

Unfortunately, because only one burrow replicate was analysed for those seasons, it is difficult to know if these values are representative of that sediment type. This is perhaps unlikely, as the three replicate subsurface samples analysed in February show a small variance, and a PL concentration similar to the majority of the samples. Hence the samples collected from subsurface samples in August and May were probably collected from microbial 'hot spots'. The values gained were an accurate measurement of PL in that sample, because an appropriately sized internal standard peak was present in the correct position.

There was little point analysing the data statistically to compare between sediment types and seasons due to small sample sizes (n=1 in all values except in February where n=2 for wall samples and n=3 for subsurface and surface samples). Also, the extremely large values for two of the samples would render the ANOVA invalid because the assumption of homogeneous variances would not be satisfied.

Table 5.3.1. Phospholipid measurements made in burrow wall, surrounding subsurface and surface sediments associated with burrows of *Biffarius arenosus*. Samples were collected from Warneet, Western Port over 4 seasons between August 1994 and May 95. Means ± 1 SE are shown for February data.

	Sampling Date			
	26 Aug 94	24 Nov 94	8 Feb 95	19 May 95
PLFA concentration (μg g dw^{-1})				
Wall	5.08	2.86	3.21 ± 0.1	6.07
Subsurface	14.44	105.08	2.45 ± 0.5	1.26
Surface	4.52	4.56	3.04 ±1.1	4.93
Cell numbers (x 10^{8} g dw^{-1})				
Wall	11.1	6.3	7.0 ± 0.1	13.3
Subsurface	31.5	230	5.4 ± 1.2	2.8
Surface	9.9	10.0	6.6 ± 2.4	10.8
% Saturated FA				
Wall	45	51	54 ± 4.9	39
Subsurface	41	55	60 ± 3.5	38
Surface	43	51	47 ± 2.9	43
% Monounsaturated FA				
Wall	35	30	30 ± 4.9	37
Subsurface	38	24	28 ± 2.3	25
Surface	38	35	32 ± 4.0	38
% Polyunsaturated FA				
Wall	2	2	3 ± 2.1	2
Subsurface	1	2	1 ± 0.6	2
Surface	4	4	2 ± 0.6	3
% Branched FA				
Wall	15	16	13 ± 2.1	19
Subsurface	18	16	9 ± 4.6	35
Surface	13	10	17 ± 1.7	15
% > C20 FA				
Wall	12	10	8 ± 0	11
Subsurface	8	10	16 ± 6.4	2
Surface	11	8	9 ± 1.7	10
16:1ω7 trans/cis ratio				
Wall	0.06	0.00	0.04	0.08
Subsurface	0.06	0.09	0.06	0.00
Surface	0.04	0.03	0.04	0.06
18:1 ω 7 trans/cis ratio				
Wall	0.05	0.04	0.06	0.06
Subsurface	0.06	0.09	0.06	0.00
Surface	0.04	0.04	0.06	0.07

Cell numbers, estimated from the PLFA concentrations, ranged between 2.8 x 10^8 and 2.3 x 10^{10} g dw^{-1} (median value 10^9 g dw^{-1}) (Table 5.3.1).

5.3.3.2 Community Structure

No noticeable differences were observed in microbial community structure between sediment types (Table 5.3.2). All samples had similar fatty acid profiles, and equivalent proportions of saturated, monounsaturated (MUFA), polyunsaturated (PUFA) and branched fatty acids (Figure 5.3.1). Palmitic acid (16:0) consistently made up the largest proportion (between 21 and 35%) in all samples, which is not surprising since it is the major component of phospholipid in membranes of nearly all microorganisms (Erwin 1973). Multivariate statistical analysis can be a useful tool for comparing microbial assemblages (Rajendran *et al.* 1992, Scholz and Boon 1993, Boon *et al.* 1996), however, it was not used in this study because there appeared to be few differences between samples, and the small sample size would lend little power to the analysis.

The PLFA profiles of all sediment types showed evidence of a prokaryote-dominated microbial community (Table 5.3.2). High concentrations of branched and mono C_{14}-C_{18} FA, low levels of PUFA and presence of the specific biomarkers such as iso-and anteiso- branched C_{15}-C_{17}, cy- branched and beta-hydroxyl branched FA, all indicate prokaryote dominance (Moriarty *et al.* 1985b, Boon *et al.* 1996). Using proportions of specific biomarkers, it was calculated that prokaryotes constitute approximately 80% in all samples (Figure 5.3.2). The exception is the May subsurface sample, where low sensitivity resulted in the smaller peaks being reduced or absent, and the larger components disproportionately larger, skewing the relative proportions of peaks to overemphasise the dominating component (prokaryotes) (Table 5.3.2).

The fatty acids 10Me16:0 and cy 17:0, comprised a small proportion of these sediment profiles, but provided evidence of the presence of sulphur-reducing bacteria (Table 5.3.2). Dowling *et al.* (1988) showed that the biomarker 10Me16:0 was a FA restricted to the PL membranes of the acetate-oxidising

Table 5.3.2. Percentage of individual fatty acids in sediment phospholipid samples collected from burrow walls, subsurface and surface sediments associated with the burrows of *Biffarius arenosus*. Samples were collected from Warneet, Western Port, over 4 seasons between August 1994 and May 1995. Means + 1 SE are shown for February data.

	26 Aug 94			24 Nov 94			08 Feb 95			19 May 95			Mean
	Wall	Subsurface	Surface	Wall	Subsurface	Surface	Wall	Subsurface	Surface	Wall	Subsurface	Surface	Percentage
14:0	0	1	0	1	1	1	1 + 0	0 + 0	0 + 0	0	3	0	0.6
i15:0	0	4	0	1	1	1	1 + 0	0 + 0	1 + 0	1	3	1	0.9
a15:0	1	2	1	1	2	1	1 + 0.7	0 + 0.6	1 + 0.6	1	14	1	2.2
15:0	1	1	1	1	2	2	2 + 0.7	0 + 0.6	1 + 0	1	2	1	1.3
i16:0	1	1	1	1	1	1	1 + 0.7	1 + 0.6	1 + 0.6	1	4	1	1.3
16:1w9c	1	1	1	1	0	1	0 + 0	0 + 0	1 + 0	1	0	1	0.7
16:1w7c	7	9	10	9	8	15	9 + 3.5	3 + 1.7	11 + 1.2	1	7	10	8.7
16:1w7t	0	1	0	0	1	0	0 + 0	0 + 0	0 + 0	6	0	1	0.3
16:1w5c	1	1	1	1	0	1	1 + 0	0 + 0	1 + 0	1	0	1	0.8
16:0	21	23	26	25	30	35	31 + 1.4	22 + 2.3	28 + 2.9	21	21	25	25.7
b-OH..14:0	1	1	1	0	0	0	0 + 0	0 + 0	0 + 0	1	0	0	0.3
10Me16:0	1	2	1	2	1	1	1 + 0	1 + 0.6	2 + 0.6	2	0	1	1.3
i17:0	2	2	1	1	1	1	1 + 0	1 + 0.6	2 + 0.6	2	0	2	1.3
a17:0	1	0	1	5	5	1	0 + 0	1 + 1.7	1 + 0.6	1	14	1	2.6
17:1 & cy 17:0	1	2	2	1	1	1	3 + 0.7	0 + 0.6	2 + 0.6	3	0	2	1.5
17:0	1	3	2	1	3	1	2 + 0	2 + 1.2	2 + 0	2	0	3	1.8
18:2w6	0	1	1	1	0	1	1 + 0	1 + 0	1 + 0	1	0	1	0.8
i18:0	2	1	1	1	1	1	1 + 0	1 + 0.6	1 + 0	2	0	1	1.1
18:1w9c	7	7	7	7	5	5	6 + 0.7	10 + 2.3	6 + 0.6	8	6	6	6.7
18:1w7c	17	16	16	10	7	11	11 + 2.8	11 + 0.6	10 + 5.2	17	12	16	12.8
18:1w7t	1	1	1	0	1	0	1 + 0	1 + 0	1 + 0	1	0	1	0.8
18:0	15	9	9	15	13	8	15 + 6.4	21 + 2.9	9 + 0.6	9	13	9	12.1
br19:1	1	1	1	1	0	0	1 + 0.7	0 + 0	1 + 0	1	0	1	0.6
b-OH..16:0	1	1	2	1	1	1	1 + 0	1 + 0.6	1 + 0	2	0	1	1.1
b-OH..i17:0	1	1	2	1	1	1	1 + 0.7	0 + 0.6	1 + 0	1	0	1	0.9
cy19:0	0	0	0	0	0	0	0 + 0	0 + 0	0 + 0	1	0	0	0.1
b-OH..a17:0 & cy19:0	2	2	1	1	1	1	1 + 0	1 + 0.6	2 + 0.6	2	0	1	1.3
diFAME	3	3	2	2	2	1	1 + 0	3 + 0.6	2 + 1.2	3	0	1	1.9
20:4w6	1	1	1	1	1	1	1 + 0	0 + 0	1 + 0	1	0	1	0.8
20:5w3	1	0	2	1	0	2	1 + 1.4	0 + 0	1 + 0.6	1	2	2	1.1
20:0	2	2	3	2	2	2	2 + 1.4	2 + 1.2	2 + 0.6	2	0	2	1.9
20:1	3	2	2	2	2	1	2 + 0	4 + 1.2	2 + 0	2	0	2	2.0
22:0	2	1	1	1	1	1	1 + 0	3 + 0.6	1 + 0.6	2	0	1	1.3
24:0	0	0	1	2	1	1	1 + 0	4 + 2.3	1 + 0	2	0	1	1.2
26:0	1	1	0	2	3	1	0 + 0	3 + 2.3	0 + 0	0	0	1	1.0
28:0	1	1	0	0	0	0	0 + 0	0 + 0	0 + 0	1	0	0	0.3

Figure 5.3.1

Proportions of saturated (solid), mono- (shaded) and polyunsaturated (open) and branched (dotted) fatty acids in burrow wall (wall), subsurface (sub) and surface (sur) sediments associated with burrows of *Biffarius arenosus*. Samples were collected from Warneet, Western Port over 4 seasons between August 1994 and May 95. Means + SE are shown for February where n = 3.

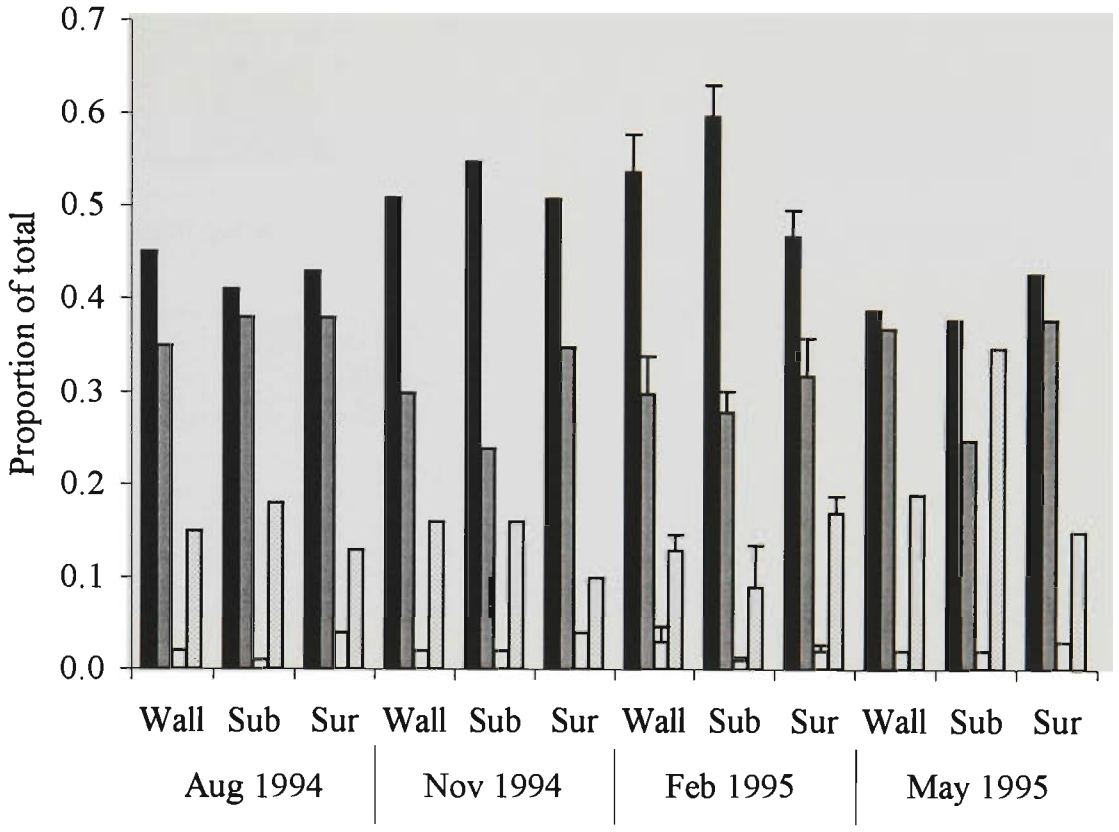
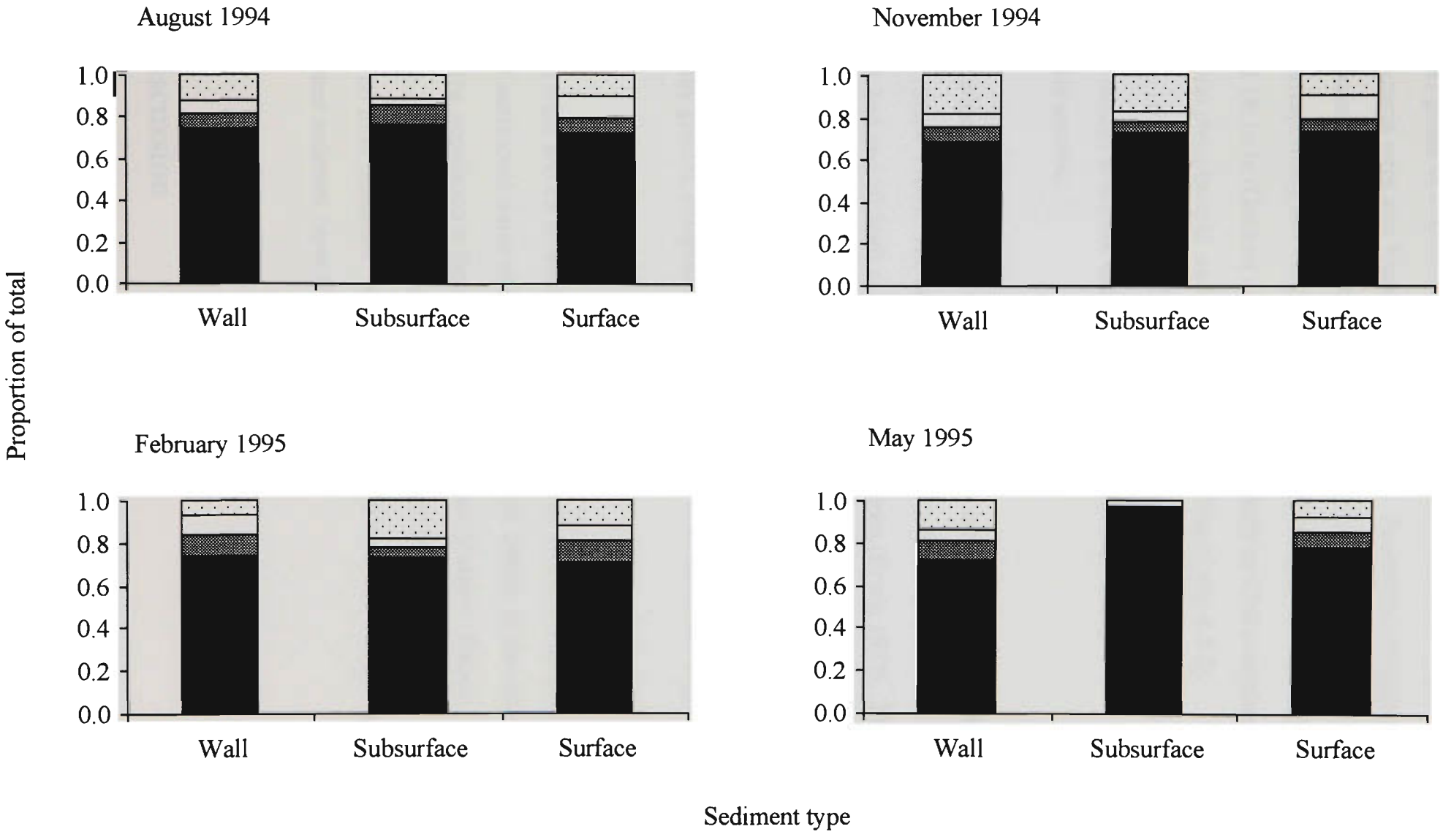

Proportion of total
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Wall Sub Sur Wall Sub Sur Wall Sub Sur Wall Sub Sur
Aug 1994
Nov 1994
Feb 1995
May 1995

Figure 5.3.2

Functional group proportions of prokaryotes (solid), sulphur-reducing bacteria (shaded), eukaryotes (open) and higher plants (dotted) found in burrow wall, subsurface and surface sediments associated with burrows of *Biffarius arenosus*. Samples were collected from Warneet, Western Port over 4 seasons between August 1994 and May 95.

Abbreviations: Pro = prokaryotes, SRB = sulphur-reducing bacteria, Euk = eukaryotes, HP = higher plants.


August 1994
November 1994
February 1995
May 1995
1.0
0.8
0.6
0.4
0.2
0.0
Wall
Subsurface
Surface
Proportion of total
Sediment type

Desulfobacter genus of sulphur-reducing bacteria. Other biomarkers, specific to anaerobic bacteria were also found in these sediments. Anaerobic assemblages typically contain high concentrations of the cyclopropyl fatty acids, cy 17:0 and cy 19:0, and corresponding low concentrations of their monounsaturate precursors 16:1ω7c and 18:1ω7c (Guckert *et al.* 1985). In this study cy 17:0 comprised 1-3% of the total phospholipid, and cy 19:0 only 1% or less (Table 5.3.2). Additionally, 16:1ω7c and 18:1ω7c comprised between 6 and 17% of the total PL, so even though anaerobic bacteria were present, the community was predominantly aerobic.

Biomarkers which indicate the presence of eukaryotes include 18:2ω6, 20:4ω6 and 20:5ω3 (Shaw 1966). The eukaryote biomarkers can be separated into 'animal' (18:2ω6 and 20:4ω6) and 'plant' (20:5ω3) series (Erwin 1973). These FA comprised between 0 and 2 % of the total PL (Table 5.3.2), suggesting that eukaryotes (fungi, protozoa, micrometazoa and diatoms and algae) were only a minor part of the microbial community. Fatty acids specific for higher plants were proportionally greater, comprising between 3 and 11 % of the total PL (Table 5.3.2).

The ratio of trans and cis isomers of the FA 16:1ω7 and 18:1ω7, is used as an indicator of nutritional status of bacteria (Guckert *et al.* 1986). If the ratio exceeds 1, the population is thought to be under a state of stress. Ratios calculated for these sediments fell in the range of 0 to 0.09 (Table 5.3.1), suggesting no food limitation. No patterns were observed when comparing the ratios between sediment types (Figure 5.3.3).

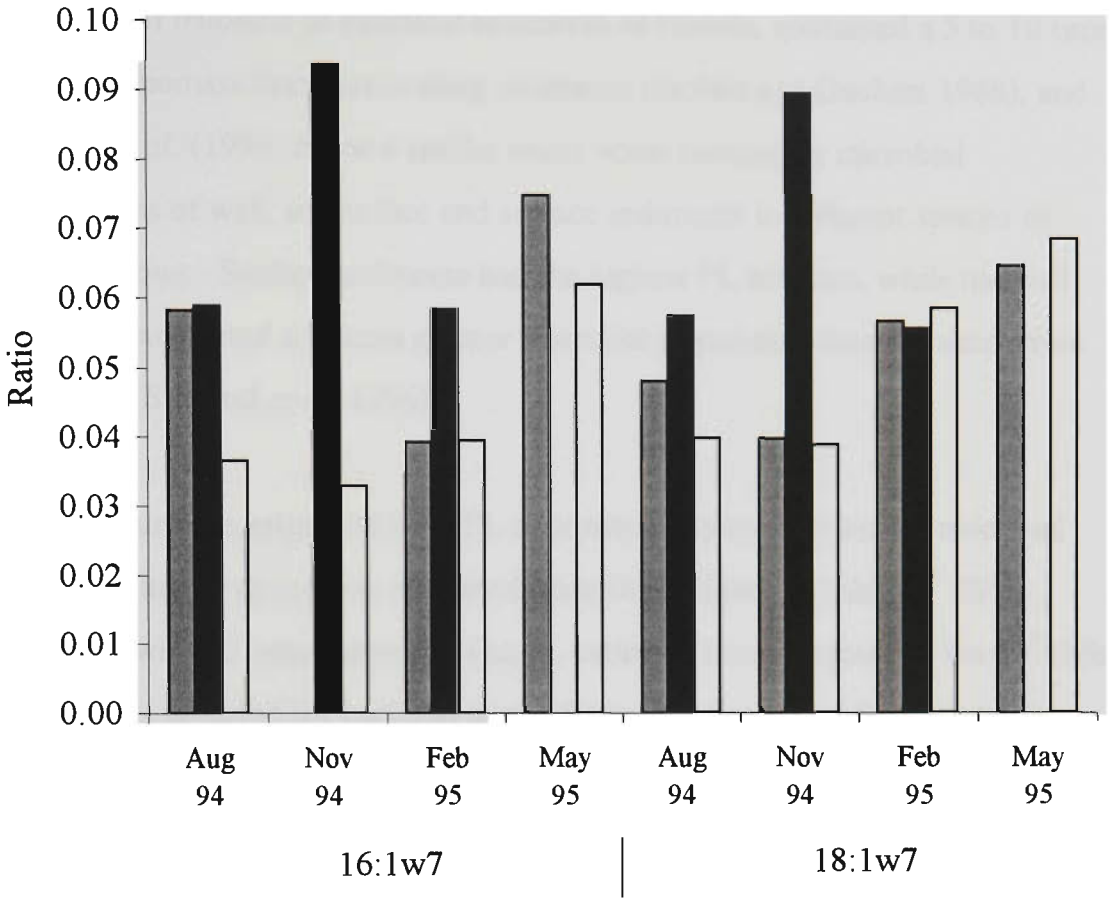
5.3.4 Discussion

5.3.4.1 Total biomass and cell count estimates

The present study showed no significant trend over sediment types, but some samples had a much higher PL biomass that others, probably due to sampling of a

Figure 5.3.3

Ratio of trans/cis isomers of the fatty acids 16:1ω7 and 18:1ω7 in burrow wall (shaded), subsurface (solid) and surface (open) sediments associated with burrows of *Biffarius arenosus*. Samples were collected from Warneet, Western Port over 4 seasons between August 1994 and May 95.


0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Ratio
Aug 94
Nov 94
Feb 95
May 95
Aug 94
Nov 94
Feb 95
May 95
16:1w7
18:1w7

microbial "hot spot". These results are quite different to the few studies that have applied the PLFA technique to investigate the effects of an irrigated burrow structure on microbial communities. Burrows constructed by *Sergio* (as *Callianassa*) *trilobata* in intertidal sediments of Florida, contained a 5 to 10 times higher PL biomass than surrounding sediments (Dobbs and Guckert 1988), and Steward *et al.* (1996) found a similar result when comparing microbial communities of wall, subsurface and surface sediments in different species of worm burrows. Surface sediments had the highest PL biomass, while the wall sediments supported a 3 times greater microbial population than the subsurface sediments (Steward *et al.* 1996).

Bacterial numbers estimated from PL concentrations were at least 3 orders of magnitude higher than those estimated from DAPI direct counts (10^8-10^{10} compared with 10^5 respectively). The PL estimate is much closer to the 10^9 cells g^{-1} sediment reported for bacterial populations in sediments and soils from environments as varied as under the ice in Antarctica to tropical estuaries (White 1993). As discussed in section 5.1.4, direct counts often underestimate bacterial populations, and factors such as cell damage during ultrasonication or use of DAPI over AO as the stain may have contributed to the underestimation of these counts.

Using biochemical methods to analyse microbial communities removes the problem of needing to separate microbes from surfaces (a problem for direct counts (Moriarty 1980)), which preserves the micro-structure of the consortia (White 1988). Additionally, gas chromatography methods used for the analysis of PLFA are both reproducible and highly sensitive, and the fatty acid recovery is quantitative (Bobbie and White 1980). However, the conversion of microbial biomass to bacterial numbers is based on the mean biomass of an average *Escherichia coli* cell (Mancuso *et al.* 1990), so if the bacteria in this study's sediments are larger or smaller than *E. coli*, then the calculated cell density will be an over or under estimate, respectively. Additionally, this study's bacteria may have quite a different PLFA to cellular carbon ratio which would add another error factor to the conversion calculation (Scholz and Boon 1993). Calculated

abundances were much larger than DAPI counts, suggesting that the bacteria may be larger than *E. coli*. Still, it is unlikely that the size difference would account for such a large discrepancy (3 orders of magnitude) between the calculated and direct counts. Even if cells had twice the diameter of *E. coli* cells, this would mean an 8 fold greater overall size, but still only an increase in density of about an order of magnitude, not the 3 orders of magnitude recorded.

5.3.4.2 Community structure

No clear patterns or differences were observed in the microbial communities inhabiting the different sediment types in this study. Because the burrow environment is more oxidising (section 4.3) it was thought that the burrow wall would have a different microbial community to the subsurface sediment. This theory was not supported by the PLFA data. All three sediment types had similar FAME profiles, proportions of saturated, unsaturated and branched fatty acids, and proportions of functional groups, and none showed any evidence of seasonal changes or physiological stress. It seems a little surprising that all samples were so evenly composed (compared to Scholz and Boon 1994, Boon *et al.* 1996). It is possible that the small number of samples analysed did not provide an accurate picture of the average communities present.

In comparison, sediment collected from the wall of *Sergio trilobata* burrows had a similar microbial community to the burrow matrix and surrounding sediments, but also contained relatively higher proportions of certain fatty acids, like 18:1ω7c (a fatty acid found in both eukaryotes and prokaryotes) and biomarkers for sulphur-reducing bacteria (Dobbs and Guckert 1988). Steward *et al.* (1996) found a similar result, with burrows sustaining a microbial assemblage intermediate to that found in the surface and subsurface sediments. Three fatty acids were restricted to the burrow wall communities, 18:1ω7c, 20:5ω3 and 20:4ω6, the first being a component in both pro- and eukaryotic membranes, and the others being biomarkers for eukaryotes (Steward *et al.* 1996).

Additionally, Dobbs and Guckert (1988) found variation between nutritional status of microbes inhabiting the different sediment types. Microbes in the burrow wall were the least physiologically stressed of the three, suggesting that the burrow provided adequate essential nutrients for the microbial inhabitants. This method of examining physiological stress in microorganisms assumes that the relationship of an increase in trans/cis ratio of the FA 16:1ω7 due to starvation, observed in *Vibrio cholerae*, holds for other species (Guckert *et al.* 1986), and therefore should be used conservatively.

The presence of small concentrations of anaerobic and sulphur-reducing bacteria (SRB) in the oxic surface sediments supports previous documentation of anoxic micro-niches in oxic sediments, especially in burrow environments (Dobbs and Guckert 1988). An irrigated burrow wall is often only a few mm thick, so anoxic and oxic sediments are closely associated (Tomaszek 1995), and both types of bacteria can exist there. Reichardt and Bussman (in Reichardt *et al.* 1991) found that SRB were not confined to anaerobic sediments, and reached high densities in the aerobic sediments as well.

The presence of FA indicative of higher plants in these sediment samples is not surprising, because the sandflat is bordered by mangrove stands and beds of seagrass. Even though both types of vegetation would contribute debris and detritus to the sandflat, their combined contribution was no greater than 9% in any one sample.

5.3.4.3 Limitations

The two major problems notable from these results were limits imposed by time restrictions and the small sample size. As outlined in the methods, this study was restricted financially to a maximum of 20 samples (analysis cost $1000/sample). On reflection, the samples chosen for the PLFA analysis were probably not the most suitable. With an aim to collect the maximum amount of data from a limited number of samples, I selected one burrow replicate from each of three seasons, and 3 burrow replicates from the fourth season. It was thought that this would

reveal any variation between burrows as well as any temporal variation related to seasons.

Two problems became evident during sample analysis. Firstly, with so few samples to run, it was impossible to adjust the dilution procedures to achieve the most suitable sensitivity for these sediments, so the initial runs were too dilute and lacked sensitivity. In the less sensitive profiles, the smaller peaks were much reduced or absent, and the larger components disproportionately larger, skewing the relative proportions of peaks, such as seen in the May subsurface sample (Table 5.3.2). A compounding factor was that the samples were very small in volume, due to small sediment samples, so it was impossible to repeat the analysis after increasing sensitivity. Ideally, preliminary samples would have been run to check sensitivity, after which the actual burrow replicates could have been analysed.

Secondly, two samples, the May and August subsurface sediments, produced extraordinarily high values (especially August) indicating that the samples were perhaps collected from 'hot spots' of microbial activity. Intertidal and marine sediments are often heterogeneous (Anderson and Meadows 1978), and microbial measures in sediments often display large variation (Kirchman *et al.* 1982, Montagna 1982) because of the associated patchiness of microorganisms (Federle *et al.* 1983). Increasing the number of replicate burrows to be analysed, would have provided results more representative of the average microbial community. In retrospect, because of the study's primary interest in differences between sediment types, more burrow replicates should have been analysed from a single sampling date, and temporal variation disregarded. This may have given a stronger result showing any differences between sediment types, rather than the weak result gained, showing some unusually high values which may or may not be indicative of the average community. To further reduce the problem of heterogeneity, larger volumes of sediment should have been sampled, homogenised and then subsampled (Montagna 1982). This sampling technique would have had only limited application to these sediment samples, because the burrow wall samples had a small size.

5.3.4.4 Further work

To obtain a clearer picture of the microbial biomass and community structure of the different sediment types within and around *Biffarius arenosus* burrows, further work incorporating the suggested improvements is recommended. Few studies have investigated microbial properties of burrow environments using the PLFA method, and the only documented study of thalassinidean burrows involved *Sergio trilobata* (Dobbs and Guckert 1988), a species with a different trophic mode from *B. arenosus*. Steward *et al.* (1996) showed that burrows of different worm species supported different microbial communities, probably due to unique burrowing and feeding ecology of the inhabiting species. If this pattern is the same for callianassids, then burrows of the different feeding types may support different microbial communities. Furthering this study would increase understanding of the interaction between feeding ecology and the burrow environment in marine sediments.

5.4 Chapter Summary

A summary of the findings is outlined in the table below.

Variable	Sediment type		
	Burrow wall	Subsurface	Surface
DAPI bacterial counts (cells g dw $^{-1}$)	1.6 x 10^6	1.4 x 10^6	1.8 x 10^6
Microbial enzyme activity (mmol g dw $^{-1}$ h^{-1})	8.7	2.7	7.9
Phospholipid bacterial abundances (cells g dw $^{-1}$)	9.4 x 10^8	6.7 x 10^9 (including high values)	9.3 x 10^8
Microbial biomass (µg PL g dw $^{-1}$)	5.1	30.8 (including high values)	4.3
Community structure	Mainly prokaryotes, some eukaryotes and evidence of higher plants	Mainly prokaryotes, some eukaryotes and evidence of higher plants	Mainly prokaryotes, some eukaryotes and evidence of higher plants

Epifluorescent counts indicated 2 major findings: very low numbers of bacteria (1-2 x 10^6 cells g dw^{-1}) and little difference in abundances among the sediment types. Microbial activity, however, was much higher in both the surface and burrow wall sediments, than in the surrounding subsurface sediments. This finding supports the idea that bacteria were more active in the surface and burrow wall sediments. Although not measured, production and growth rates would probably follow the same pattern.

It was disappointing that low replication reduced the power of the PLFA biomass results, with the presence of the two exceptionally high values confirming that spatial rather than temporal burrow replication would have given results that were more representative of the average environment. This analysis could be used as a pilot study, with further investigation into differences between sediment type using a larger number of replicate burrows to reduce problems associated with variability. One useful result of the PLFA data was the calculation of a cell density estimate for comparison with the DAPI counts. The estimate of bacterial abundance from PLFA was much closer to previously published values suggesting that the DAPI counts were a marked underestimation of in situ abundance. Unfortunately no reason for this result was clearly identified.

In light of the results documented by Dobbs and Guckert (1988) and Steward *et al.* (1996), it was interesting that microbial community structure did not differ greatly among the three sediment types. Burrow structures are known to create a mosaic of biogeochemical microenvironments within and around the burrows (Aller 1988, Fenchel 1996), and so are able to support a diverse range of microorganisms. If sediments were not highly reducing and shrimp density was high, then the biogeochemical 'halo' surrounding individual burrows may overlap, and the microbial assemblage may be relatively consistent throughout the sediments. This may explain the results seen here, but seems unlikely, given the differences in redox potential among burrow wall, subsurface and surface sediments (section 4.3).

CHAPTER 6

GENERAL CONCLUSIONS

6.1 Summary of findings

The work presented in this thesis was conducted with five aims in mind:

1. to describe the structure and functional morphology of burrows of *Biffarius arenosus*;
2. to determine the feeding ecology of the species;
3. to describe the physiochemical characteristics of the burrow environment;
4. to determine whether the microbial characteristics of the burrow environment differ from the surrounding sediments; and
5. to quantify the effect of burrowing activities on the diffusive flux of a non-reactive tracer across the water-sediment interface.

The structure of *Biffarius arenosus* burrows indicated that the species was probably a deposit-feeder and that it used its burrow primarily to gain access to subsurface food supplies. Despite individual variation, all burrows possessed the same components: a U-shaped surface connection and a complex series of tunnels and chambers arranged in an irregular spiral shape. Several of the burrow features facilitate deposit-feeding: spiralling tunnels and chambers which allow maximal exploitation of the subsurface food supply, and the U-shaped section and additional opening shafts which assist with passive and active irrigation and oxygenation of water in the tunnels. Some burrow features suggested that the shrimps make use of seagrass/algal harvesting and filter-feeding to also gain nutrition (Nickell and

Atkinson 1995), but it seems unlikely that *B. arenosus* would utilise these two feeding modes because access to the sediment surface is hindered by the small number of burrow openings and constricted opening shafts. Moreover, the features indicative of filter-feeding could be as equally important for burrow irrigation. Overall, the burrow structure was most similar to that of burrows of other deposit-feeding species (eg. *Callianassa bouvieri*, Dworschak and Pervesler 1988).

A series of experimental and observational investigations of the feeding ecology of *Biffarius arenosus* confirmed that the species was a deposit feeder. The shrimps' digestive tracts were packed with sediment, unidentifiable brown organic matter and occasionally some identifiable material derived from vascular plants and some diatom frustules. The presence of plant material in the guts indicated that the species may also collect some food material from the sediment surface (ie. seagrass/algal-harvesting), but laboratory investigations showed that shrimps did not leave their burrows to scavenge food even when sediment food resources were very poor. Identifying the primary food source proved difficult using traditional food preference experiments, but multiple stable isotope analysis, using $\delta^{13}C$ and $\delta^{15}N$, identified the likely carbon sources of *B. arenosus* as seagrass and seagrass epiphytes. Mangrove material was concluded to be an unlikely food source, and could contribute < 25% of the carbon assimilated by the shrimps.

Overall, the physiochemical and microbial properties of the *Biffarius arenosus* burrow environment were more similar to the surface sediments than the surrounding subsurface sediments. This confirms that, in some respects, the burrow wall can be considered an extension of the surface sediments and thus the water-sediment interface. Organic carbon was not concentrated in the burrow walls, a finding consistent with the lack of an obvious mucus lining and a sediment particle size distribution similar to surrounding sediments. Burrow wall colour indicated more oxidising conditions than the surrounding subsurface sediments, a feature that was confirmed by redox measurements.

The more oxidising conditions in the burrow wall enhanced microbial activity (measured as FDA hydrolysis) relative to surrounding reducing sediments, but not bacterial abundance (DAPI counts) or microbial biomass (total phospholipid contents). That similar numbers of bacteria were found in the burrow wall, surrounding subsurface and surface sediments is consistent with the finding that organic carbon content did not differ between these three sediment zones. The presence of both aerobic and anaerobic microorganisms in all sediment samples (as indicated by PLFA profiles) confirmed the presence of a mosaic of microhabitats in and around the burrow wall. This result supports previous documentation of sediment heterogeneity, especially around irrigated burrow structures (Dobbs and Guckert 1988, Tomaszek 1995).

Burrowing activity of *Biffarius arenosus* resulted in the disruption of the sediment matrix, with relatively larger particles being selectively ejected from the burrows, and the enhancement of the diffusive flux of tracer over the increased water-sediment interface. Activity, identified by the ejection of sediment from the burrow, occurred only when burrows were submerged, a result consistent with the shrimps' need for regular ventilation of burrow waters during periods of high activity and therefore oxygen consumption. A 44% increase in the water-sediment interface surface area due to the presence of *B. arenosus* burrows, in combination with an increased irrigation activity during high tide, promoted the flushing of burrow waters and the renewal of burrow wall concentration gradients, thereby elevating the diffusive flux of tracer (D_2O) from the sediments by 400%.

6.2 Concluding remarks

This study shows that *Biffarius arenosus* has a large impact on the sediment and nutrient dynamics of the intertidal zones of Western Port. The species is abundant (bay-wide density is 12 individuals m^{-2} (Coleman and Poore 1980)), and the presence of *B. arenosus* burrows increases the surface area of the sediment-water interface by over 40%. This shrimp species appears not to concentrate organic matter in its burrow wall, nor to store caches of plant material or other organic

matter. The absence of these practices limits the shrimps' influence on decomposition in the sediments. However, the physical structure of the burrows and the bio-irrigating activity of the shrimps would stimulate aerobic decomposition in the sediments by extending the water-sediment interface and its associated oxidising conditions to depth. This was reflected in the redox status of these three zones of sediment. Additionally, the introduction of oxygen via burrow irrigation would enhance oxic microbial-mediated processes such as nitrification, which in combination with an elevated diffusive flux of solutes from the sediments into the burrow waters, would have far-reaching effects on sediment nutrient cycling and the liberation of nutrients into the overlying water.

This thesis provides one of the first detailed accounts of the interaction between burrowing fauna and the biogeochemistry of sediments in Australia. Considerable recent interest in the environmental condition of coastal waters (eg. Harris *et al.* 1996) has clearly demonstrated that very little is known about the relationship between local burrowing fauna and sediment nutrient dynamics. This thesis is a preliminary step in addressing this issue.

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