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ORIGINAL ARTICLE

Bioturbating shrimp alter the structure and diversity of bacterial communities in coastal marine sediments

Bonnie Laverock[1,2], Cindy J Smith[2], Karen Tait[1], A Mark Osborn[2], Steve Widdicombe[1] and Jack A Gilbert[1]

[1]*Plymouth Marine Laboratory, Plymouth, UK and* [2]*Department of Animal and Plant Sciences, The University of Sheffield, Sheffield, UK*

Bioturbation is a key process in coastal sediments, influencing microbially driven cycling of nutrients as well as the physical characteristics of the sediment. However, little is known about the distribution, diversity and function of the microbial communities that inhabit the burrows of infaunal macroorganisms. In this study, terminal-restriction fragment length polymorphism analysis was used to investigate variation in the structure of bacterial communities in sediment bioturbated by the burrowing shrimp *Upogebia deltaura* or *Callianassa subterranea*. Analyses of 229 sediment samples revealed significant differences between bacterial communities inhabiting shrimp burrows and those inhabiting ambient surface and subsurface sediments. Bacterial communities in burrows from both shrimp species were more similar to those in surface-ambient than subsurface-ambient sediment ($R = 0.258$, $P < 0.001$). The presence of shrimp was also associated with changes in bacterial community structure in surrounding surface sediment, when compared with sediments uninhabited by shrimp. Bacterial community structure varied with burrow depth, and also between individual burrows, suggesting that the shrimp's burrow construction, irrigation and maintenance behaviour affect the distribution of bacteria within shrimp burrows. Subsequent sequence analysis of bacterial 16S rRNA genes from surface sediments revealed differences in the relative abundance of bacterial taxa between shrimp-inhabited and uninhabited sediments; shrimp-inhabited sediment contained a higher proportion of proteobacterial sequences, including in particular a twofold increase in Gammaproteobacteria. Chao1 and ACE diversity estimates showed that taxon richness within surface bacterial communities in shrimp-inhabited sediment was at least threefold higher than that in uninhabited sediment. This study shows that bioturbation can result in significant structural and compositional changes in sediment bacterial communities, increasing bacterial diversity in surface sediments and resulting in distinct bacterial communities even at depth within the burrow. In an area of high macrofaunal abundance, this could lead to alterations in the microbial transformations of important nutrients at the sediment–water interface.

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Introduction

Marine sediments harbour complex and diverse microbial communities, which have a critical role in driving carbon and nutrient cycling to support marine ecosystems (Torsvik *et al.*, 2002; Horner-Devine *et al.*, 2004; Lozupone and Knight, 2007). For example, it is estimated that up to 80% of the nitrogen required by photosynthetic organisms in shallow seas is derived from microbial regeneration of organic nutrients in sediment (Dale and Prego, 2002). Laterally and at large scales (cm to km, globally), the structure, composition and diversity of microbial communities are directly influenced by variation in environmental factors, including temperature, salinity, organic matter composition and concentration, sediment type and pH (Wilde and Plante, 2002; Köster *et al.*, 2005; Hughes Martiny *et al.*, 2006; Lozupone and Knight, 2007). Across smaller environmental scales (mm to cm), microbial community composition and function can vary considerably, in particular, with increasing sediment depth, driven primarily by changes in redox conditions and the availability of nutrients and organic matter (Jensen *et al.*, 1993; Urakawa *et al.*, 2000; Llobet-Brossa *et al.*, 2002; Köpke *et al.*, 2005; Köster *et al.*, 2008; Böer *et al.*, 2009; Jansen *et al.*, 2009).

Correspondence: B Laverock, Molecular Biology Facility, Plymouth Marine Laboratory, Prospect Place, Plymouth PL1 3DH, UK.
E-mail: bonver@pml.ac.uk
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The physical mixing and irrigation of the sediment by benthic macrofauna, a process known as bioturbation, has an important influence on the structure and diversity of benthic microbial communities. For example, infaunal burrow walls can have 10-fold higher numbers of bacteria compared with surrounding sediment (Papaspyrou *et al.*, 2005). Such increases are thought to result from several factors including the greater availability of organic material, the presence of biopolymers within the burrow wall and an extension of the oxic–anoxic interface, all of which affect redox reactions and solute transport (Kristensen, 2000). One group of bioturbators of particular interest is the thalassinidean decapods. These shrimp occur globally, with the distribution of the Callianassidae, Upogebiidae, Axioidea and Callianideidae families peaking at between 30 and 40 species in the tropical regions (20°N and 5°S), whereas temperate regions typically contain <10 species within each family (Dworschak, 2000). Thalassinideans are one of the most active groups of burrowing macrofauna in coastal sediments, and they create large, complex burrows that can increase the sediment surface area by up to 9 m^2 for every m^2 of the sediment surface (Griffis and Suchanek, 1991).

A major control on the redox potential of the burrow wall is the occurrence of irrigation events during which the shrimp beat their pleopods (walking legs) to flush oxygenated water through the burrow (Stamhuis and Videler, 1998). Such events are vital for the sustenance of microbial redox reactions, particularly those coupled across oxic–anoxic interfaces. For instance, Stief *et al.* (2004) showed that the almost constant irrigation activities of filter-feeding mayfly larvae led to consistently high oxygen levels within their burrows, which increased both the production and consumption of nitrate within burrow walls. Thalassinideans exhibit a high level of plasticity in their irrigation behaviour (Nickell and Atkinson, 1995; Berkenbusch and Rowden, 2000). Whereas deposit feeders may spend a large proportion of their time under hypoxic conditions deep within their burrow, suspension or filter feeders rely on more regular burrow irrigation and their burrows tend to be well oxygenated throughout (Nickell and Atkinson, 1995; Howe *et al.*, 2004; Webb and Eyre, 2004). The deposit feeder *Callianassa subterranea* (Montagu, 1808) and the suspension feeder *Upogebia deltaura* (Leach, 1815) are two important species of thalassinidean shrimp that dominate the soft sandy-mud sediments in Plymouth Sound (Parry *et al.*, 2003) and can reach densities up to 60 individuals per m^2 in European shallow seas (Tunberg, 1986; Rowden and Jones, 1994; Christiansen, 2000; Dworschak, 2000). *U. deltaura* draws approximately three times more water through the burrow than *C. subterranea* (149.5 ± 35.5 compared with 50.3 ± 33.6 ml h^{-1}; Nickell, 1992), although both species are known to be tolerant of intermittently hypoxic conditions (Forster and Graf, 1995; Astall *et al.*, 1997). A schematic of their differing burrow structures is shown in Figure 1.

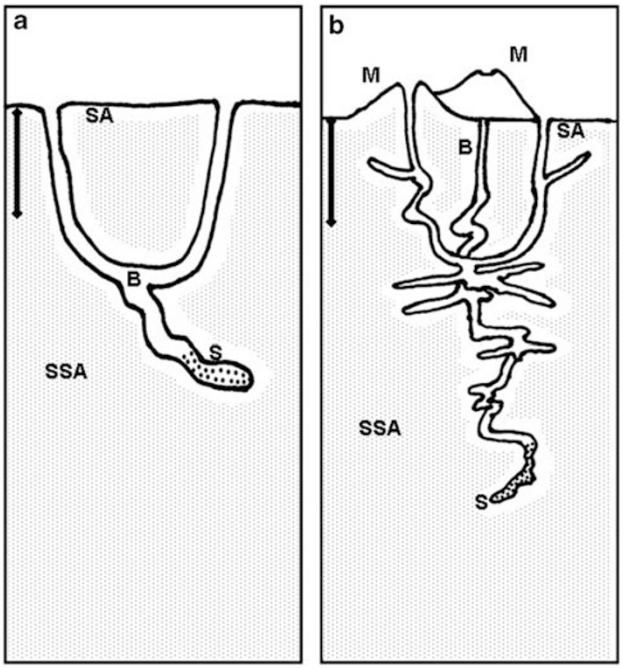

Figure 1 Burrow cross sections for (**a**) *U. deltaura* and (**b**) *C. subterranea*, showing shrimp burrows (B, unshaded) with mound (M) and sump (S) locations, as well as the ambient sediment surface (SA) and subsurface (SSA, shaded). The 'burrow entrance' extended from 0 to 3 cm sediment depth, whereas the 'mid-burrow' was the area between 3 cm and the sump. Ambient sediment was taken at least 5 cm away from any burrow sediment or mounds. Illustrations are two-dimensional generalisations of typical burrows for each species, based on observations during this study, and drawn after burrow types one and five as identified by Griffis and Suchanek (1991). Scale bar = 10 cm.

In this experiment, we have investigated whether the presence of these two important bioturbating shrimp species alters the structure and composition of bacterial communities. Specifically, we have investigated, first, whether the presence of bioturbating shrimp affects the structure of sediment bacterial communities within the burrow and the surrounding ambient sediment; and second, whether bacterial community structure changes with sediment and burrow depth. Finally, sequencing of cloned 16S rRNA genes was used to investigate variation in the taxonomic composition and diversity of bacterial communities within shrimp-inhabited and uninhabited sediments.

Materials and methods

Site and sample description

Sandy-mud sediment (40% sand (2–0.063 mm); 60% mud (<0.063 mm)) was collected on 29 November 2007 using a box corer from a site $\sim$100 m north of the breakwater in Plymouth Sound (50.338

N, 04.148 W; 10 m water depth). This site is heavily populated by both species of shrimp as part of a diverse macrofaunal community containing other large bioturbating species (Kendall and Widdicombe, 1999; Parry *et al*., 2003). The top layer of sediment (~10 cm depth) was carefully removed and the remaining subsurface sediment was sorted manually to remove large burrowing organisms. Any *C. subterranea* and *U. deltaura* recovered were placed in individual plastic mesh containers and immersed in continuously flowing seawater. Once all large burrowing macrofauna had been removed, the subsurface sediment was used to fill 14 plastic cores (65 cm deep, internal diameter 30 cm) to a depth of 40–45 cm, and the 10-cm-thick surface layer overlaid to create a total sediment depth of 50–55 cm. Cores were transferred to the benthic mesocosm of the Plymouth Marine Laboratory. The mesocosm consists of four interconnected plastic tanks (1 m^3), filled with 1 μm filtered seawater. A closed circulation system through an external 1 μm filter provides flow-through to each tank. Each tank also contains an internal pump to facilitate mixing of the water and to promote laminar flow across the surface of the tank. Cores were randomly distributed between the four tanks, topped up with seawater and left to settle for a 24-h period before shrimp were introduced. In 12 of the cores, a single shrimp of either *U. deltaura* or *C. subterranea* was added. The remaining two cores were left uninhabited and considered as 'control' cores. All cores were maintained for a minimum of 4 months under dimly lit conditions at 15–18 °C and at a salinity of 35‰.

Sediment samples for DNA extraction and analysis were taken between April and June 2008; subsequent statistical analyses revealed that there was no temporal effect on total bacterial community structure between sampling dates. The overlying water in each core was drained carefully before the core was removed from its mesocosm tank. Sediment samples (1 ml) were taken using the barrel of a 2.5 ml syringe. From the surface of each core, 2 ml of sediment was collected and 1 ml each assigned to separate tubes, representative of the top ~1.5 cm oxidised layer. 'Ambient' (undisturbed) sediment samples were then taken at 6 cm depth intervals down to 30 cm. A hydraulic pump was used to extrude sediment from the core incrementally, and a sharp-edged stainless steel plate was used to section the core horizontally at each depth. A horizontal transect was assigned to bisect the core across its centre, and 'ambient' samples were taken every 2 cm along the transect at every depth (15 samples per depth; depicted in Figure 2), taking care to position the transect to avoid mounds and burrow openings and walls. Shrimp burrow samples were taken using a sterile spatula to scrape the oxidised (lighter coloured) sediment from the burrow wall at 3 cm depth intervals until the burrow ended. Sediment samples were immediately transferred to 2 ml Eppendorf (Eppendorf AG, Hamburg, Germany) tubes and frozen in liquid nitrogen before storage at −80 °C.

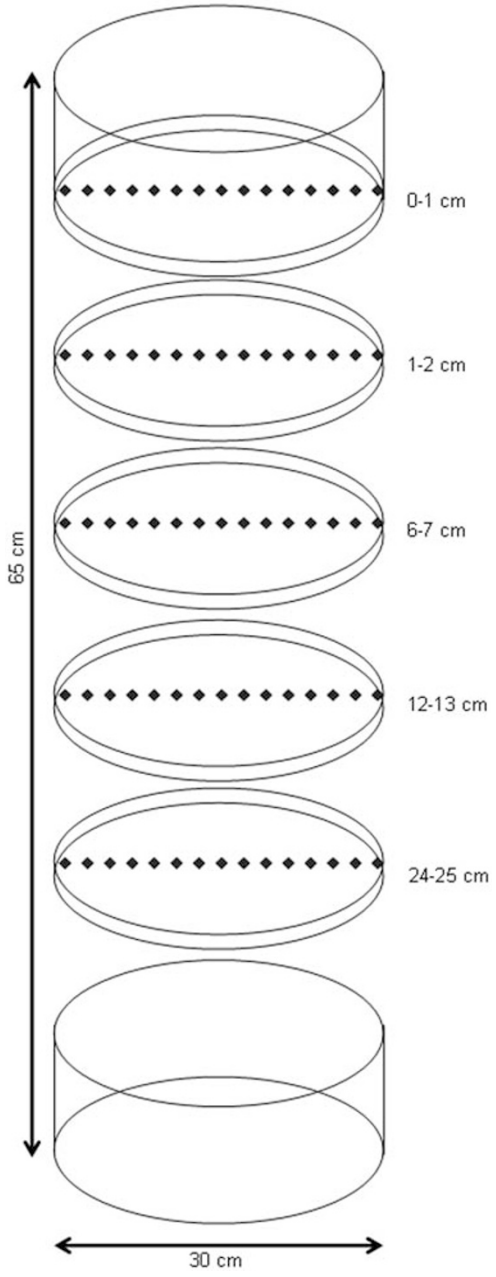

Figure 2 Schematic to show the procedure used for sectioning and sampling sediment cores. Cores were sliced as indicated using a stainless steel plate. Samples (1 ml) of ambient sediment were taken every 2 cm across a horizontal transect, as indicated (♦) using a 2 ml syringe. Burrow samples (not shown) were taken every 3 cm depth to the end of the burrow.

Three cores for each of the two shrimp species and one control core were randomly selected for subsequent molecular analyses. Sediments from the shrimp-inhabited cores were assigned to one of the five categories: 'surface ambient' (SA) sediment was from the top 2 cm of the core and at least 5 cm away from any burrow openings or surface mounds; 'subsurface ambient' (SSA) sediment was any

sediment below 2 cm and at least 5 cm from any burrow structure; 'burrow' (B) sediment was taken from the oxidised sediment on the inside wall of the burrow (unshaded), which varied in thickness between 0 and 1 cm; 'mound' (M) samples were taken from the darker coloured mound sediment immediately adjacent to burrow exit holes; and 'sump' (S) samples were taken from the part of the burrow identified as the sump, if present (Figure 1). The two burrow types shown in Figure 1 are generalisations. In this study, *U. deltaura* burrows had between two and three entrance or exit holes, whereas *C. subterranea* burrows had between two and four. The number of mounds per *C. subterranea* burrow can also vary, but was generally between two and three; there was always at least one burrow entrance hole that did not have an associated sediment mound. The sump is defined as a blind-ending shaft at the base of the burrow, frequently containing loose organic and mineral debris and faecal material (Nickell and Atkinson, 1995). In these cores, it was recognised as the area containing looser, coarser material with higher liquid content than the compacted burrow walls. Defined in this way, there were often several portions of burrow assigned the label 'sump'. In *U. deltaura*-inhabited cores, the depth of the sump varied between individual burrows, with a minimum depth of 15 cm and a maximum depth of 30 cm, whereas in *C. Subterranea*-inhabited cores, the sump occurred at a minimum depth of 18 cm and a maximum depth of 32 cm; again, this varied between individual burrows. In *U. deltaura* cores 3, 5 and 12, the maximum burrow depths were 18, 30 and 30 cm, respectively. In *C. subterranea* cores 9, 10 and 19, the maximum burrow depths were 30, 24 and 32 cm, respectively. Sediment from the uninhabited core was either 'surface control sediment' collected from the top 2 cm of the core or 'subsurface control sediment' collected at depths below 2 cm. Samples used for this study are described in Table 1.

DNA isolation

Sediment samples were homogenised by stirring with a sterile metal spatula and then 0.5 g was added to a 2 ml Eppendorf tube together with 0.5 ml of 0.1 M sodium phosphate buffer (pH 8.0) and 0.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1; pH 4.0). Samples were lysed using 0.3 g each of 212–300 and 710–1180 μm glass beads (Sigma-Aldrich, Gillingham, UK). The lysis step consisted of two cycles of bead beating at 2000 r.p.m. for 30 s using a MoBio Vortex Genie with adaptor (Cambio, Cambridge, UK), with a 30 s interval on ice. The lysate was separated from sediment by centrifugation at 13 200 r.p.m. for 5 min at 4 °C. The upper aqueous layer was transferred to a clean Eppendorf tube containing 0.5 ml of chloroform/isoamyl alcohol (24:1), mixed and centrifuged for a further 5 min. The upper aqueous phase was again transferred to a fresh tube and nucleic acids were precipitated with 2 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) at −20 °C overnight before centrifugation at 13 200 r.p.m. for 30 min. The resulting pellet was washed twice with 70% ethanol, air dried and resuspended in 100 μl of sterile filtered Milli-Q water (Millipore, Bedford, MA, USA). Extraction of DNA was confirmed by agarose gel electrophoresis.

Terminal-restriction fragment length polymorphism analysis

PCR amplification of 16S rRNA gene fragments was performed using the primer pair 63F (5′-CAGGCCTA ACACATGCAAGTC-3′) and 1389R (5′-ACGGGCGGT GTGTACAAG-3′) (Marchesi *et al.*, 1998, Osborn

Table 1 Mesocosm sediment samples used for T-RFLP and sequencing analyses

Core No.	*Bioturbator*	*T-RFLP (229 samples)*		*Sequencing (21 samples)*
		Depth of ambient samples	*Depth of burrow samples*	
1	None	0, 6, 12 and 24 cm [72][a]		4 cm intervals across surface transect [7+2[b]]
9	*Callianassa*	0, 6, 12, 18, 24 and 30 cm [6]	0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 cm [25]	3 mound samples, pooled 1 sample from burrow entrance [2]
10	*Callianassa*	0, 6, 12, 18 and 24 cm [5]	0, 3, 6, 9, 12, 15, 18, 21 and 24 cm [21]	2 mound samples, pooled 1 sample from burrow entrance [2]
19	*Callianassa*	As core 10 [5]	0, 3, 6, 9, 12, 15, 18, 21, 24 and 30 cm [25]	4 mound samples, pooled 1 sample from a mound that differed in colour to the others [2]
3	*Upogebia*	0, 6, 12, 18, 24 and 30 cm [6]	3, 6, 9, 12, 15, and 18 cm [10]	No surface burrow samples collected
5	*Upogebia*	As core 3 [6]	0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 cm [22]	4 samples from burrow entrances [4]
12	*Upogebia*	As core 3 [6]	As core 5 [20]	2 samples from burrow entrances [2]

Abbreviation: T-RFLP, terminal-restriction fragment length polymorphism.
[a]Numbers in square brackets indicate the total number of samples used within each category.
[b]For the sequencing study, one ambient sediment sample was used for each of the shrimp species *C. subterranea* and *U. deltaura*. These samples each consisted of a pool of three ambient surface samples taken from the three replicate cores for that species.

et al., 2000). The forward primer was labelled at the 5′ end with the phosphoramidite fluorochrome 5-carboxyfluorescein (6-FAM). One microlitre of a 10^{-1} dilution of sediment DNA was added to a 50 µl PCR mixture containing 5 × PCR buffer (Promega, Southampton, UK), 2 mM $MgCl_2$, 0.2 mM dNTPs, 5 µg of bovine serum albumin, 2.5 U of GoTaq Flexi DNA polymerase (Promega) and 1 µM of each primer. PCR conditions were as described previously (Osborn *et al.*, 2000), but with a final extension step at 72 °C for 7 min. PCR products were visualised by agarose gel electrophoresis and then purified using SureClean purification kit (Bioline, London, UK). Purified product (5 µl) was then digested separately with 20 U of restriction enzyme *Alu*I in a total volume of 15 µl at 37 °C for 3 h. Digested products (0.5 µl) were mixed with deionised formamide (9 µl) and 0.5 µl of carboxy-X-rhodamine-labelled GS500 internal size standard (Applied Biosystems, Warrington, UK). Samples were denatured by heating at 95 °C for 3 min and immediately transferred to ice. Samples were electrophoresed using an ABI3730 (Applied Biosystems) at 15 V for 20 min in a 36 cm array containing POP7 polymer (Applied Biosystems). Resulting electropherograms were initially analysed using GeneMapper 3.7 software (Applied Biosystems) and subsequently using T-Align (Smith *et al.*, 2005). All data were normalised to exclude terminal-restriction fragments (T-RFs) that contributed $<0.5\%$ to the community profile, and square root-transformed to approximate normal distribution. Similarity percentage analyses were performed on transformed T-RF data in Primer 6.1 (Clarke and Gorley, 2006) to investigate the percent contribution of individual T-RFs to the similarity matrix and whether variations in bacterial community structure were attributable either to changes in the relative abundance or to the presence or absence of specific T-RFs. Non-metric multidimensional scaling ordination, analysis of similarity and all other multivariate analyses were also carried out in Primer 6.1 (Clarke, 1993) using the Bray–Curtis resemblance matrix to compare the presence and relative abundance of T-RFs (peaks) in each sample profile. All reported R values have a significance level of $P<0.01$, except where indicated.

Clone library construction and sequencing

Clone libraries were constructed for surface ambient sediment, surface mound sediment (for *C. subterranea*) and 'burrow entrance' sediment (for both species), which were taken from the burrow walls at 0–2 cm depth. Clone libraries were prepared from PCR-amplified 16S rRNA gene fragments using the primer pair 9bfm (5′-GAGTTTGATYHTGGCTCAG-3′) and 1512uR (5′-ACGGHTACCTTGTTACGACTT-3′; Mühling *et al.*, 2008). One microlitre of a 10^{-1} dilution of sediment DNA was added to a 25 µl PCR mixture containing 5 × PCR buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 µg of bovine serum albumin, 1.25 U of GoTaq Flexi DNA polymerase (Promega) and 0.2 µM of each primer. PCR cycle conditions were as above. PCR products were cloned using the pGEM-T Easy Vector System I cloning kit according to the manufacturer's instructions (Promega). Transformants were selected on Luria–Bertani agar plates containing ampicillin (50 mg ml^{-1}), X-gal (40 mg ml^{-1}) and 0.1 M isopropyl-β-D-thiogalactopyranoside. White colonies were screened by PCR using the vector primers M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′; Invitrogen, Paisley, UK) and the resulting PCR products were sequenced directly using an ABI3730. Sequences were aligned by ClustalW in MEGA 4 (Molecular Evolutionary Genetics Analysis; Tamura *et al.*, 2007) and base pairs were checked manually using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequence identity matrices were produced using BioEdit, and then input into DOTUR (Schloss and Handelsman, 2005) to obtain Chao1 and ACE richness indices, operational taxonomic unit abundance and rarefaction data. All sequences were classified into phylum and class taxonomic level using the Ribosomal Database Project Naive Bayesian rRNA Classifier Version 2.0 (Wang *et al.*, 2007). Sequences with a confidence threshold of $<80\%$ were marked as unclassified. All unclassified sequences were manually aligned against the National Center for Biotechnology nulceotide (NCBI nucleotide) database to infer taxonomy based on the most significant basic local alignment search tool alignment with taxonomic information. All sequences were then aligned using ClustalW, and the phylogenetic relationship of the remaining unclassified sequences was inferred from neighbour-joining trees with 500 bootstrap replicates.

Results

Burrow bacterial communities are distinct from surface and subsurface ambient sediment communities, but not from each other

Terminal-restriction fragment length polymorphism profiles were obtained from 229 samples digested with *Alu*I, yielding a total of 229 different T-RFs. The mean number (± s.d.) of T-RFs per profile was 40 ± 3.65. Multidimensional scaling ordination analysis revealed considerable overlap between the structure of the bacterial communities from shrimp-inhabited and uninhabited sediment cores (Figure 3), which nevertheless showed a clear separation into the three sediment groups: 'burrow', 'surface ambient' and 'subsurface ambient' sediment ($R=0.258$). When considering cores irrespective of sediment sample category, bacterial communities present in cores burrowed by *U. deltaura* and *C. subterranea* barely separated from each other ($R=0.082$). Bacterial communities in the control core were clearly separate from those in *U. deltaura*-inhabited cores ($R=0.291$); however, they were less

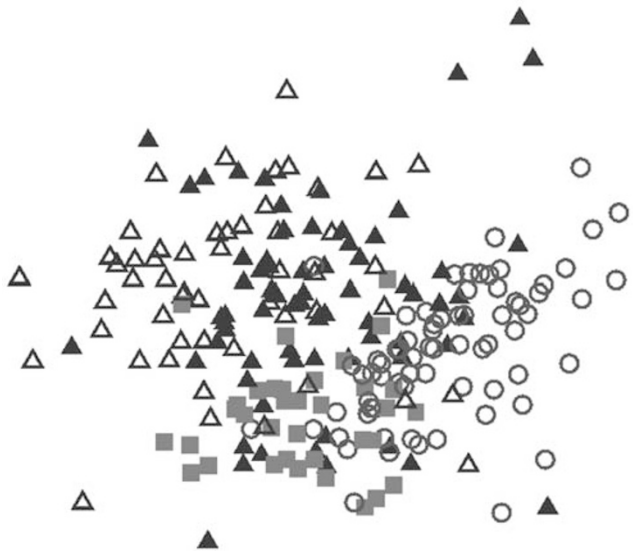

Figure 3 Non-metric multidimensional scaling (MDS) ordination of a Bray–Curtis resemblance matrix calculated from square-root-transformed T-RF relative abundances for 229 sediment bacterial communities profiled by terminal-restriction fragment length polymorphism (T-RFLP) analysis using *Alu*I. Filled triangles, *C. subterranea* burrow sediment; hollow triangles, *U. deltaura* burrow sediment; filled squares, ambient surface sediment; hollow circles, ambient subsurface sediment. Two-dimensional stress = 0.21.

distinct from the bacterial communities in *C. subterranea*-inhabited cores ($R = 0.142$).

Subsequently, the bacterial communities present within surface and subsurface ambient sediments were compared. In all three treatments (control, *U. deltaura*-inhabited and *C. subterranea*-inhabited), surface and subsurface ambient sediment communities were distinct from each other ($R = 0.368$). The difference between ambient surface and subsurface communities was more pronounced within bioturbated sediments than in the uninhabited control sediment ($R = 0.583$; $P < 0.007$, $R = 0.543$; $P < 0.013$ and $R = 0.283$ for *U. deltaura*- and *C. subterranea*-inhabited cores and for the control core, respectively). There was no significant difference in the ambient subsurface sediment communities between the shrimp-inhabited or uninhabited cores ($R = 0.086$; $P < 0.07$). Within surface sediment samples, separation was only seen between bacterial communities in *U. deltaura*-inhabited cores, and those in uninhabited cores ($R = 0.327$; $P < 0.03$).

Shrimp-inhabited cores were then analysed individually to investigate the potential differences between 'ambient' and 'burrow' sediments. All bioturbated cores showed significant differences between the bacterial communities present within burrow sediment and those in subsurface ambient sediment ($R = 0.355$), with the exception of core 10 containing *C. subterranea* ($R = -0.006$; $P < 0.44$). In contrast, there was no significant difference between the burrow communities and those in surface sediments in shrimp-inhabited cores ($R = -0.096$, $P < 0.77$). However, when compared with the control core, burrow communities were distinct from those present in both surface and subsurface uninhabited sediments across all depths (Table 2).

Variation in bacterial community structure between individual burrows

In the *C. subterranea*-inhabited sediment (cores 9, 10 and 19), there was greater dissimilarity between bacterial communities inhabiting the different burrows than between the communities inhabiting the ambient sediment in each of the cores ($R = 0.195$ and 0.097, respectively; $P < 0.097$). In the *U. deltaura*-inhabited cores (cores 3, 5 and 12), differences between ambient sediment bacterial communities were only found between two of the three cores (cores 3 and 5; $R = 0.298$; $P < 0.011$), whereas differences were observed in burrow sediment communities between cores 12 and 3 ($R = 0.200$; $P < 0.02$), and cores 3 and 5 ($R = 0.369$).

Bacterial communities from *U. deltaura*- and *C. subterranea*-inhabited burrows were compared and were found to be only weakly different ($R = 0.143$). Similarity percentage analysis revealed that all such differences were found to be a result of changes in the relative abundance of individual T-RFs rather than changes in the presence or absence of T-RFs. Where variation was observed between bacterial communities in individual burrows of the same shrimp species, 87–99% of differences were because of the changes in the relative abundance of individual T-RFs.

Burrow bacterial communities vary with burrow depth

The bacterial community structure within shrimp burrows was investigated at 3 cm depth intervals from 0 to 30 cm. Depth-specific variation in community structure was seen in *U. deltaura*-inhabited burrows ($R = 0.215$; $P < 0.023$). Burrow communities could be broadly partitioned between three locations (Figure 1): the 'burrow entrance' (0–3 cm), the 'mid-burrow' and the 'sump' (global $R = 0.279$; $P < 0.016$). The greatest difference in bacterial community structure was observed between the burrow entrance and the sump ($R = 0.73$; $P < 0.044$).

In samples from *C. subterranea* burrows, significant changes in bacterial community structure with depth were only observed between samples taken at 3 and 24 cm, and between samples taken at 6 and 24 cm ($R = 0.357$; $P < 0.021$ and $R = 0.399$; $P < 0.012$, respectively), where the samples at 24 cm were among those labelled as the 'sump'. Bacterial communities present within *C. subterranea* ejection mounds did not differ from those in the burrow as a whole ($R = -0.083$; $P < 0.72$), but were quite distinct when compared pairwise with communities from individual depths, in particular, when compared with the 24 cm 'sump' community ($R = 0.452$; $P < 0.012$).

Table 2 ANOSIM comparisons of bacterial communities in burrow and ambient sediment in sediment mesocosms inhabited by two species of bioturbating shrimp

	R *value*	*Significance,* P%
Global separation into the three sediment groups: burrow, surface ambient and subsurface ambient	0.258	0.1
Separation of all burrow sediment and subsurface ambient sediment (from *U. deltaura* and *C. subterranea* cores)	0.355	0.1
Separation of all burrow sediment and surface ambient sediment (from *U. deltaura* and *C. subterranea* cores)	−0.096	77.0
Comparing subsurface ambient communities from shrimp-inhabited and uninhabited cores	0.086	7.0
Variation between depth profiles in the control core only (Figure 3)	0.339	0.1
Comparing surface and subsurface ambient sediment		
Global comparison across all three treatments (control, *U. deltaura* and *C. subterranea*)	0.368	0.1
In *U. deltaura*-inhabited cores	0.583	0.7
In *C. subterranea*-inhabited cores	0.543	1.3
In the control (uninhabited) core	0.283	0.1
Comparing C. subterranea and U. deltaura cores		
Global separation of *C. subterranea* and *U. deltaura* treatments, irrespective of sediment type	0.082	0.1
Separation of *C. subterranea* and *U. deltaura* treatments, considering burrow sediment only	0.143	0.1
C. subterranea		
Global test between *C. subterranea* and the control core, irrespective of sediment type	0.142	0.1
Comparison between all *C. subterranea* burrows	0.195	0.1
Global test for differences between *C. subterranea* burrow sediment and control surface and subsurface sediment	0.254	0.1
Burrow vs control ambient surface sediment	0.150	0.3
Burrow vs control ambient subsurface sediment	0.277	0.1
U. deltaura		
Global test between *U. deltaura* and the control core, irrespective of sediment type	0.291	0.1
Comparison between the surface communities from *U. deltaura* cores and the control core	0.327	3.0
Global test for differences between *U. deltaura* burrow sediment and control surface and subsurface sediment	0.447	0.1
Burrow vs control ambient surface sediment	0.355	0.1
Burrow vs control ambient subsurface sediment	0.574	0.1

Abbreviations: ANOSIM, analysis of similarity; T-RF, terminal-restriction fragment; T-RFLP, terminal-restriction fragment length polymorphism. ANOSIM *R* values were derived from Bray–Curtis resemblance matrices calculated from relative abundances of 16S rRNA T-RFs in T-RFLP community profiles.

Similarity percentage analysis revealed that in *U. deltaura* burrows, 24% of the variability between communities at depths of 0 and 18 cm was because of the presence or absence of T-RFs, whereas the remaining 76% of variability was because of the change in the relative abundance of individual T-RFs between depths (average dissimilarity 47%). In *C. subterranea* burrows, only 10% of the variability between communities at depths of 0 and 18 cm was because of the changes in the presence or absence of T-RFs (average dissimilarity 44%).

Ambient sediment bacterial communities vary with depth

Within the uninhabited control core, there was considerable overlap between all sediment samples; nevertheless, there was a significant change in bacterial community structure with depth ($R = 0.339$; Figure 4). The bacterial communities became progressively different with increasing distance between samples, with the exception of the communities present at depths of 12 and 24 cm (Primer RELATE test for seriation, $\rho = 0.419$; Table 3). Similarity percentage analysis revealed that 80–97% of the differences in bacterial community structure between any two depths were because of the variation in the relative abundance of T-RFs rather than of T-RF presence or absence. The greatest variability in bacterial communities was seen between samples taken at depths of 0 and 24 cm, where 14% of this variation was because of the differences in the presence or absence of T-RFs.

Bacterial communities from shrimp-inhabited surface sediments have increased taxon richness compared to those from control sediment

Clone libraries of PCR-amplified 16S rRNA gene sequences were constructed from 21 surface ambient and burrow sediment samples from shrimp-inhabited and uninhabited (control) cores (Table 1). A total of 857 sequences were generated from these libraries and grouped into operational taxonomic units. At 99% nucleotide identity, the Chao1 and ACE indices of taxon richness were three and four times higher within *C. subterranea*- and *U. deltaura*-inhabited sediment, respectively, than in control ambient sediment, whereas at 97% nucleotide

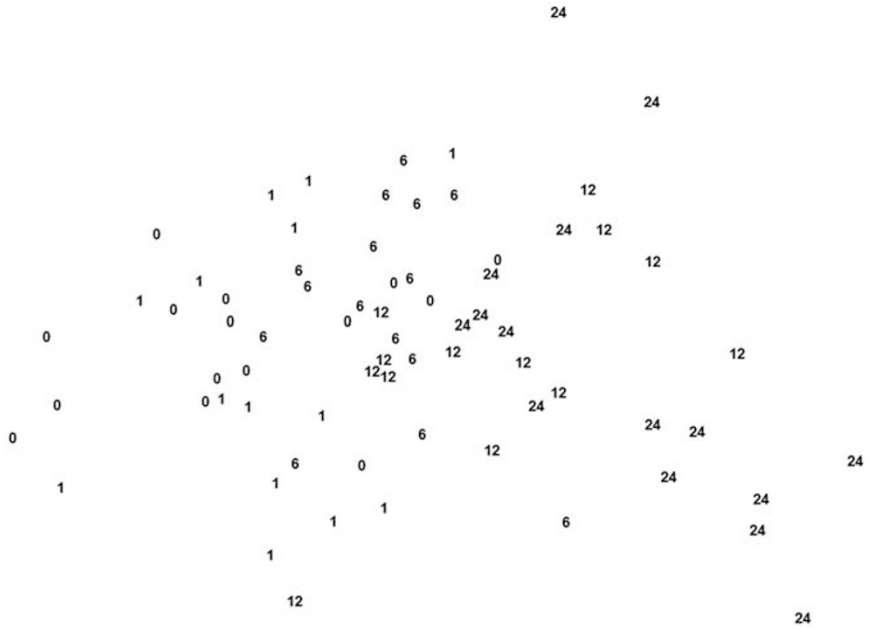

Figure 4 Multidimensional scaling ordination (MDS) ordination of a Bray–Curtis resemblance matrix calculated from square-root-transformed T-RF relative abundances for control sediment bacterial communities profiled by terminal-restriction fragment length polymorphism (T-RFLP) analysis using *Alu*I. Numbers indicate the depth in cm at which samples were taken. Two-dimensional stress = 0.17.

Table 3 ANOSIM pairwise comparisons of bacterial community structure at different depths in an undisturbed control sediment core

Groups	*Distance apart (cm)*	R *statistic*	*Significance level* P
All groups (global test)		0.339	<0.001
0, 1	1	−0.004	<0.452
1, 6	5	0.26	<0.001
0, 6	6	0.286	<0.001
6, 12	6	0.314	<0.001
1, 12	11	0.33	<0.001
0, 12	12	0.395	<0.001
12, 24	12	0.124	<0.025
6, 24	18	0.494	<0.001
1, 24	23	0.613	<0.001
0, 24	24	0.62	<0.001

Abbreviations: ANOSIM, analysis of similarity; T-RF, terminal-restriction fragment; T-RFLP, terminal-restriction fragment length polymorphism.
ANOSIM *R* values were derived from Bray–Curtis resemblance matrices calculated from relative abundances of 16S rRNA T-RFs in T-RFLP community profiles. Samples are arranged by increasing distance (depth) between samples.

identity, shrimp-inhabited sediment had twofold higher taxon richness than uninhabited sediment (Table 4). *C. subterranea*- and *U. deltaura*-inhabited sediment also showed higher taxon evenness than control sediment at both 97% and 99% nucleotide identity (Table 4).

For taxonomic classification (phylum and/or class levels), sequences were grouped by treatment (control, *U. deltaura*-inhabited and *C. subterranea*-inhabited sediment; Table 5). Irrespective of sediment type, all groups were dominated by sequences related to members of the phylum Proteobacteria, although the shrimp-inhabited sediment groups contained higher relative abundances of these sequences. Conversely, sequences related to the phylum Planctomycetes were present at higher relative abundance in the uninhabited surface sediment samples. Among the shrimp-inhabited treatment groups, the *C. subterranea* libraries contained higher relative abundances of Gammaproteobacteria-related sequences, whereas the *U. deltaura* core libraries contained greater numbers of Acidobacteria.

Discussion

In this study, we have found that bacterial communities inhabiting the burrow walls of *U. deltaura* and *C. subterranea* are distinct from those in the surrounding surface and subsurface sediment in terms of their community structure, and that the bacterial communities within bioturbated sediment had increased taxon richness when compared with those in uninhabited sediment. In addition, we have observed that these bacterial communities vary significantly between different functional burrow sections, most notably between the mid-burrow and the sump communities. We suggest that although these depth-based observations are shrimp species specific, nevertheless the differences in total bacterial community structure between the burrows of the two species of shrimp were not as great as expected.

Table 4 Diversity and richness indices and taxon number estimates for surface bacterial communities within uninhabited and shrimp-inhabited sediment mesocosms

Mesocosm treatment	*No. of sequences*	*99% nucleotide identity*				*97% nucleotide identity*			
		No. of OTUs	*Pielou's evenness*	*Chao1*	*ACE*	*No. of OTUs*	*Pielou's evenness*	*Chao1*	*ACE*
Control	313	256	0.98782	763.64	759.77	236	0.97896	614.84	696.08
U. deltaura	275	262	0.99659	2855.75	3077.11	242	0.99007	1413.8	1465.94
C. subterranea	269	251	0.99504	2084	2175.21	234	0.98810	1605.56	1469.68

Abbreviation: OTU, operational taxonomic unit.
Chao1 and ACE indices and OTUs were identified at 97% and 99% nucleotide identity using DOTUR (Schloss and Handelsman, 2005). Pielou's evenness (Pielou, 1966) was calculated from OTU abundance matrices using the diverse test in Primer 6.1.

Table 5 Taxonomic classification of surface sediment samples, comparing control ambient sediment to shrimp-inhabited sediment

Taxon	*Control*		*U. deltaura*		*C. subterranea*	
	No. of OTUs	*RA (%)*[a]	*No. of OTUs*	*RA (%)*[a]	*No. of OTUs*	*RA (%)*[a]
Acidobacteria	18	5.8	18	6.5	7	2.6
Actinobacteria	6	1.9	13	4.7	11	4.1
Bacteroidetes	38	12.1	41	14.9	38	14.1
Candidate division OP11	24	7.7	2	0.7	3	1.1
Candidate division WS3	8	2.6	1	0.4	1	0.4
Chloroflexi	30	9.6	23	8.4	17	6.3
Deferribacteres	5	1.6	1	0.4	0	0.0
Nitrospirae	5	1.6	4	1.5	2	0.7
Planctomycetes	57	18.2	35	12.7	22	8.2
Proteobacteria	92	29.4	112	40.7	135	50.2
alpha-	*8*	*2.6*	*6*	*2.2*	*21*	*7.8*
gamma-	*39*	*12.5*	*55*	*20.0*	*60*	*22.3*
delta-	*41*	*13.1*	*42*	*15.3*	*44*	*16.4*
epsilon-	*4*	*1.3*	*9*	*3.3*	*10*	*3.7*
Verrucomicrobia	5	1.6	3	1.1	7	2.6
Unclassified	25	8.0	22	8.0	26	9.7
Total	313		275		269	

Abbreviation: OTU, operational taxonomic unit.
The percent contribution to the total sequence library is indicated alongside the number of OTUs assigned to each phylum or proteobacterial class. Italicised values are sub-divisions of Proteobacteria, and as such do not contribute to the total values quoted below.
[a]Relative abundance of taxon within 16S rRNA gene libraries from this treatment.

In previous studies, the level of dissimilarity between different sediment categories is often dependent on the ecology of the organism studied. Bacterial communities from the burrows of single species of nereidid worm, thalassinidean shrimp and fiddler crab (Lucas *et al.*, 2003; Papaspyrou *et al.*, 2005; Bertics and Ziebis, 2009, respectively) were found to be more similar to those in the surrounding subsurface sediment than to surface communities. In contrast, in this study the bacterial communities inhabiting the burrow wall more closely resembled those in surface rather than subsurface sediment. Similar observations have been made for polychaete worm burrows (Steward *et al.*, 1996) and other thalassinidean shrimp burrows (Bertics and Ziebis, 2009). Such relationships between burrow and surface communities may be a consequence of similarities in their physicochemical environments, at least for some species (Bird *et al.*, 2000; Bertics and Ziebis, 2009); although previous studies have cautioned that the burrow should not be seen as a simple extension of the sediment surface (Papaspyrou *et al.*, 2005). These various findings suggest that different burrowing organisms vary in the influence they exert on sediment bacterial communities and emphasise that holistic studies on bioturbation should not assume that the burrows of all species will exhibit the same biogeochemical properties. This conclusion is consistent with earlier multispecies studies on bioturbating organisms (Aller *et al.*, 1983; Christensen *et al.*, 2000; Papaspyrou *et al.*, 2006).

It was expected that there may be some level of inter- and even intra-species heterogeneity in

shrimp behaviour, which would in turn impact on the biogeochemical and microbial signature of the burrow (Aller *et al.*, 1983; Berkenbusch and Rowden, 2000; Bertics and Ziebis, 2009). Visually, the burrows of *U. deltaura* and *C. subterranea* are very different, and the two species show differing burrow-building and irrigation behaviours. However, this study has shown that the bacterial communities inhabiting both types of shrimp burrow vary only weakly from each other. Consequently, this study would suggest that the biggest single influence on microbial community structure and diversity is the presence or absence of burrows. Although species-specific differences in burrow morphology and feeding activity seem to have a comparatively lesser role, these activities are still significant. There is also likely to be some level of abiotic control. For instance, callianassid shrimp show intraspecific plasticity in burrow morphology according to prevailing environmental conditions, such as temperature and organic content (Berkenbusch and Rowden, 2000; Rowden and Jones, 1995). Marinelli *et al.* (2002) found that the irrigation frequency of mimic burrows had no effect on the microbial community structure in sediment surrounding the burrow. Rather, they suggest that the burrow inhabitant's behaviour (for example, as a controller of burrow wall composition) is a more important determinant of microbial community structure within the burrow wall. For example, thalassinidean shrimp are known to sort sediment particles using their mouth parts and compact the finer particles into the burrow wall, especially in those species that maintain long-term burrow structures, such as the upogebiids (Coelho *et al.*, 2000). In addition, many thalassinidean shrimp excrete mucus to aid in the compaction of the burrow wall sediment and may 'garden' their burrow by incorporating organic matter into the walls (Astall *et al.*, 1997; Coelho *et al.*, 2000; Dworschak *et al.*, 2006; Koller *et al.*, 2006). It can be envisaged that the presence of this material may influence the structure and diversity of bacterial communities within the burrow walls.

Thalassinidean shrimp therefore actively alter both the physical structure and the chemical environment within the sediment (Astall *et al.*, 1997), and it may be predicted that this has a significant impact on the microbial communities inhabiting the sediment. In the case of *C. subterranea*, the sediment surrounding the burrow may be regularly disturbed as the shrimp fills in old tunnels and constructs new ones (Stamhuis *et al.*, 1997). The intermediate disturbance hypothesis predicts that in areas where there is an intermediate level of 'disturbance' (a discrete event that disrupts the ecosystem, changes resource availability or alters the physical environment), higher levels of biodiversity are likely to be promoted by the coexistence of otherwise competing species (Connell, 1978). Widdicombe *et al.* (2000) found that the impact on diversity within eukaryotic communities because of the presence of one or more macrofaunal bioturbators was dependent on both the density and the specific behaviour of the bioturbator. They suggested that the community response was a trade-off between the increased resources likely to be made available by bioturbation (organic matter and nutrient-rich solutes) and the severity of the actual physical disturbance. In a natural community high in bioturbator activity, such as Plymouth Sound, there is likely to be an interaction between the effects of the different bioturbators, which may also be temporally changeable.

The effects on the microbiota are more complex still. It is thought that the irrigation and burrow wall 'grooming' (maintenance) and 'gardening' (feeding) activities performed by thalassinidean shrimp could contribute to creating environmental microniches within the burrow (Astall *et al.*, 1997; Coelho *et al.*, 2000; Abed-Navandi *et al.*, 2005), which may influence the microbial signature at different burrow depths and between individual burrows. Niche separation could be one of the most important controls on microbial diversity (Ramette and Tiedje, 2007). The level of microbial species or genotypic diversity in an environment is likely to be disturbed by two independent factors: the lowered fitness of specialists because of environmental perturbation and the disruption of resource partitioning in complex communities (Parnell *et al.*, 2009). The highest level of genotypic diversity tends to occur in communities of intermediate species richness, subject to neither of these extremes. On the basis of the model of the relationship between genotypic and species diversity in natural bacterial communities worldwide in the study by Parnell *et al.* (2009), the surface communities studied here are of intermediate species richness; however, it is evident that the bioturbated sediment has both higher species richness and greater genotypic diversity, suggesting that the specific levels of disturbance provided by the shrimp may offer the opportunity for functional specialisation while also allowing the complementary use of the resources (for example, organic matter and nutrient-rich solutes) provided by the burrow environment. Parnell *et al.* (2009) suggest that the maintenance of genotypic diversity in a fluctuating environment could contribute to the maintenance, function and stability of the environment. It is clear that these burrowing shrimp may qualify as 'ecosystem engineers' (Jones *et al.*, 1994) due to their impact on the physical environment and the biodiversity, and possibly functioning, of the ecosystem (Meysman *et al.*, 2006).

It may be anticipated that these physical disturbances, combined with dynamic environmental conditions within the burrow, will lead to temporal and spatial variation in the structure of bacterial communities inhabiting burrow walls (Kristensen, 2000; Furukawa, 2001). Indeed, Kristensen and Kostka (2005) proposed that the highly variable

environmental conditions within macrofaunal burrows support unique communities of microorganisms. In this study, we found that each individual burrow exhibited an individual microbial fingerprint that was nevertheless more similar to other burrows (of either shrimp species) than to the surrounding sediment. It would be interesting to observe the effects of fluctuating levels of shrimp behaviour (with season and environment) on the maintenance of this burrow diversity, and the subsequent effects on nutrient cycles.

In investigating bacterial community structure, the burrow has, to date, been considered as a single environment in which bacterial communities are relatively stable with time compared with ambient surface sediment (Kristensen and Kostka, 2005; Papaspyrou *et al.*, 2005). Hence, traditionally only single burrow samples between 5 and 20 cm depth (where specified) and single-depth ambient subsurface samples are isolated for the study. Recently, Bertics and Ziebis (2009) found that in thalassinidean shrimp burrows, the microbial communities in samples from 1, 2 and 8 cm burrow depth all clustered with surface sediment communities. Our study suggests a more complex scenario in which the bacterial communities inhabiting burrows of *U. deltaura* and *C. subterranea* vary in addition with burrow depth. This effect was most pronounced in *U. deltaura* burrows in which marked differences were observed in the bacterial communities present in the burrow entrance, the mid-burrow and the sump. The sump may potentially contain a distinct bacterial community due to variation in the concentration of organic substrates, localised changes in sediment characteristics or reduced irrigation of the sump in comparison to the wider burrow system.

The similarity between bacterial community structure in burrow sediment and surface sediment from other burrowed cores, but not from the control core, suggests that the presence of the burrow affects not only the bacterial communities present within the direct burrow wall perimeter, but also within the surrounding sediment, particularly in cores inhabited by *U. deltaura*. In an area naturally dense in thalassinidean shrimp, this could have a substantial impact on the overall sediment microbiology and consequently influence biogeochemical processes within the wider system (Aller and Aller, 1998). In support of this, Bertics and Ziebis (2009) found that as the combined bioturbation activities of thalassinidean shrimp and fiddler crabs increased, so did the *in situ* microbial abundances and oxidation–reduction potential. Such effects have also been observed in sediments inhabited by lugworms, which resulted in changes in the pore-water chemistry of the entire permeable sediment (Volkenborn *et al.*, 2007). Nevertheless, recent investigations have noted the differences between sediment characteristics *in situ* and mesocosm sediments (Papaspyrou *et al.*, 2007), and it would be beneficial to confirm the conclusions of this study by comparison with *in situ* exclusion studies.

Several earlier studies have indicated that the burrow wall can harbour greater number of bacterial cells than the ambient sediment (Papaspyrou *et al.*, 2005; Kinoshita *et al.*, 2008) and can function as a site for enhanced microbial metabolic activity (Phillips and Lovell, 1999). The phylogenetic analyses performed herein suggest that bioturbated sediment may also have higher diversity and species richness than ambient sediment and that the presence of *U. deltaura* or *C. subterranea* may result in shrimp-specific differences in bacterial community composition. It is possible that such changes may in addition influence the function and activity of bacterial communities within the burrow. For example, the presence of *U. deltaura* in sediments has been shown to lead to significant increases in total denitrification rates (Howe *et al.*, 2004), while numbers of ammonia-oxidising bacteria were greater at certain depths within a polychaete burrow than in the surrounding sediment (Satoh *et al.*, 2007).

In conclusion, we have found that the presence of bioturbating shrimp in sediments can lead to changes in the structure and diversity of marine bacteria sediment communities. We have observed these changes not only in burrow wall sediments, but also in more distant ambient sediment communities, in particular at the sediment surface. Such community changes in response to bioturbation may have wider ecological significance within marine shelf sediments. Bioturbation physically disturbs the sediment, and the findings of this study imply that this will affect the biodiversity (and hence, by inference, the function) of microbial communities within the sediment. The key role of bioturbators in nutrient cycling is well established (Jones *et al.*, 1994; Meysman *et al.*, 2006). The shrimp species studied here are thought to impact on nitrogen cycling, as the sediment surface and burrow walls provide an important interface for coupled nitrification–denitrification reactions (Aller *et al.*, 1983). It is clear from previous studies that the burrow environment experiences physicochemical conditions distinct from the surrounding sediment (Aller *et al.*, 1983; Bird *et al.*, 2000; Furukawa, 2001). In highly productive areas naturally dense in thalassinidean shrimp, such as temperate and tropical coastal regions, these shrimp could make a significant contribution to coupled benthic–pelagic nutrient exchange. An important next step in our investigations will be to integrate further examination of the genetic diversity and functional potential of the bacteria in burrow walls with biogeochemical observations to provide insight into how the two are linked. The distinct microbial communities present in the burrow wall may provide significant genetic potential for the cycling of key nutrients between the sediment and the water column.

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