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ORIGINAL ARTICLE

Biodiversity of benthic microbial communities in bioturbated coastal sediments is controlled by geochemical microniches

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We used a combination of field and laboratory approaches to address how the bioturbation activity of two crustaceans, the ghost shrimp *Neotrypaea californiensis* and the fiddler crab *Uca crenulata*, affects the microbial diversity in the seabed of a coastal lagoon (Catalina Harbor, Santa Catalina Island, CA, USA). Detailed geochemical analyses, including oxygen microsensor measurements, were performed to characterize environmental parameters. We used a whole-assemblage fingerprinting approach (ARISA: amplified ribosomal intergenic spacer analysis) to compare bacterial diversity along geochemical gradients and in relation to subsurface microniches. The two crustaceans have different burrowing behaviors. The ghost shrimp maintains complex, deep-reaching burrows and permanently lives subterranean, supplying its burrow with oxygen-rich water. In contrast, the fiddler crab constructs simpler, J-shaped burrows, which it does not inhabit permanently and does not actively ventilate. Our goal was to address how varying environmental parameters affect benthic microbial communities. An important question in benthic microbial ecology has been whether burrows support similar or unique communities compared with the sediment surface. Our results showed that sediment surface microbial communities are distinct from subsurface assemblages and that different burrow types support diverse bacterial taxa. Statistical comparisons by canonical correspondence analysis indicated that the availability of oxidants (oxygen, nitrate, ferric iron) play a key role in determining the presence and abundance of different taxa. When geochemical parameters were alike, microbial communities associated with burrows showed significant similarity to sediment surface communities. Our study provides implications on the community structure of microbial communities in marine sediments and the factors controlling their distribution.
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Introduction

The burrowing, ventilation and foraging activity of benthic macrofauna organisms affects key ecosystem processes of marine sediments, including organic matter remineralization, nutrient cycling, biogeochemical interactions and benthic–pelagic fluxes (Rhoads, 1974; Aller, 1982, 1988, 1994; Kristensen *et al.*, 1991, 2000; Gilbert *et al.*, 1998, 2003; Banta *et al.*, 1999). Microbial abundances and activities have been shown to increase due to the complex biogeochemical interactions induced by bioturbation activity (Hansen and Kristensen, 1998; Lohrer *et al.*, 2004; Kogure and Wada, 2005). The construction of burrows increases the sediment–water interface, offering additional surfaces for microbial colonization and chemical reactions (Aller and Aller, 1986; Meyers *et al.*, 1987; Reichardt, 1989; Grossman and Reichardt, 1991; Marinelli *et al.*, 2002). The transport of particles (bioturbation) and the flushing of burrows (bioirrigation) create 3-dimensional geochemical zonation patterns with substantial changes of redox-conditions and the formation of temporally and spatially dynamic microenvironments. Oxidized microhabitats often occur next to reduced sediment compartments thus allowing a tighter coupling of redox reactions

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(for example, nitrification—denitrification) (Mayer *et al.*, 1995; Pelegrí and Blackburn, 1996; Tuominen *et al.*, 1999; Svensson *et al.*, 2001; Dollhoph *et al.*, 2005). The flushing of burrows with oxygen-rich water by a deep-burrowing *thalassinidean* shrimp has been shown to transport oxygen as deep as 80 cm into the sediment (Ziebis *et al.*, 1996a), whereas penetration depth of oxygen by molecular diffusion is typically only a few mm in coastal sediments (Revsbech *et al.*, 1980; Glud *et al.*, 1994). Only a thin film of surface sediment is generally oxic, containing molecular oxygen and allowing aerobic respiration. Below this zone the sediment is anoxic. The oxidized zone, characterized by a positive redox potential and the availability of other electron acceptors (for example, nitrate, ferrous iron), can extend deeper into the sediment.

One of the key questions in benthic microbial ecology is whether similar environmental conditions support the same microbial communities. One hypothesis is that the microbial assemblages in the oxic and oxidized zones surrounding the burrows are equivalent to communities in surface sediments. In contrast, it has been suggested that burrow walls support unique microbial communities that differ considerably from those found at the surface (Kristensen and Kostka, 2005; Papaspyrou *et al.*, 2005, 2006). The question remains how microbial communities within burrows compare to the ambient sediment and to the sediment surface.

There are very few studies that have addressed the impact of habitat heterogeneity on microbial diversity in sediments (Hewson *et al.*, 2007), mainly because it remains a challenge to quantify the large number of abiotic and biotic factors that shape the microbial habitat on different spatial and temporal scales. The majority of marine studies on microbial diversity have been performed in the water column (Hewson and Fuhrman, 2004; Hannig *et al.*, 2006; Pommier *et al.*, 2007; Fuhrman *et al.*, 2008), with a focus on whether bacteria exhibit biogeographical patterns. However, the processes that govern these microbial distribution patterns in the field are still poorly understood (Suzuki and DeLong, 2002; Castro-Gonzalez *et al.*, 2005; Fuhrman *et al.*, 2008). Sediment systems harbor even higher abundances and a greater diversity of microorganisms (for example, Curtis *et al.*, 2002; Torsvik *et al.*, 2002), yet they remain sadly understudied (Jørgensen and Boetius, 2007). The correlation between habitat complexity and microbial diversity remains to be determined (Fierer, 2008). Bioturbated sediments provide ideal opportunities to compare microbial communities in relation to environmental parameters.

Fiddler crabs and ghost shrimp are among the most abundant bioturbating macrofauna in coastal areas all over the world. Their burrows have been acknowledged as important conduits for chemical exchange between the water column and the sediment, linking the activity of these crustaceans to nutrient recycling, organic matter degradation and primary productivity (for example, Kostka *et al.*, 2002; Papaspyrou *et al.*, 2005). In this study, we examined the impact of the bioturbation activity of the ghost shrimp *Neotrypaea californiensis* and the fiddler crab *Uca crenulata* on microbial community compositions in the sediment.

Methods

Study site

The investigations were carried out in a shallow lagoon located in Catalina Harbor, Catalina Island, CA, USA (33° 25.23′ N, 118° 19.42′ W) about 35 km southwest of Los Angeles, USA (Figure 1). The head of Catalina Harbor is a shallow (<2 m), low energy area of fine-grained sand, surrounded by beach and a gentle plain. Below the low-water mark, the sediments become more silty. The average tidal range is 1.1 m and tides are mixed, with the higher

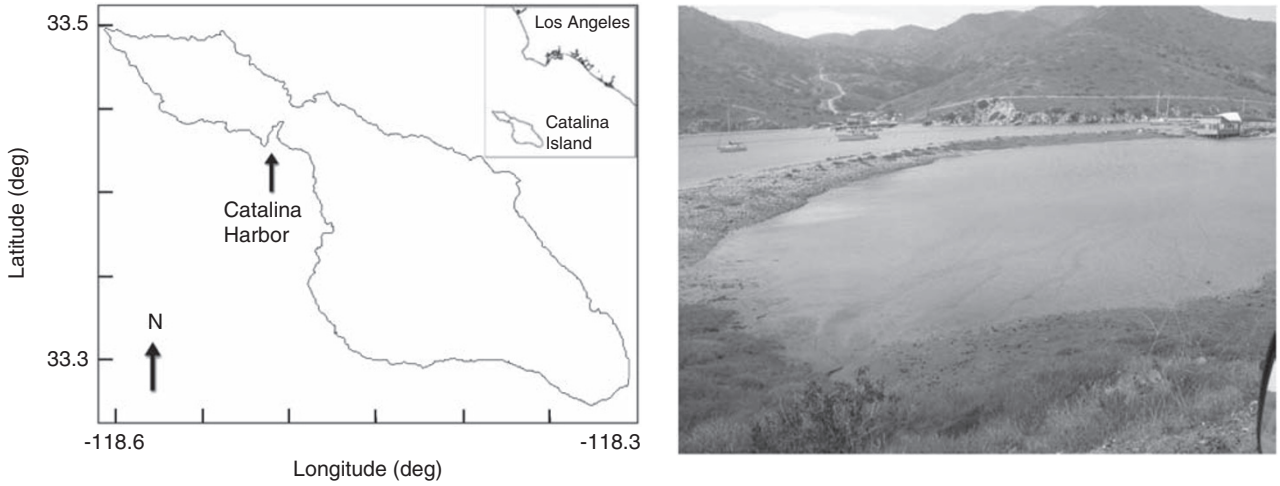

Figure 1 All samples were collected in Catalina Harbor (pictured right) on Santa Catalina Island (indicated by an arrow on the left), which is located 35 miles off the southern coast of Los Angeles, CA, USA.

high water preceding the lower low water (Colbert *et al.*, 2008a, b).

Over the course of the experiment (June–August 2005), water temperature was typically 18 °C and salinity was 34.5‰. The two most abundant burrowing macrofauna were the Mexican fiddler crab *Uca crenulata*, Lockington, 1877 (Crustacea: Decapoda: Ocypodoidea) and the bay ghost shrimp *Neotrypaea californiensis,* Dana, 1854 (Crustacea: Decapoda: Thalassinidea), earlier known as *Callianassa californiensis* (Manning and Felder, 1991). Both species are typical inhabitants of intertidal areas along the west coast of North America and occur in high abundances of greater than 200 individuals m^{-2}. They are representatives of two species-rich superfamilies, *Ocypodoidea* (Rafinesque, 1815) and *Thalassinoidea* (Latreille, 1831) (Martin and Davis, 2001), which occur worldwide with ~100 (Bisby *et al.*, 2007) and more than 500 known taxa (Dworschak, 2000), respectively.

Ghost shrimp (*Thalassinidean)* have increasingly attracted attention in benthic ecology because of their significant burrow architectures and their influence on biogeochemical processes and microbial communities (Aller and Dodge, 1974; Waslenchuk *et al.*, 1983; Forster and Graf, 1992, 1995; Ziebis *et al.*, 1996a, b; Huettel *et al.*, 1998; Kinoshita *et al.*, 2003). Their burrows usually consist of a conspicuous upper Y or U-shape structure, which allows for easy flushing with overlying water. Burrows continue vertically into the sediment and often exhibit several galleries of interconnected chambers at distinct sediment depths (for example, Ziebis *et al.*, 1996a). The deepest burrows reported to date reached 3 m (Pemberton *et al.*, 1976). Most known species remain subsurface their entire life and maintain semi-permanent burrows that are constantly reworked. *N. californiensis* inhabits intertidal areas stretching from Alaska to Baja California. Its burrow architecture is complex, extending down to ~76 cm with several branches with openings to the surface (MacGinitie, 1934; Brenchley, 1981; Swinbanks and Murray, 1981).

Fiddler crabs have a distinctively different burrowing behavior. They maintain simple J-shaped burrows, which usually have a single entrance and continue into the sediment at a 45° angle down to a depth of ~20 cm, ending in a terminal chamber. They leave their burrows frequently during low tide to forage on algae, bacteria and detritus on the sediment surface (Zeil *et al.*, 2006). *U. crenulata* is found along the west coast of North America from Santa Barbara, California to Central Mexico. Although these two burrowing crustaceans inhabit the same area, the difference in behavior, burrow construction and foraging has most likely a vastly different ecological impact and effect on benthic microbial communities.

At our study site, shrimp burrows reached an average depth of ~20 cm, which seemed to be limited by a cohesive sediment layer found at that depth within the lagoon. Shrimp burrows consisted of several branches with three to four openings to the surface. Pumping activity of the permanently subterranean shrimp could be observed in the field by sediment ejections from the burrow openings. Crab burrows extended to a maximum of 10 cm depth and consisted of a single J-shaped tube. Fiddler crabs were often seen at the sediment surface or sitting at their burrow opening.

Sampling

A total of six sampling sites were chosen at 2-m intervals along a transect perpendicular to the shoreline, starting at the water's edge (0 m) and continuing to a distance of 10 m. Sites were named according to their distance from shore (0, 2, 4, 6, 8, 10 m). An additional location in the same area (2 m parallel to the transect), but with no apparent macrofauna bioturbation activity served as a control site (non-bioturbated zone). Except for the 0 m site there was a visible photosynthetic mat at the sediment surface. The bioturbation intensity at each site was determined by counting the number of burrows within a 25 × 25 cm frame (10 replicates per distance*)*. Four sediment cores were collected closely together at each of the 6 sites and at the control site for geochemical and microbiological analyses, using acrylic core liners (*ø* 5.4 cm, length 30 cm).

Microsensor profiling

Oxygen concentrations and penetration depths were determined during high and low tide conditions at the control, the 4 m and 10 m sites. *In situ* profiling was performed during low tide using a small benthic lander, to which modified micromanipulators were attached. Microsensors were connected to battery-powered picoammeters and signals were recorded directly on a laptop computer. During high tide sediment cores were collected and microprofiles were measured in intact sediment cores directly after retrieval. Microprofiles of oxygen were measured in vertical intervals of 250 μm using Clark-type amperometric oxygen sensors (Revsbech and Jørgensen, 1986; Revsbech, 1989; Unisense, Aarhus, Denmark). Microprofiles of sulfide with amperometic microelectrodes (Jeroschewsky *et al.*, 1996) were also performed to a depth of 5 cm, yet no sulfide was detected and the measurements are not further discussed. Profiles of redox potential using microsensors were performed in 250-μm steps in cores containing burrows. For all measurements sensors were attached to motorized micromanipulators (Märzhäuser, Wetzlar, Germany), and driven vertically into the sediment in μm to mm steps, controlled by a computer. Signals were amplified and transformed to millivolt (mV) by a 2-channel picoammeter (PA 2000) (Unisense), and directly recorded on a computer using the software Profix

(Unisense). Redox-potential sensors were connected to portable or table-top pH/mV meter (WTW-pH340, WTW GmbH, Weilheim, Germany; pH-m210 Meter Lab, Radiometer Analytical, Lyon, France). Oxygen measurements were also performed directly inside burrows by positioning the tip of the electrode inside a burrow and recording the change in oxygen concentration over time. Narrow aquaria were set up to observe the burrowing behavior and to perform detailed measurements in and around burrows. Two narrow aquaria (dimensions: $40 \times 30 \times 3$ cm) were filled with sieved sediment (500 μm) from the study site. The first aquarium served as a control and did not contain a ghost shrimp, whereas to the second aquarium one adult shrimp was added. The aquaria were kept in the laboratory under running seawater and a simulated natural light cycle for 6 weeks. At this time the shrimp had established a burrow system and oxygen profiles were measured in vertical profiles (250 μm depth increments) in 2 cm horizontal intervals along the width of both aquaria. Fiddler crabs were also kept in aquaria. After they had established a simple burrow, they usually stayed at the surface or at the entrance of their burrows. Oxygen profiles measured in and around crab burrows revealed no oxygen transport into the burrows.

Geochemical and microbiological analyses

All four cores were sliced in 1-cm intervals under nitrogen atmosphere and each section was subsampled for further processing. All subsamples for one site (pore water and sediment) were taken from the exact same core. A second core was taken as a back up. Small samples of sediment (0.5 cm^3) were frozen at −20 °C for the analyses of iron, a second subsample (0.5 cm^3) was immediately frozen at −80 °C for molecular biological studies. Exactly one cm^3 of sediment was fixed in 9 ml of filter sterilized (0.2 μm) formaldehyde/seawater solution (4% v/v) for the enumeration of microorganisms. Pore-water was collected using a pore-water press (KC Denmark, Silkeborg, Denmark) and samples (3 ml) for ammonium and nitrate analyses were frozen immediately. The remainder of the sediment section was used for the determination of porosity and organic content (Loss on ignition, LOI). Two sediment cores were taken directly targeting burrows of *U. crenulata* and *N. californiensis* for direct sampling of burrow linings for microbiological analyses.

Porosity was determined by drying a known volume of sediment at 65 °C for 24 h. The loss on ignition (LOI) was determined after combusting samples at 450 °C for 24 h. Ferric and ferrous iron concentrations in sediment samples were analyzed following procedures described by Lovley and Philips (1987), with modifications described by Kostka and Luther III (1994) and Thamdrup *et al.* (1994). This extraction procedure allows for the quantification of microbially reducible ferric iron in aquatic sediments (Lovley and Philips, 1987). Pore-water ammonium concentrations were determined by flow injection analysis modified for small sample volumes (100 μl sample) (Hall and Aller, 1992). The sum of nitrate and nitrite was determined spectrophotometrically after reduction of samples with spongy cadmium (Jones, 1984). The procedure was modified for the analyses of small sample volumes (0.5–1 ml). The small sample sizes allowed relatively high spatial resolution of the analyses.

Microbial abundances in the sediment were determined by direct counts of stained cells (acridine orange) using epifluorescence microscopy following the enumeration protocol by Epstein and Rossel (1995). For comparing microbial community structures the PCR-based whole-community fingerprinting approach ARISA (amplified ribosomal intergenic spacer analysis) (Fisher and Triplett, 1999; Hewson and Fuhrman, 2004; Brown *et al.*, 2005) was applied. ARISA amplifies the intergenic spacers between the 16S and 23S rRNA genes using a fluorescent primer. Results are displayed as the amount of different PCR products of specific fragment length, which correspond to 'operational taxonomic units' (OTUs) (Brown and Fuhrman, 2005). DNA was extracted from 100 mg of sediment using the Qbiogene (Bio101) Soil DNA kit (Qbiogene Inc., Carlsbad, CA, USA). Extracted DNA was quantified using PICO Green fluorescence (Molecular Probes Inc., Eugene, OR, USA) and diluted to 10 ng cm^{-3}. PCR was performed using the universal primer 16S-1392F (5′-G[C/T]ACACACCGCCCGT-3′) and the bacterial primer 23S-125R labeled with a 5′ TET (5′-GGGTT[C/G/T]CCCCATTC[A/G]G-3′). The 50-μl PCR combined 200 nM of each primer with 1 × PCR buffer, 2.5 mM $MgCl_2$, 250 μM of each deoxynucleotide, 2.5 units of Taq polymerase (Promega, Madison, WI, USA) and 40 nM BSA (Sigma, St Louis, MO, USA, catalog no. A-7030). Thermocycling consisted of a 5-min heating step at 94 °C, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 56 °C for 30 s and extending at 72 °C for 45 s, and finished with a 10-min extension step at 72 °C. The products were purified using a Clean & Concentrator kit (Zymo Research Corp., Orange, CA, USA), quantified using PICO Green fluorescence and duplicates of 10 ng of purified products from each sample were run on an ABI 377XL automated slab gel sequencer (Applied Biosystems, Foster City, CA, USA). Results were analyzed using the ABI Peak Scanner software where each peak displayed in an electropherogram represented one OTU and the peak area represented the amount of the OTU present. Peak Scanner outputs were transferred to Microsoft Excel (Seattle, WA, USA) spreadsheets for subsequent analysis and binning as described by Hewson and Fuhrman (2006). The program $Primer_6$ (PRIMER-E Ltd, Lutton, UK) was used to further analyze the data sets and to perform a cluster analysis comparing the similarity

(Bray-Curtis) of microbial communities found at different locations. Prior to clustering, OTU data was logarithmically transformed (log $(x+1)$) to achieve a normal distribution. PC-ORD (MJM Software Design, Gleneden Beach, OR, USA) was used to perform a canonical correspondence analysis (CCA) to determine the relationships between microbial communities and environmental parameters (ter Braak, 1986, 1995; Cetinic *et al.*, 2006).

Results

Bioturbation activity

Based on the average number of burrow openings m^{-2}, bioturbation intensity increased with distance from the shoreline ($R^2 = 0.9478$, $P < 0.001$) to a total of 870 burrow openings m^{-2} at 10 m distance (Figure 2a). We distinguished three general areas of varying bioturbation intensity: 1. Low bioturbation, corresponding to ~100 burrow openings m^{-2} at 0 m distance, 2. Moderate bioturbation, corresponding to a number between 260 and 400 burrow openings m^{-2} at the 2 m and 4 m sites and 3. High bioturbation, reaching 560–870 burrow openings m^{-2} at the 6 m, 8 m and 10 m sites. *U. crenulata* burrows remained constant with distance from shore (~170 burrow openings m^{-2}) except for the shoreline where only ~42 burrow openings m^{-2} were counted. In contrast, the abundance of *N. californiensis* burrows increased linearly with distance from 60 burrows m^{-2} at the 0 m site to 640 burrows m^{-2} at the distance of 10 m (Figure 2a). Using a simple calculation, based on the average number of burrows for each species and the mean dimensions of the burrows (crab burrow: ~10 cm deep, ø 2.5 cm, shrimp burrow: ~20 cm deep, ø 1 cm) this leads to an increase of the sediment–water interface by ~600%. The tidal range and dry exposure of the near shore sites might be an explanation for the increase of bioturbation intensity.

Organic content and porosity. LOI (in % dry weight) varied little (0.84–2.8%) with depth or along the transect, except for the 8 and 10 m sites, where in the top 2 cm values reached 17.5% of the dry weight (Figure 2b). The photosynthetic mat at the sediment surface appeared to be thicker in this region, possibly contributing to a higher organic content. At the other sites, LOI was slightly higher in the top 2 cm (2.8%) than deeper in the sediment. Porosity was highest at the sediment surface (0.3–0.55) at all sites except for the control site where porosity stayed constant (0.2) throughout the sediment column (Figure 2c). Porosity decreased below the surface and stayed at values between 0.22 and 0.25.

Geochemical zonation

Microsensor studies. During both high and low tide conditions, oxygen penetration into the sediment increased from the non bioturbated zone at the 0 m site (~1 mm) to deepest oxygen penetration (>6 mm) within the area of high bioturbation (10 m distance) (Figures 3a, b). Oxygen concentrations and penetration depths were slightly higher during low tide, decreased rapidly within the first mm at all sites but stayed at elevated concentrations deeper into the sediment at the bioturbated sites. During high tide the oxygen profile measured at the 10 m site showed subsurface maxima indicating oxygen transport deeper into the sediment probably within burrows. Detailed oxygen measurements in narrow aquaria (Figures 3c and d) showed deepest oxygen penetration to a depth of 3–4 mm in the sediment without shrimp (Figure 3c). The contour lines describe the heterogeneity of the sediment surface and show photosynthetic activity at the sediment–water interface with highest oxygen concentrations at the interface. In the second narrow aquarium deeper oxygen transport was documented within burrows and across burrow walls (Figure 3d, 25 cm width) creating radial gradients of oxygen concentration to a distance of ~2 mm. Vertical oxygen profiles measured directly within a burrow opening of the ghost shrimp *N. californiensis* compared with the sediment 5 cm away from the burrow (Figure 4a) illustrated also deep penetration (>2 cm) of oxygen-rich water within the burrow compared with oxygen penetration into the sediment by molecular diffusion (3 mm). Oxygen concentrations measured at a depth of 3 cm inside the burrow over a period of 35 min (Figure 4b) illustrated that oxygen concentrations were maintained at constant high levels (150–250 μM). These measurements were repeated for several burrows showing the similar pumping activity. Vertical profiles of redox-potential (mV) measurements, depicted as a contour plot (Figure 4c), showed an oxidized zone (positive redox-potential) of ~2 cm surrounding a burrow. Similar measurements were performed on fiddler crab burrows, yet we were not able to document or measure an intrusion of oxygen-rich water into the crab burrow. No dissolved sulfide was detected in any of the field or laboratory measurements, despite the fact that sulfate reduction has been previously measured (unpublished data) in the exact same area. One explanation might be a precipitation of dissolved sulfide as iron sulfide.

Distribution of redox species. The distributions of the redox couples ferric/ferrous iron and nitrate/ammonium are illustrated as contour plots along the transect (Figures 5a–d). The profiles measured at the control site are not included in the contour plots. Here, the oxidized form of iron (Fe III) was only present in the top 2 cm, whereas reduced iron (Fe II) increased with depth to concentrations of 1200 μM cm^{-3} wet sediment. Similarly, nitrate was only present just below the sediment surface (20 μM), whereas ammonium showed a typical increase with depth starting at 7 μM at 1 cm sediment

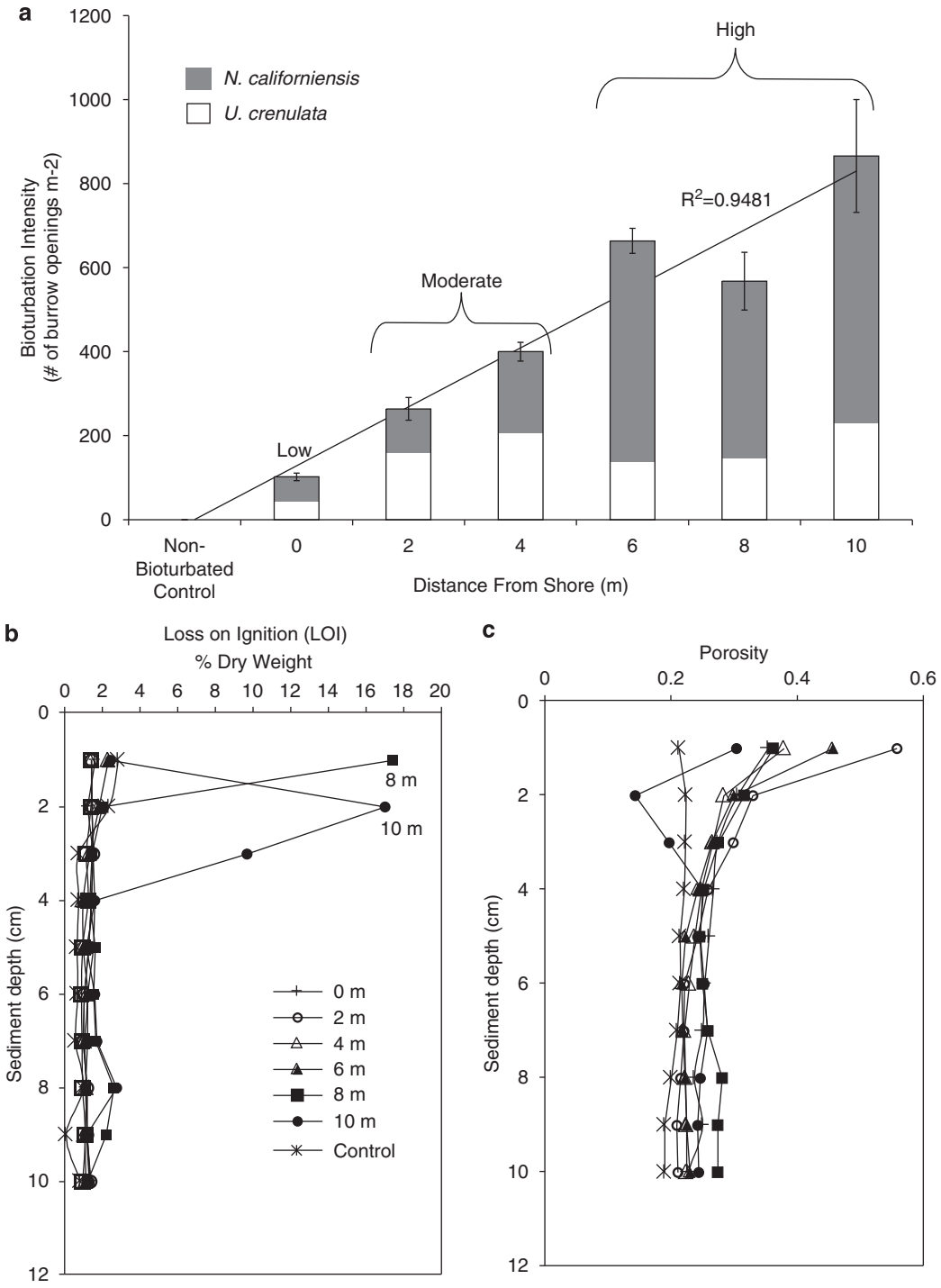

Figure 2 (**a**) Bioturbation intensity was determined along a 10-m transect perpendicular to the coastline in Catalina Harbor. The number of burrow openings was counted in 2-m intervals (0, 2, 4, 6, 8, 10 m) using a frame (25 × 25 cm) and compared with a non-bioturbated control site. Ten frames were counted per site, the bars indicate the mean number of burrows per m^2 and error bars represent the s.e.m. There was a linear increase of the total number of burrows with distance. Burrows created by the ghost shrimp *N. californiensis* (gray bars) were distinguished from burrows by the fiddler crab *U. crenulata* (white bars). (**b**) As a measure of organic content the loss on ignition (LOI) was determined in 1-cm depth intervals in sediment cores collected at the 6 sites and the control area. LOI is illustrated as the percentage of the sediment dry weight. (**c**) On the same cores sediment porosity was determined as the weight loss after drying (60 °C, 24 h) and is expressed as parts of 1.

depth and increased to 110 μM at 10 cm sediment depth. In contrast, a comparison of oxidized and reduced species of iron and nitrogen along the transect showed a patchy distribution reflecting the presence of burrows and bio-irrigation activity. The distribution of ferric iron at the 0–4 m sites was

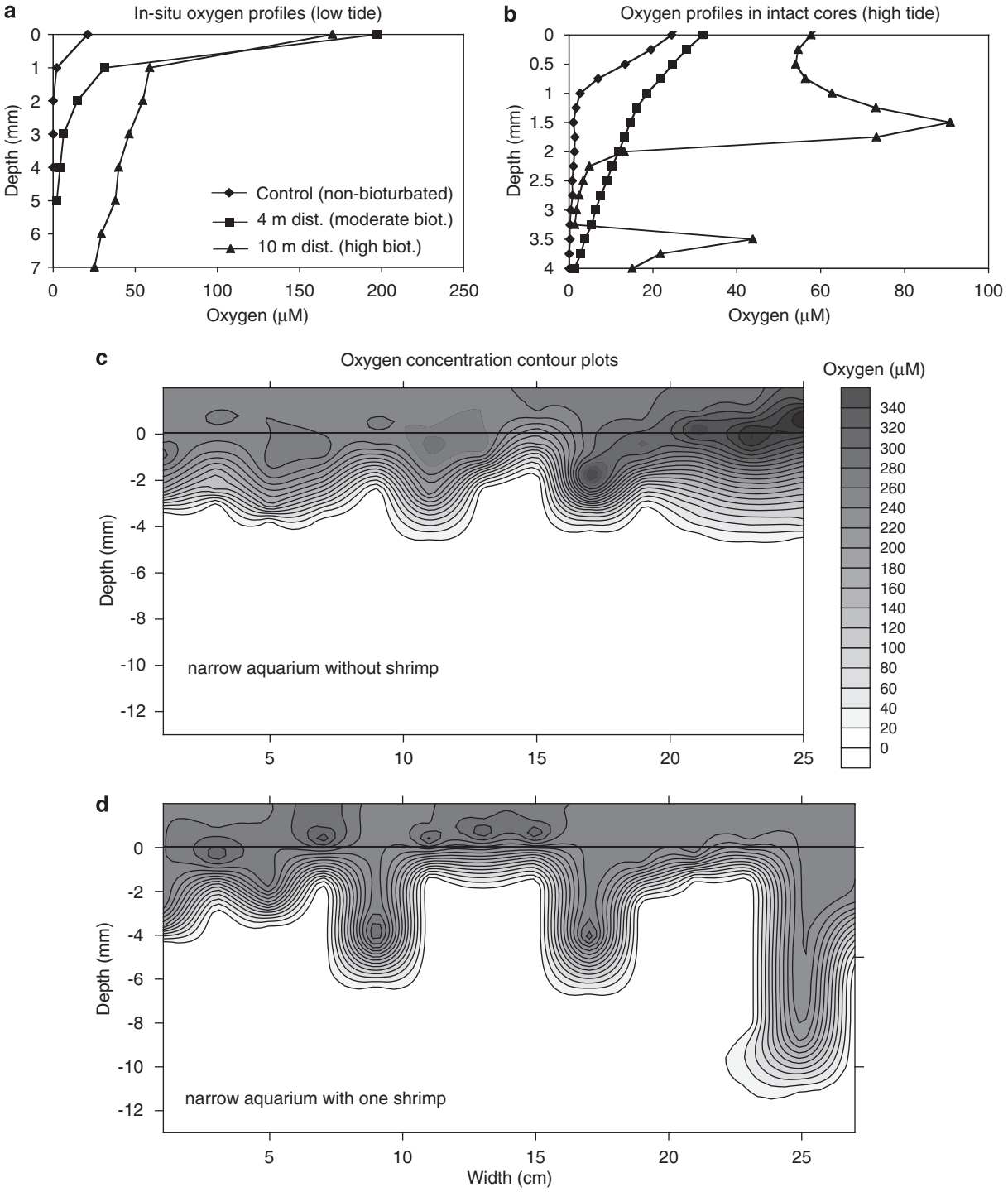

Figure 3 (**a** and **b**) Oxygen microprofiles were measured *in situ* at three locations in the field, in the non-bioturbated control area, at the 4 m site (moderate bioturbation) and at the 10 m site (high bioturbation). *In situ* microprofiles were measured during low tide (**a**) using a battery-powered, portable microsensor system. During high tide, cores were collected and profiles were performed in intact cores immediately after retrieval (**b**). (**c**, **d**) Contour plots of oxygen concentration are illustrated for microprofiles measured in a narrow aquarium without a burrowing shrimp (**c**) and in a second aquarium which contained one adult shrimp (**d**). The aquaria were kept in the laboratory for 6 weeks. At this time the shrimp had established a burrow system and oxygen profiles were measured in both systems in vertical profiles (250 μm depth intervals) in 2 cm horizontal increments along the width of the aquaria. The contour lines represent concentration intervals of 20 μM.

similar to the control site, with high concentrations occurring in the top 2 cm (Figure 5a). With increasing distance (6, 8 and 10 m) and increasing bioturbation activity, this sharp surface peak disappears and ferric iron is present in fairly high concentrations also at greater depths. A deeper reaching zone of

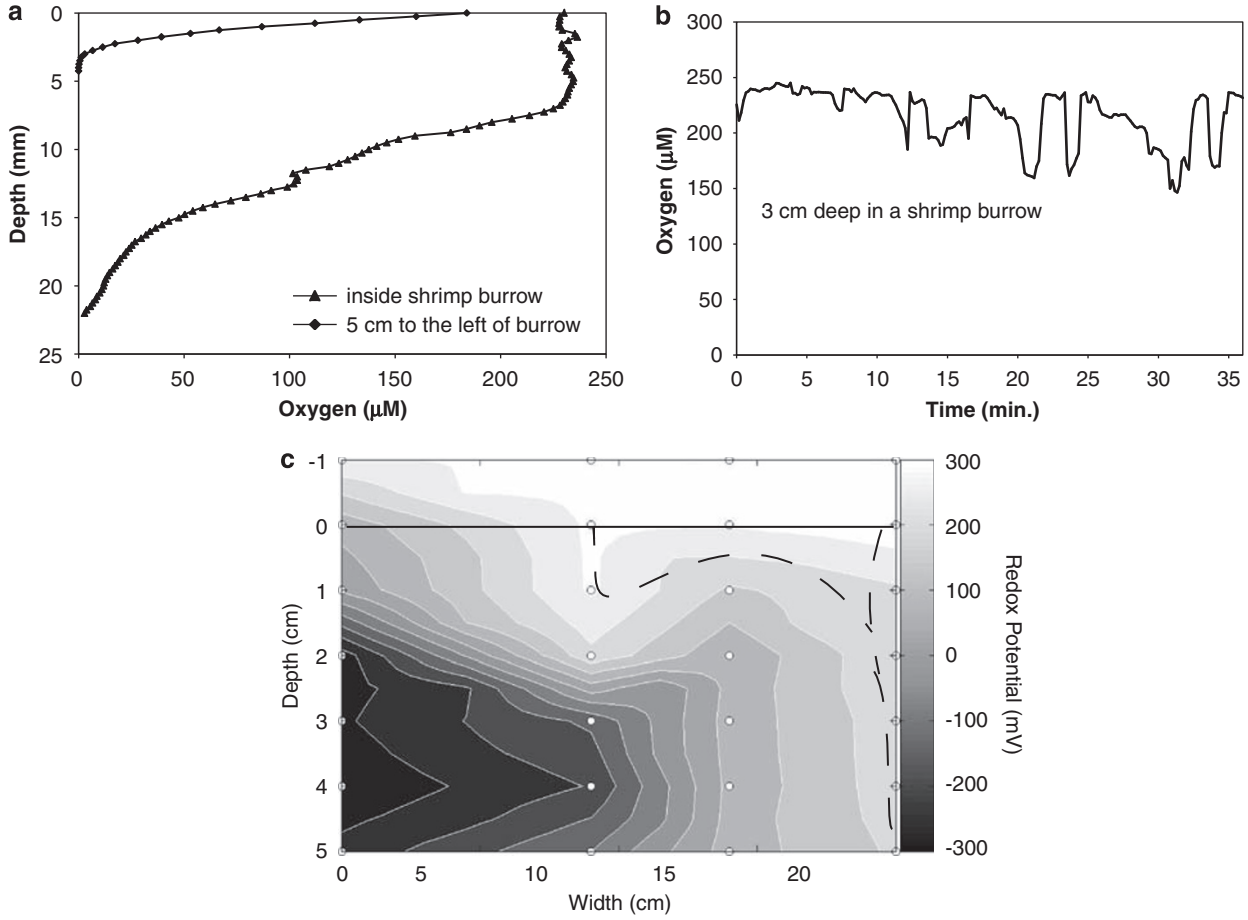

Figure 4 (**a**) Oxygen was measured directly in a burrow opening and compared with a profile in ambient sediment 5 cm to the left of the burrow. (**b**) The change of oxygen concentration over time was monitored in a shaft of a shrimp burrow at 3 cm depth. (**c**) A 2D contour plot of redox potential was created from profiles performed around a shrimp burrow. The dashed lines depict the position of the burrow. The contour lines represent increments of 50 mV.

oxidized iron is especially apparent at the 6 m site. In a similar pattern the subsurface (2–3 cm) peak in ferrous iron concentration, which is still evident at the 0–2 m sites, disappeared along the transect. The subsurface decrease in ferrous iron at the 6–10 m sites seemed to mirror the zones with an increase in ferric iron. Similarly a decrease of ammonium at the 6–8 m sites was reflected in an increase of subsurface nitrate concentrations, especially at 6 m distance. Oxidation effects are primarily evident at the 6–10 m sites, which were characterized by dense populations of mainly ghost shrimp (Figure 2a).

Microbiology

Cell counts. Cell numbers were comparably low at the control site (Figure 6a) and remained rather constant (1.5×10^9 cells cm^{-3} sediment) throughout the 10 cm. In contrast, microbial abundances were up to a magnitude higher in the bioturbated areas (Figures 6b and c), reaching highest values at 10 m distance (20×10^9 cells cm^{-3} sediment). At the 0–4 m sites the depth profiles of cell showed highest values toward the sediment surface and a decrease with depth. At the 6 m–10 m sites cell numbers were generally highest at the surface and decreased rapidly to a depth of 5 cm, except for the 6 m site, where cell numbers were highest at 3 cm depth. Abundances remained more or less constant below 5 cm depth at values higher than at the control site ($3–5 \times 10^9$ cells cm^{-3} sediment).

Clustering and similarity analysis of community fingerprints. Statistical analyses based on a Bray-Curtis similarity comparison of bacterial-community fingerprints as measured by ARISA in 1 cm, 2 cm and 8 cm sediment depths at all sites along the transect, showed a very distinct cluster of all surface microbial communities (Figure 7a). This cluster was distinct from the communities found at 2 cm and 8 cm, which showed a similarity of 40–65% to one another. A separate cluster for communities found at 2 cm and 8 cm depth showed a high similarity (>80%) between both depths. Highest similarity was found between samples collected in the bioturbated areas 2–10 m distance, whereas the communities at 0 m were less similar.

A comparison of the microbial communities sampled directly at the burrow walls of the two species *U. crenulata* and *N. californiensis* at the

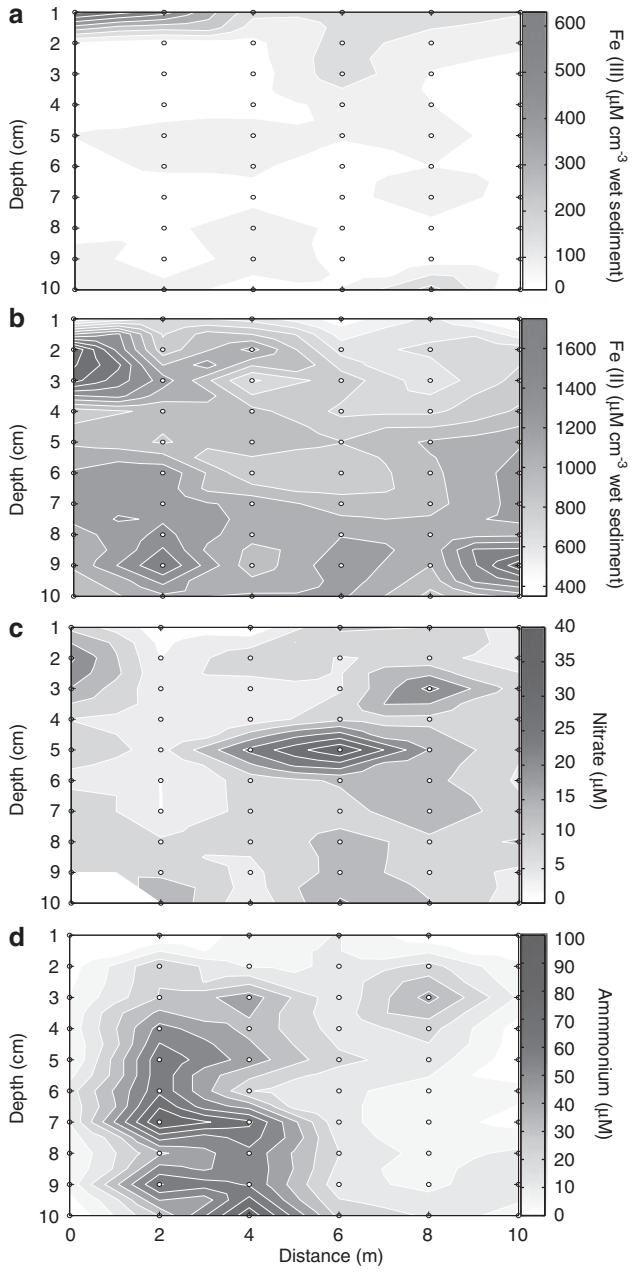

Figure 5 Ferric iron (**a**), ferrous iron (**b**), nitrate (**c**) and ammonium (**d**) concentrations were analyzed from sediment and pore-water samples collected at each site along the transect down to a depth of 10 cm and are displayed as 2D contour plots. Contour lines represent increments of 45 μM cm^{-3} wet sediment (ferric iron), 100 μM cm^{-3} wet sediment (ferrous iron), 5 μM (nitrate) and 10 μM (ammonium), respectively.

three depths (1 cm, 2 cm and 8 cm) showed separate clusters for the two burrow systems with less than 30% similarity between them (Figure 7b). The communities which formed at all three depths at the wall of the mud shrimp burrow showed highest similarity to the surface communities (Figure 8). In contrast, the communities in the crab burrow at all depths were more closely related to deeper sediment communities, although the communities at 1 cm and 2 cm depth appear to form a separate cluster.

A CCA biplot was used to compare the community fingerprinting data with environmental parameters. The CCA technique creates an ordination diagram where axes are created by a linear combination of environmental variables (ter Braak, 1986, 1995). The eigenvalue for each axis generated by CCA indicates how much of the variation seen in species data can be explained by that axis. In this case, Axis 1 explained 34.3% of the variance in species data (eigenvalue = 0.473) and Axis 3 explained 4.9% of the variance (eigenvalue = 0.067). Although Axis 2 explained 11.4% of the variance the *P*-value was >0.05 and so was not displayed. The right side of the ordination diagram is predominately occupied by surface samples with the exception of two samples taken at 2 cm depth at the 6 and 10 m sites. Whereas on the left side of the plot all remaining samples from depths 2 and 8 cm were located. Therefore, the CCA biplot supported the distinction between communities from the surface sediment and deeper sediment layers (Figure 9). The length of an environmental line (ferric iron, ferrous iron, nitrate, ammonium) represents the extent of species distributions change along that environmental variable. The orientation of the line represents the gradient of the environmental variable. The closer an environmental arrow is to an axis, the stronger is the correlation between that variable and the axis. There was a strong positive correlation of Fe (III) with Axis 1 ($r = 0.899$), where the majority of surface samples were found. The plot also indicated a strong negative correlation of Fe (II) and ammonium with Axis 1 ($r = -0.764$ and $r = -0.580$, respectively) into the direction of the deeper samples. Nitrate correlated strongest with Axis 3 but also had a stronger correlation with Axis 1 ($r = 0.292$) than all other environmental variables, which might be an indication that different environmental parameters contribute to the effect of nitrate on local microbial communities.

Discussion

Bioturbation activity and microbial communities

Despite the regional and global importance of macrofauna sediment reworking, reasonable estimates of bioturbation exist only for a limited set of conditions and regions of the World (Henderson *et al.*, 1999; Teal *et al.*, 2008). Although it has been known that burrowing organisms affect sediment biogeochemistry, the interactions are often complex and detailed investigations on the effect of geochemical microzonations on microbial diversity are scarce (Kostka *et al.*, 2002; Lucas *et al.*, 2003; Matsui *et al.*, 2004; Papaspyrou *et al.*, 2006). This is not surprising because studies on microbial diversity in marine sediments have also only just begun (Mußmann *et al.*, 2005; Wilms *et al.*, 2006; Wu *et al.*, 2008). The impact of bioturbation is species-specific and activity dependent (for example,

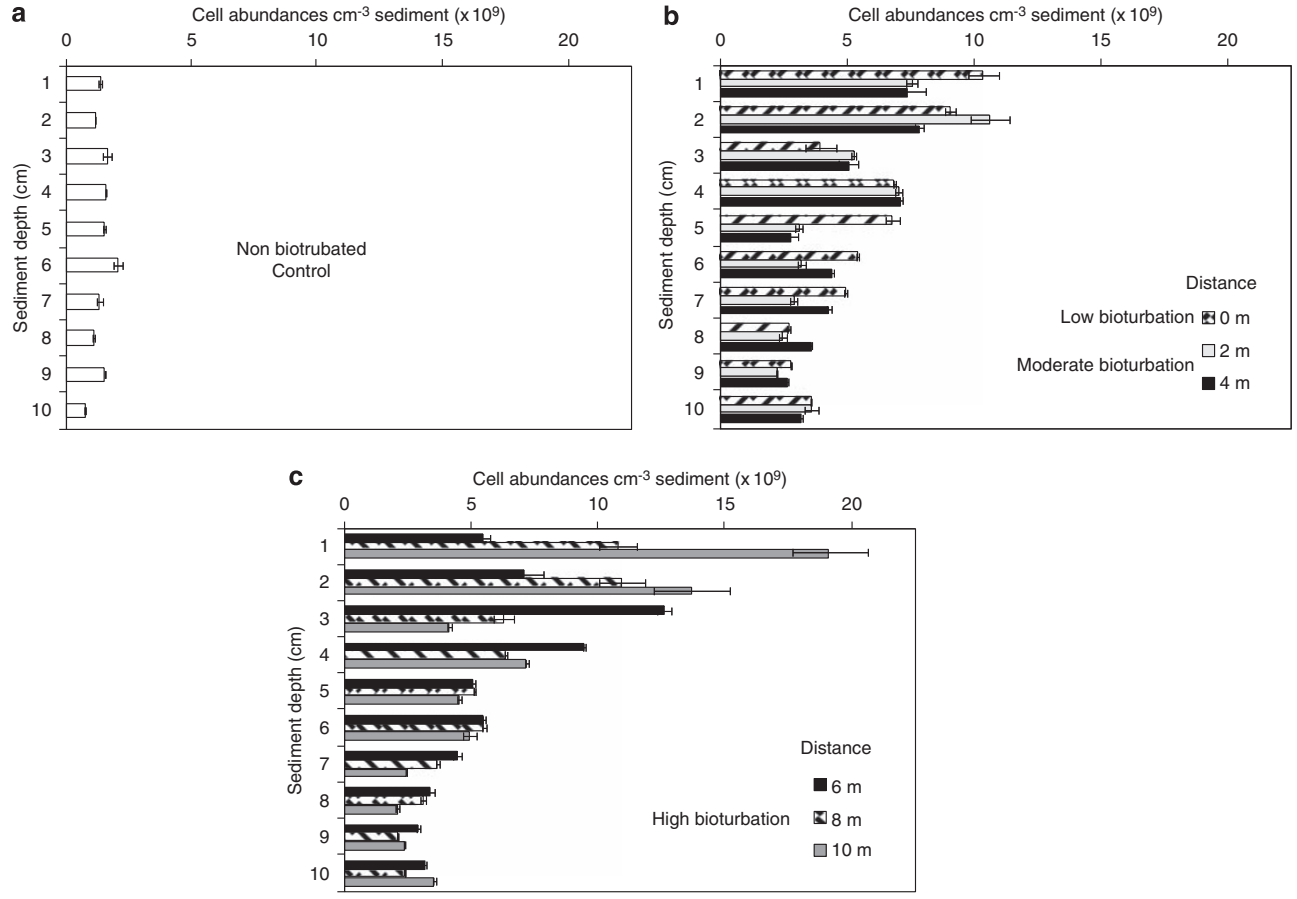

Figure 6 Cell counts (Acridine Orange Direct Counts) were performed for each site down to a depth of 10 cm in 1-cm vertical resolution. Cell abundances per cm^3 of sediment were compared for the non-bioturbated control site (**a**), the sites characterized by low to moderate bioturbation (0 m, 2 m and 4 m) (**b**) and the highly bioturbated sites (6 m, 8 m and 10 m) (**c**). Triplicate filters were prepared and counted for each sample and error bars indicate the s.d. for each sample.

Pelegrí and Blackburn, 1996; Christensen *et al.*, 2000; Marinelli *et al.*, 2002; Solan and Kennedy, 2002; Mermillod-Blondin *et al.*, 2004), therefore microbial communities associated with specific burrow structures most likely also show variability.

Our observations demonstrate that the two species of sediment dwelling crustaceans in the shallow lagoon of Catalina Harbor exhibit different bioturbation activities, which in turn have contrasting effects on the subsurface microbial communities associated with the burrows. Although the ghost shrimp lives permanently subterranean, is constantly reworking its burrow and flushing it, to maintain a certain oxygen level within the burrow; the fiddler crab builds a simple burrow in which it resides only occasionally and does not actively ventilate it. Oxygen transport into the shrimp burrow as well as into the surrounding sediment led to oxidation effects, which were documented also in the field studies by a decrease of reduced compounds and extension of oxidized conditions deeper into the sediment (Figure 5).

A key question in benthic microbial ecology has been whether distinct microhabitats harbor distinct microbial assemblages. This issue has been much discussed in microbial ecology and in the emerging field of microbial biogeography. Do habitats with similar environmental conditions promote similar microbial communities? It has been suggested (Kristensen and Kostka, 2005), that even if geochemical conditions in burrows are equivalent to the sediment surface, the microbial communities in and around burrow walls are most likely unique. This hypothesis has been explained by the great temporal variability of environmental conditions inside the burrows, but a greater physical stability of the burrow itself compared with a frequently disturbed sediment surface.

On the basis of our investigations, we suggest that the geochemical conditions at and around the burrow walls of *N. californiensis* burrows were very similar to the sediment surface (Figures 3d and 4c). Our community fingerprinting results (Figure 8) indicate that the oxic and oxidized zones associated with the shrimp burrow support microbial communities that are very similar to those found in the top 1 cm of the sediment (similarity of ~55–70%). At all sample depths (1 cm, 2 cm and 8 cm) inside the burrows, the ARISA fingerprints clustered within the surface communities found at all sites and are

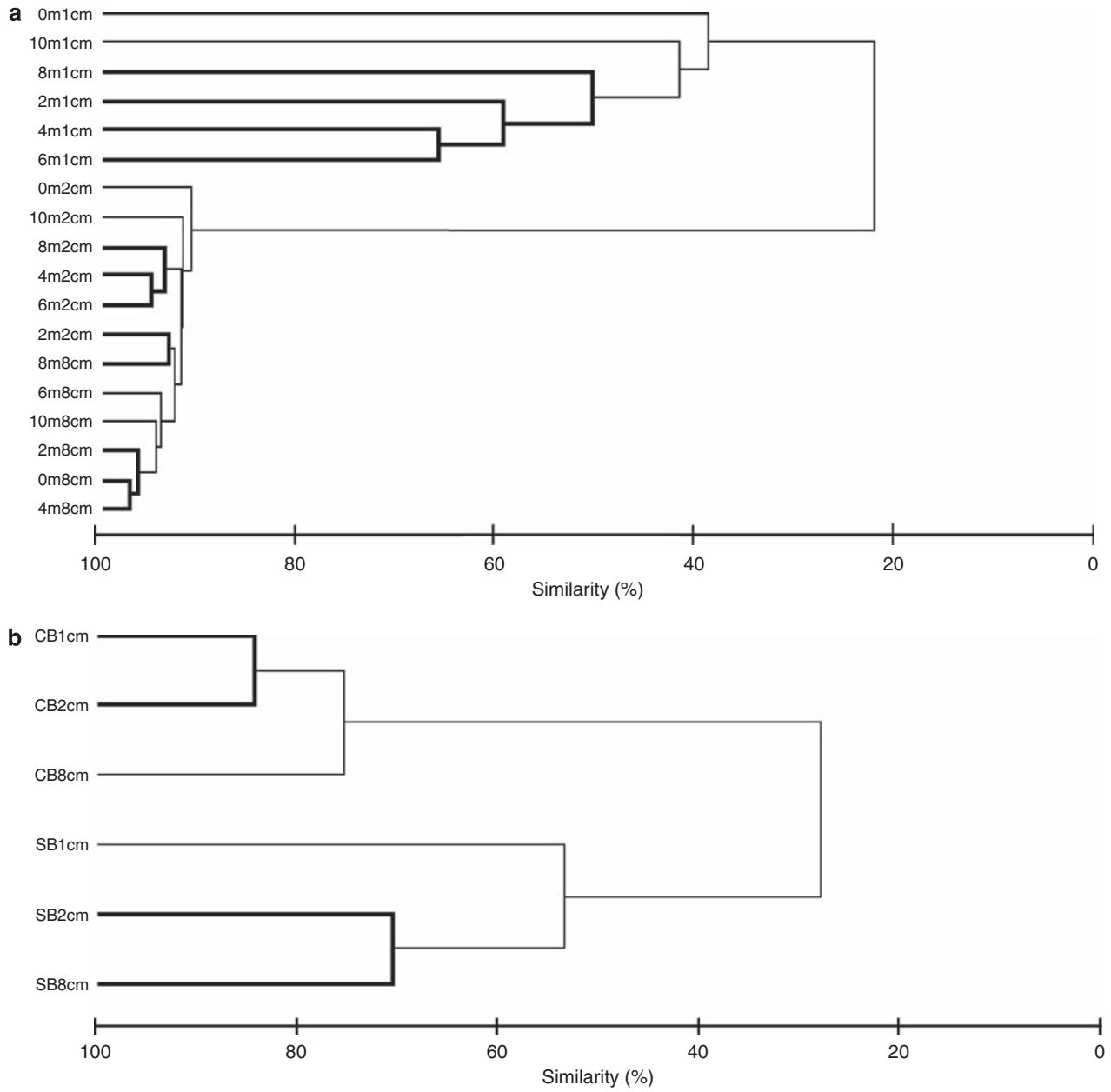

Figure 7 Microbial diversity was determined for sediment depths 0–1 cm, 1–2 cm and 7–8 cm from each bioturbated site along the transect (**a**), as well as for samples directly from the wall lining of *U. crenulata* and *N. californiensis* burrows at these same three depths (**b**). A cluster analysis was performed for all of these communities using the Bray-Curtis similarity index after log $(x+1)$ transformation, and is represented as a dendrogram. Bold lines highlight sample clusters that are not statistically different. The samples are named according to the distance in m (x) and the depth in cm (y) where they were taken (xmycm). CB stands for 'Crab Burrow' and SB stands for 'Shrimp Burrow' and the number indicates the depth in cm.

very different from communities deeper in the sediment.

In contrast to the constantly bio-irrigating ghost shrimp, fiddler crabs may be called 'temporary' bioturbators. They establish a burrow but do not live below the surface. In the field, they are most often seen at the sediment surface or guarding the entrance of their burrow and do not seem to spend long periods within the burrows. We were not successful in measuring oxygen in any of the crab burrows and assume that they are not actively ventilating their burrows. The communities found within the crab burrows differed from the ones in the shrimp burrows (Figure 7b), showing more similarity to subsurface communities (Figure 8). At 8 cm depth the crab burrow communities did not differ significantly from any of the communities found at the same depth from all other sites. Interestingly, the crab burrow communities at 1 cm and 2 cm depth seemed to form a unique cluster, although showing closer similarity to subsurface than surface communities. This might support the

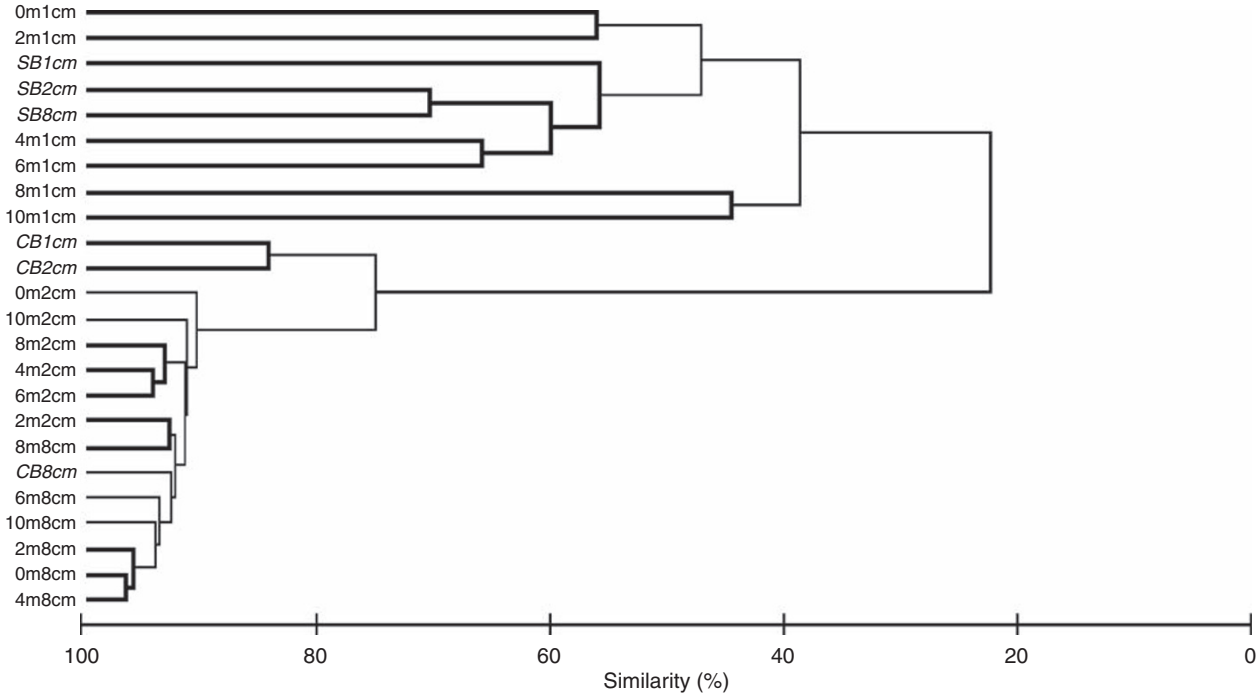

Figure 8 The Bray-Curtis dendrogram combines the similarity data shown in Figure 7 for both sediment and burrow wall samples. Samples clustered with bold lines are not statistically different.

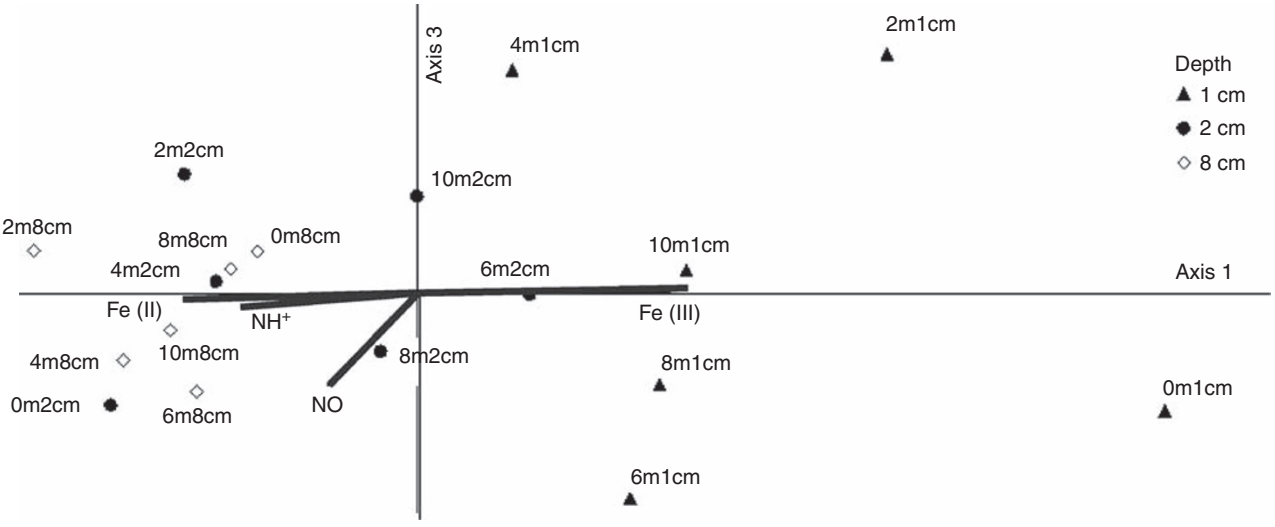

Figure 9 An ordination diagram displaying the first and third axis of a canonical correspondence analysis was created using those locations with both species (ARISA) and environmental (NO_3^{2-}, NH_4^+, Fe (II) and Fe (III)) data. Samples are grouped according to their depth (1 cm, 2 cm and 8 cm) and displayed with different symbols as indicated in the legend.

hypothesis by Kristensen and Kostka (2005), who suggested that burrows support unique communities. Burrowing behavior is species-specific and to understand the effect of bioturbation on microbial communities one has to take into account the ecology of the macrofauna organisms.

Papaspyrou *et al.* (2005) studied the bacterial communities in burrows of the ghost shrimp *Pestarella tyrrhena* (Decapoda: Thalassinidea). They found a 10-fold increase of bacterial abundance associated with the burrow wall, which they attributed to the higher organic content compared with the surrounding sediment. They did not measure oxygen at the burrow wall or the surrounding sediment. Their study by molecular fingerprints (DGGE) of the bacterial communities suggested that the bacterial composition of the burrow wall was more similar to the ambient anoxic sediment. The

authors therefore suggested that burrow walls have distinct properties and should not be considered merely as a simple extension of the sediment surface. Organic content and the quality thereof is certainly a structuring component for microbial communities. Oxygen is one of the strongest parameters affecting microbial communities in sediments, as it is the most favorable electron acceptor in the oxidation of carbon. It divides the communities into assemblages that vary in their oxygen requirements and tolerances (aerobes, anaerobes). The question is what are the key parameters structuring microbial communities. Another question is how to define 'similar'. In our example, we define the similarity between burrow walls and their surrounding as the similarity in the oxic and oxidized conditions. We do not dispute that burrow walls may represent unique environments. It depends on a combination of factors and may vary among different burrow types, depths and compartments as well as oxidation events. Our study suggests that burrows can support similar microbial populations compared with the surface if the environmental parameters, that is, redox conditions are equal.

The argument that free-living microbes are ubiquitous (Finlay, 2002) and environmental parameters determine their presence has been suggested by the 'Baas-Becking' hypothesis (Baas-Becking, 1934: 'everything is everywhere and the environment selects'). This assumption has been controversial for much of the last century (that is, reviewed in Hedlund and Staley, 2003; Ge *et al.*, 2008) and recent studies have shown that the richness or diversity of microorganisms might be more complex (McArthur *et al.*, 1988; Horner-Devine *et al.*, 2004). Salinity, temperature or nutrient gradients have been identified to change aquatic microbial communities (Ward *et al.*, 1998; Crump *et al.*, 2004) and a more recent hypothesis has been that microbial composition and diversity patterns reflect the influences of both past events and contemporary environmental variations (Martiny *et al.*, 2006; Ge *et al.*, 2008). Hewson and Fuhrman (2004) found by comparing the diversity and richness of bacterio-plankton along a salinity gradient, that some taxa (or OTUs) are specific to distinct environments whereas others have a ubiquitous distribution from river to sea. So both might be true, there are microorganisms that are everywhere and others which are endemic. A lot more work needs to be done to address this.

Few studies on microbial diversity have been performed in benthic systems (Llobet-Brossa *et al.*, 1998; Köpke *et al.*, 2005; Córdova-Kreylos *et al.*, 2006; Wilms *et al.*, 2006; Hewson *et al.*, 2007; Liang *et al.*, 2007) and studies on the effect of bioturbation on microbial community structure remain extremely scarce (Plante and Wilde, 2004; Papaspyrou *et al.*, 2005, 2006). Environmental parameters vary quickly, especially in coastal sediments. To adequately address the factors controlling biodiversity in sediments, the environmental parameters have to be measured at a resolution that accounts for this variability, which is unfortunately not always possible. Some laboratory and field studies (Korona *et al.*, 1994; Haubold and Rainey, 1996; Rainey and Travisano, 1998; Zhou *et al.*, 2002; Treves *et al.*, 2003; Horner-Devine *et al.*, 2004) suggested that environmental 'patchiness' played a role in the maintenance of the microbial diversity in soils and sediments. Patchiness comprises the complexity of environmental conditions that can vary along multiple spatial and temporal scales. Our examples show that the stability of environmental conditions over time supports the establishment of specific microbial assemblages. One remaining question is how quickly these communities develop as a result of varying redox conditions.

There are a number of different approaches to study the types of microbes present in an environment and their abundance. We used ARISA (Fisher and Triplett, 1999; Hewson and Fuhrman, 2004; Brown *et al.*, 2005; Fuhrman, 2008) as a PCR-based community finger-printing method to characterize and compare the richness of different taxa (OTU's). This method results in data of discrete numbers, such as the total fluorescence of a specific fragment length, thus permitting statistical comparisons of species (OTU) richness. ARISA is a relatively inexpensive and fast way to compare bacterial community structures by analyzing the lengths of the intergenic spacers between 16S and 23S rRNA genes present in almost all bacteria. Therefore, archaea are not considered in this approach and some groups like the *Planktomycetes* might not be included in the analyses, if they lack linked 16S and 23S genes (Fuhrman, 2008). Nevertheless, this assemblage fingerprinting approach allows comparison of microbial richness along environmental gradients with a phylogenetic resolution, that is within 98% of 16S rRNA sequence identity, near the range widely considered to be bacterial 'species' or 'ecotypes' (Fuhrman, 2008).

Effect of bioturbation on biogeochemical cycles

The combined effect of the bioturbation activity of the two investigated crustaceans on the biogeochemistry in the shallow lagoon of Catalina Harbor showed an increase of microbial abundances (Figures 6a–c) at all sediment depths as bioturbation intensity increased (Factor 10 or higher). The overall increase of oxidized chemical compounds (ferric iron and nitrate, Figures 4a and c) reflects a significant increase of oxidation-reduction potential within the sediment. The statistical analyses (Figure 9) suggested that the availability of ferric iron correlated with all surface (1 cm) microbial fingerprints. Whereas the abundance of reduced nitrogen and iron species correlated with assemblages found deeper in the sediment. More investi-

gations are needed to correlate environmental parameters with microbial diversity and activity. Another important next step in our investigations will be to determine the metabolic activities associated with distinct microniches. Microbial processes enhanced or induced by bioturbation activity may significantly contribute to element cycling at the seafloor.

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