



FEATURE ARTICLE

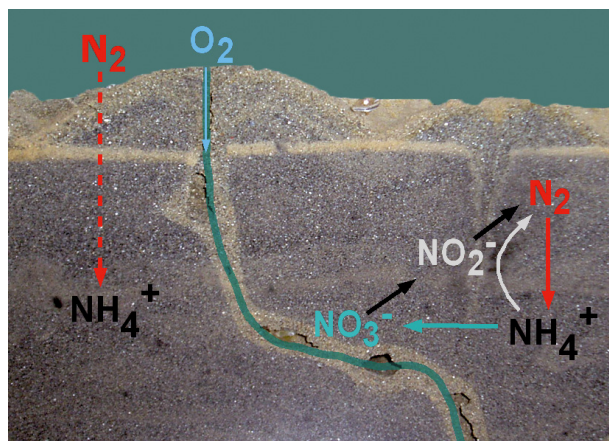
# Burrowing deeper into benthic nitrogen cycling: the impact of bioturbation on nitrogen fixation coupled to sulfate reduction

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**ABSTRACT:** Biological dinitrogen ( $N_2$ ) fixation is the primary input of fixed nitrogen (N) into the marine biosphere, making it an essential process contributing to the biological functions of all organisms. Because biologically available N often limits marine productivity, microbial processes leading to its loss and gain (e.g. denitrification and  $N_2$  fixation, respectively) play an important role in global biogeochemical cycles. Bioturbation is known to influence benthic N cycling, most often reported as enhancement of denitrification and a subsequent loss of  $N_2$  from the system.  $N_2$  fixation has rarely been addressed in bioturbation studies. Instead, sedimentary  $N_2$  fixation typically has been considered important in relatively rare, localized habitats such as rhizosphere and phototrophic microbial mat environments. However, the potential for  $N_2$  fixation in marine sediments may be more widespread. We show here that nitrogenase activity can be very high (up to  $5 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ h}^{-1}$ ) in coastal sediments bioturbated by the ghost shrimp *Neotrypaea californiensis* and at depths below 5 cm. Integrated subsurface  $N_2$ -fixation rates were greater than those previously found for un-vegetated estuarine sediments and were comparable to rates from photosynthetic microbial mats and rhizospheres. Inhibition experiments and genetic analysis showed that this activity was mainly linked to sulfate reduction. Sulfate-reducing bacteria (SRB) are widespread and abundant in marine sediments, with many possessing the genetic capacity to fix  $N_2$ . Our results show that  $N_2$  fixation by SRB in bioturbated sediments may be an important process leading to new N input into marine sediments. Given the ubiquity of bioturbation and of SRB in marine sediments, this overlooked benthic  $N_2$  fixation may play an important role in marine N and carbon (C) cycles.



Burrowing shrimp introduce oxygen deep into their burrows, oxidizing the surrounding sediment, where  $N_2$ -fixation leads to the production of new bioavailable nitrogen.

Image: W. Ziebis and V. J. Bertics

**KEY WORDS:** Bioturbation · Crustaceans · Marine sediment · Microniche · Nitrogen fixation · Sulfate reduction

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## INTRODUCTION

Temperate coastal and estuarine sediments are generally considered to be areas of net nitrogen (N) loss through the consumption of fixed N by denitrification and the efflux of dinitrogen gas ( $N_2$ ) (Christensen et al.

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1987, Gilbert et al. 1995, 1997, 1998, Hulth et al. 2005).  $N_2$  fixation is rarely considered in N budgets of these systems despite numerous studies of benthic  $N_2$  fixation in other habitats that have focused on photosynthetic microbial mats or sediments vegetated by seagrasses and marsh plants (Capone 1983, 1988, Herbert 1999, Carpenter & Capone 2008). These studies demonstrate that not all benthic systems are exclusively areas of denitrification, meaning that in some areas  $N_2$  fixation is also occurring. Whether the N fixed through  $N_2$  fixation is enough to replace the N lost through denitrification is still unknown and mostly likely habitat-dependent. Additionally, little attention has been given to deeper sediment layers, perhaps because of generally increasing concentrations of ammonium ( $NH_4^+$ ), a known inhibitor of  $N_2$  fixation (Yoch & Whiting 1986), with depth. However,  $N_2$  fixation has been shown to occur in sediments even at elevated  $NH_4^+$  concentrations both in the field and experimental  $NH_4^+$  additions (up to 700  $\mu M$ ) (McGlathery et al. 1998).

Many coastal sediments have a high abundance of burrowing infauna (Teal et al. 2008) with bioturbation (movement of particles) and bioirrigation (movement of fluids) having direct consequences on organic matter degradation, biogeochemical processes, and nutrient cycling (Aller 1982, Aller & Aller 1986, Kristensen 2000). Still, macrofauna burrows have rarely been studied as sites for  $N_2$  fixation because of the general view that bioirrigation leads to the transport and presence of high levels of oxygen, an inhibitor of the nitrogenase protein (Stewart 1969, Postgate 1998). However, because oxygen transport across burrow walls is limited by molecular diffusion and because oxygen is rapidly consumed, oxygen penetration into the surrounding sediment is only a few millimeters (Ziebis et al. 1996, Zorn et al. 2006). Beyond these thin oxic zones, sedimentary materials may be oxidized to a distance of several centimeters, reflecting a positive redox potential and the availability of other electron acceptors (e.g. nitrate and ferric iron). Nitrification may decrease  $NH_4^+$  concentrations and denitrification may occur just beyond the depth of  $O_2$  penetration (Canfield 1993). These 2 processes involving N have been shown to occur in bioturbated sediments (Binnerup et al. 1992, Gilbert et al. 1995), resulting in a loss of  $N_2$  from the benthic system. The anaerobic oxidation of ammonium with nitrate (anammox) (Thamdrup & Dalsgaard 2002, Dalsgaard et al. 2005) may represent another pathway that contributes to benthic  $N_2$  production. Combining the possible effects of bioturbation on geochemical zonation, lowered  $NH_4^+$  concentrations, denitrification, and loss of  $N_2$  via anammox, there may exist widespread subsurface niches where N is locally limited and that are favorable for  $N_2$  fixation. Alternatively, diazotrophs may continue to fix  $N_2$  in the

presence of fixed N (e.g. Holl & Montoya 2005), the reasons for which are not understood.

Availability of organic matter may also be enhanced due to burrowing activity (Aller 1982, Aller & Aller 1986). The localized availability of organic matter can create 'hot spots' of elevated microbial activity, often causing bioturbated sediments to be characterized by a heterogeneous, 3-dimensional geochemical zonation pattern (Rhoads 1974, Aller 1982, 2001, Kristensen 2000). One such activity that can be enhanced within these 'hot spots' is sulfate reduction (Goldhaber et al. 1977). Sulfate-reducing bacteria (SRB) are widespread in marine sediments and can be responsible for  $\geq 50\%$  of organic carbon remineralization, with especially high rates of remineralization in organic rich coastal sediments (Jørgensen 1982, Canfield 1993, Thamdrup & Canfield 1996). Because many SRB have the genetic ability to fix  $N_2$  (Zehr et al. 1995) and have been shown to fix  $N_2$  in other benthic environments (Nielsen et al. 2001, Steppe & Paerl 2002), subsurface 'hot spots' associated with macrofaunal burrows may provide ideal conditions for  $N_2$  fixation to occur.

The bay ghost shrimp *Neotrypaea californiensis* Dana, 1854 (Crustacea: Decapoda: Thalassinidea, see Fig. 1), previously known as *Callinassa californiensis* (Manning & Felder 1991), is an abundant burrowing crustacean found in intertidal areas along the western coast of North America from Alaska to Baja California (MacGinitie 1934). *N. californiensis* belongs to a cosmopolitan group of decapods, Thalassinideans, known to maintain deep-reaching burrows (some species up to 3 m deep; Pemberton et al. 1976) that significantly impact geochemical gradients and influence biogeochemical processes worldwide (Ziebis et al. 1996, Dworschak 2000). This bay ghost shrimp spends the majority of its time subsurface, constructing a burrow (up to ~80 cm deep) that is highly branching, often with several openings to the surface (MacGinitie 1934, Swinbanks & Murray 1981). *N. californiensis* is known to constantly rework its burrow, adding new tunnels, extending burrow depth, or closing off old chambers, all while simultaneously deposit-feeding and actively irrigating its burrow (MacGinitie 1934, Brenchley 1981). *N. californiensis* feeds on detritus that is transported with the ventilation current, as well as removing material from the burrow wall, which is often cemented by mucus that provides a rich substrate for microbial growth ('wall grazing') (MacGinitie 1934, Griffis & Suchanek 1991). Additionally, it has been suggested that deposit-feeding thalassinidean shrimp store organic material (e.g. seagrass fragments and other suspended particles) in burrow chambers (Griffis & Chavez 1988) and that they later graze on the microbes growing on this material, a feeding mode that was termed 'gardening' (Griffis & Suchanek 1991).

These foraging strategies suggest an enhancement of organic matter availability in *N. californiensis*-inhabited areas that could possibly support elevated levels of linked  $N_2$  fixation and sulfate reduction. To test the hypothesis that  $N_2$  fixation, possibly linked to sulfate reduction, occurs in and around ghost shrimp burrow systems, we carried out experiments in Catalina Harbor, California, a shallow intertidal lagoon previously shown to be intensely bioturbated by *N. californiensis* (Bertics & Ziebis 2009).

## MATERIALS AND METHODS

**Field site.** Investigations were carried out in an intertidal lagoon located in Catalina Harbor, Catalina Island, California (33° 25.23' N, 118° 19.42' W) from June to October 2007 and revisited in May 2008. The lagoon is a shallow (<2 m), low-energy area consisting of muddy sand (majority of grains being <500  $\mu\text{m}$ ). Tides at this location were mixed, with the higher high water preceding the lower low water, and had a range of ~1.7 m (Colbert et al. 2008). At each sampling time, the water temperature was typically 18 to 20°C and salinity was 34.5‰. Additionally, because summer and fall months are generally considered dry in Southern California, and were so during our investigations, freshwater input was negligible.

At this site, *Neotrypaea californiensis* burrows reach ~20 cm deep into the sediment (Bertics & Ziebis 2009). Additionally, each burrow has multiple branches and typically 3 to 4 openings to the sediment surface. The burrows consist of shafts (~1 cm diameter) and chambers (~2 cm diameter) that the shrimp maintains and frequently flushes with oxygen-rich water. Different microniches associated with the burrow system are evident from the coloration of the sediment (Fig. 1). Burrow walls are generally light-colored in contrast to the surrounding sediment, reflecting, for example, the presence of oxidized forms of iron. Dark microniches, indicative of reduced conditions and the presence of iron sulfides, occur where burrows have been abandoned or where organic material might have been stored (Fig. 1).

Three intertidal sampling areas in Catalina Harbor were chosen for detailed investigation based on differing levels of ghost shrimp burrow density (number of burrow openings  $\text{m}^{-2}$ ). It has been previously shown that ghost shrimp burrow density in this Catalina Harbor lagoon increases with distance from shore (Bertics & Ziebis 2009). Therefore, we selected an area close to shore (i.e. below the high-water mark), another area ~2 m offshore, and another area ~10 m offshore. The burrow density at each sampling location was determined by counting the number of burrow openings

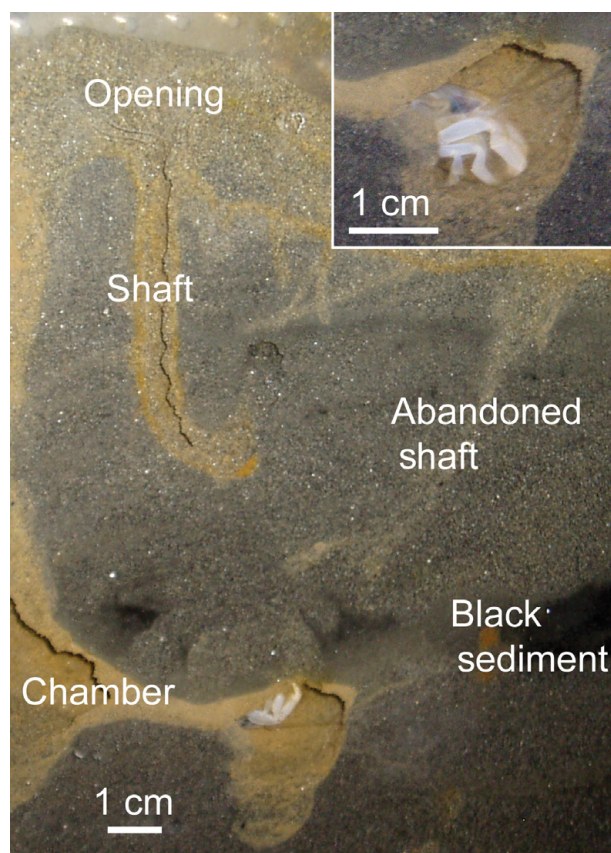


Fig. 1. *Neotrypaea californiensis* burrow system. A bay ghost shrimp can be seen in its burrow (upper right corner), with an oxidized, light-colored region adjacent to the burrow chamber. Specific burrow areas of interest are labeled in the main photograph

within a 25 × 25 cm frame with 10 replicates counted. In 2007, each of these distinct areas differed greatly in their *Neotrypaea californiensis* bioturbation activities, making our 3 sampling locations a non-bioturbated zone, a zone with medium bioturbation intensity, and a highly bioturbated zone, with 0, ~600 (581 ± 61.5), and ~1800 (1792 ± 33.3) burrow openings  $\text{m}^{-2}$  respectively (± SE). For ease of reading, these locations will further be termed 0(07)-BOM (where 07 means 2007), 600-BOM, and 1800-BOM respectively (BOM standing for burrow openings  $\text{m}^{-2}$ ). It should be noted that the non-bioturbated area, while lacking macrofauna bioturbation, contains more organic carbon input from a surrounding wet land and coarser sediment grains that could allow tidal movements and wave action during rough weather to influence some transport within these sediments. During additional investigations in 2008, the lagoon was less bioturbated than the previous year, with 0, ~120 (122.7 ± 4.8) and ~320 (324 ± 6.1) burrow openings  $\text{m}^{-2}$  respectively at the same 3 locations. These locations will further be termed 0(08)-

BOM (where 08 means 2008), 120-BOM, and 320-BOM respectively. The *N. californiensis*-bioturbated areas also had a very visible photosynthetic microbial mat at the surface in 2007, while in 2008 the mat was present but much less developed.

**Sampling and geochemical analyses.** Four parallel sediment push cores ( $\varnothing$  diam. 5.4 cm and length 30 cm) were collected during high tide from each sampling location, both in 2007 and 2008 (Fig. 2). Each core was collected randomly with no specific orientation to *Neotrypaea californiensis* burrow openings so as to avoid bias; however, each core was large enough that at least 1 burrow opening was present in each of the cores collected from the bioturbated areas. Two cores were sliced in 1 cm intervals under  $N_2$  atmosphere within a portable glove bag and each section was subsampled for further geochemical processing as follows. Pore water was collected from each interval of the first core by centrifugation (10 min at  $5000 \times g$ ) using 50 ml Macrosep<sup>®</sup> Centrifugal Devices (Pall Corporation, Life Sciences). The recovered pore water (~3 ml) was immediately frozen at  $-20^\circ\text{C}$  for later determination of  $\text{NH}_4^+$  by flow injection analysis modified for small sample volumes (Hall & Aller 1992). On the second core, porosity was determined by drying a known volume of sediment at  $65^\circ\text{C}$  for 24 h. Dried samples were combusted at  $450^\circ\text{C}$  for 24 h and total organic carbon (TOC) was calculated as the loss on ignition (LOI). Additionally, in 2007, a subsample of 2  $\text{cm}^3$  for each depth interval was frozen at  $-80^\circ\text{C}$  for a subsequent *nifH* gene survey.

**Catalina Harbor nitrogenase activity (NA).** The third sediment core from each location was sliced in 1 cm intervals and analyzed for NA using the acetylene reduction assay (Capone 1993). Acetylene ( $\text{C}_2\text{H}_2$ ) is a substrate analog of  $\text{N}_2$  gas and is preferentially reduced by the conventional (Mo-based) nitrogenase enzyme to ethylene ( $\text{C}_2\text{H}_4$ ), which is easily quantified by gas chromatography. Nitrogenase has a natural side reaction of  $\text{H}_2$  production in a 1:1 stoichiometry with  $\text{N}_2$  reduction.  $\text{H}_2$  production, which results in the futile

consumption of reducing equivalents, does not occur in the presence of  $\text{C}_2\text{H}_2$  with all reducing equivalents shunted to  $\text{C}_2\text{H}_2$  reduction. Hence, the theoretical ratio of  $\text{C}_2\text{H}_2$  reduction to  $\text{N}_2$  fixation is 4:1 (Postgate 1998). Different diazotrophic systems have mechanisms to minimize  $\text{H}_2$  loss, resulting in deviations from the theoretical 4:1 ratio. For instance, the marine cyanobacteria *Trichodesmium* spp. exhibit ratios closer to 3:1 as determined by direct  $^{15}\text{N}_2$  comparisons (Orcutt et al. 2001, Capone et al. 2005) and with particulate organic nitrogen (PON) accumulation in cultures of *Trichodesmium* spp. (Mulholland & Capone 2001). Calibration of the  $\text{C}_2\text{H}_2$  reduction method in organic rich sediments is problematic because of the high detrital N content (see Capone 1988) and has been undertaken with conflicting results (see Jones 1974 versus Seitzinger & Garber 1987).  $\text{C}_2\text{H}_2$  has been found to inhibit a number of physiological groups including nitrifiers, methylotrophs, and methanogens and the method should be applied with caution in environments where those groups are important (Oremland & Capone 1988).

In the present study, duplicate 6  $\text{cm}^3$  (2007) or triplicate 7.6  $\text{cm}^3$  (2008) sediment samples from each depth horizon were placed in 74 ml (2007) or 35 ml (2008) serum vials flushed with  $\text{N}_2$ . Each vial was injected with 5 ml (2007) or 3 ml (2008) of  $\text{C}_2\text{H}_2$  to saturate the nitrogenase enzyme and the increase in  $\text{C}_2\text{H}_4$  was assayed over a 48 h period, while NA was linear, on a gas chromatograph with a flame ionization detector. Over this time period, a total of 7 time points were taken, including sampling at time zero. At each time point, the headspace of each vial was sampled and directly injected into the gas chromatograph. Incubations were kept in the dark and at *in situ* temperature ( $20^\circ\text{C}$ ).  $\text{N}_2$ -fixation rates were calculated from the NA results using a conversion factor of 3  $\text{C}_2\text{H}_4$ : 1  $\text{N}_2$ . To compare NA across the same depth at the 3 locations, a 1-way ANOVA was performed using PASW Statistics 18 (SPSS), along with a Tukey post hoc test to determine which of the means were significantly different.

To determine the effect of light on NA in 2007, duplicate 1  $\text{cm}^3$  sediment samples from the surface of all 3 sampling locations were collected using 5 ml syringes with the tip cut off. Each sample was placed into a 74 ml serum vial (not flushed with  $\text{N}_2$ ) and injected with 5 ml of  $\text{C}_2\text{H}_2$ . Samples were kept in the sunlight during the day and were also kept on a water table so that the vials were kept at a constant temperature. Statistical comparisons between light and dark samples from the same sampling location were performed using a 1-tailed *t*-test.

**Inhibition experiment.** Inhibition experiments were carried out on the fourth core using 20 mM sodium molybdate ( $\text{Na}_2\text{MoO}_4$ ), a known sulfate reduction inhibitor (Oremland & Capone 1988).  $\text{MoO}_4^{2-}$  inhibits

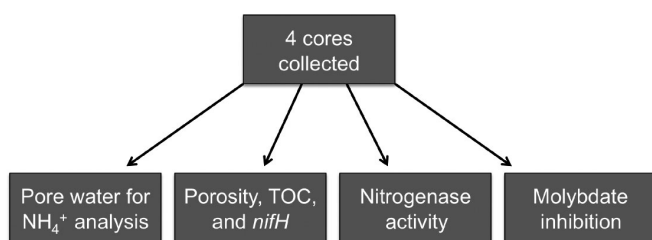


Fig. 2. Flow chart of sampling scheme for 2007. Four cores were collected at each of the 3 sampling locations and divided up as shown, with each arrow representing 1 core. All sampling was done under a  $\text{N}_2$  atmosphere. TOC: total organic carbon

sulfate reduction by uncoupling energetic metabolism and depleting ATP pools in the ATP sulfurylase reaction (Taylor & Oremland 1979) and, possibly, by interfering with sulfate transport (Newport & Nedwell 1988). Because  $N_2$  fixation requires a large amount of energy, those sulfate reducers performing  $N_2$  fixation are unable to do so due to depleted energy pools.  $Na_2MoO_4$  was added to sediment slurries from the 0–5 and 5–10 cm horizons at each sampling location in September–October 2007. To explore a potential link between  $N_2$  fixation and sulfate reduction, sulfate reduction rates (SRRs) and NA were measured for both inhibited and non-inhibited sediment slurries, with non-inhibited samples being treated the same way as the inhibited samples except for the addition of  $Na_2MoO_4$ .

To measure SRR, 6 parallel samples (5 cm<sup>3</sup> of sediment) from the 5 cm sediment slurries (0–5, 5–10 cm), were placed into 25 ml Hungate tubes while under a  $N_2$  atmosphere in a laboratory glove box. Two ml of anoxic seawater, with or without  $Na_2MoO_4$  (triplicates of both), was added to each tube. The tubes were then capped immediately (butyl rubber stoppers) and sealed with aluminum caps. Each tube was then injected with 6  $\mu$ l (170 kBq) of the tracer  $^{35}SO_4^{2-}$  and mixed thoroughly. Samples were incubated for 24 h in the dark and at *in situ* temperature. After this time, sulfate reduction was stopped by adding 20 ml of 20% zinc acetate and SRRs were determined using cold chromium distillation for radiolabeled sulfide (Fossing & Jørgensen 1989, Kallmeyer et al. 2004). To compare SRR across the same depth at the 3 locations, a 1-way ANOVA was performed using PASW Statistics 18, along with a Tukey post hoc test to determine which means were significantly different. Statistical comparisons of SRR between inhibited and uninhibited samples from the same sampling location were performed using a 1-tailed *t*-test.

To measure the effect of  $Na_2MoO_4$  addition on NA, another set of 6 Hungate tubes was prepared from the same sediment slurries that were used for the SRR measurements. Triplicate inhibited and non-inhibited samples were prepared following the same protocol as described for SRR except for the addition of the radio-tracer. Each sample was then injected with 2 ml of  $C_2H_2$ , incubated in the dark and at *in situ* temperature, and assayed for an increase in  $C_2H_4$  over a 48 h period (Capone 1993). Statistical comparisons of NA between inhibited and uninhibited samples from the same sampling location were performed using a 1-tailed *t*-test.

***nifH* genetic analysis.** Terminal restriction fragment length polymorphism (T-RFLP) was applied to a gene encoding for a subunit of nitrogenase (*nifH*) to determine which samples should be further investigated with clone libraries. Briefly, *nifH* genes from sediment DNA (20 ng reaction<sup>-1</sup>) were amplified by nested PCR using fluorescently labeled degenerate primers (Hewson &

Fuhrman 2006). Amplicons (~370 bp) were gel-purified and quantified before digestion of 200 ng of amplified product with *Hae*II (Hewson & Fuhrman 2006). T-RFLP profiles were used to select 3 samples for cloning and sequencing. These samples came from an area of low SRR and high NA (1800-BOM, 1 cm), an area of high SRR and high NA (600-BOM, 8 cm), and an area of high SRR and low NA (0(07)-BOM, 8 cm). Sequences were aligned with closest GenBank tblastx matches from cultured representatives to construct a neighbor-joining tree with Kimura correction in ARB (©Lehrstuhl für Mikrobiologie, TU München) as described by Ludwig et al. (2004). Sequences were submitted to GenBank under accession numbers HM219669–HM219842.

**Laboratory experiments.** Detailed investigations (August and November 2007) of a *Neotrypaea californiensis* burrow system were performed using narrow aquaria (40 × 30 × 3 cm) that were kept in the laboratory at the USC Wrigley Institute for Environmental Studies located on Catalina Island. At each investigation time, 1 aquarium was filled with homogenized sediment collected from the study site that was sieved through a 500  $\mu$ m sieve to remove macrofauna. One adult shrimp was placed in each of the narrow aquaria, which was then submerged in a larger tank that was continuously flushed with fresh seawater, allowing for the surface of the narrow aquaria to be supplied with oxygen-rich water. This setup was maintained for ~2 mo, allowing the shrimp to establish their burrow systems. The development of the burrows was documented by digital photography (Olympus Stylus digital camera model u10D, S300D, u300D) and tracked on transparencies placed against each aquarium wall. After the burrow system was well established, pore water was collected from individual burrow structures using a 5 ml syringe for the analysis of  $NH_4^+$  (Hall & Aller 1992); ambient sediment pore water was not collected here. Bulk pore water analysis of  $NH_4^+$  was performed in August 2007. The front wall of each aquarium was carefully removed to allow for direct sampling of these selected burrow areas for NA measurements using the  $C_2H_2$  reduction method. These different areas consisted of (1) the burrow opening to the surface, (2) burrow chambers, (3) burrow shafts or the narrow vertical portions of the burrow that led from shallow chambers to the burrow openings, (4) an abandoned burrow shaft (abandoned for ~1 mo), (5) the ambient surface sediment that did not appear to be directly associated with the burrow, and (6) conspicuous dark sediment niches that were usually found in close proximity to the burrows (Fig. 1). To collect samples, 1 ml syringes (diam. 2 mm) with the tip cut off were used to 'core' the areas of interest so that sampling extended no more than 2 to 3 mm beyond the burrow structure. This targeted sampling approach allowed only single incubations for the

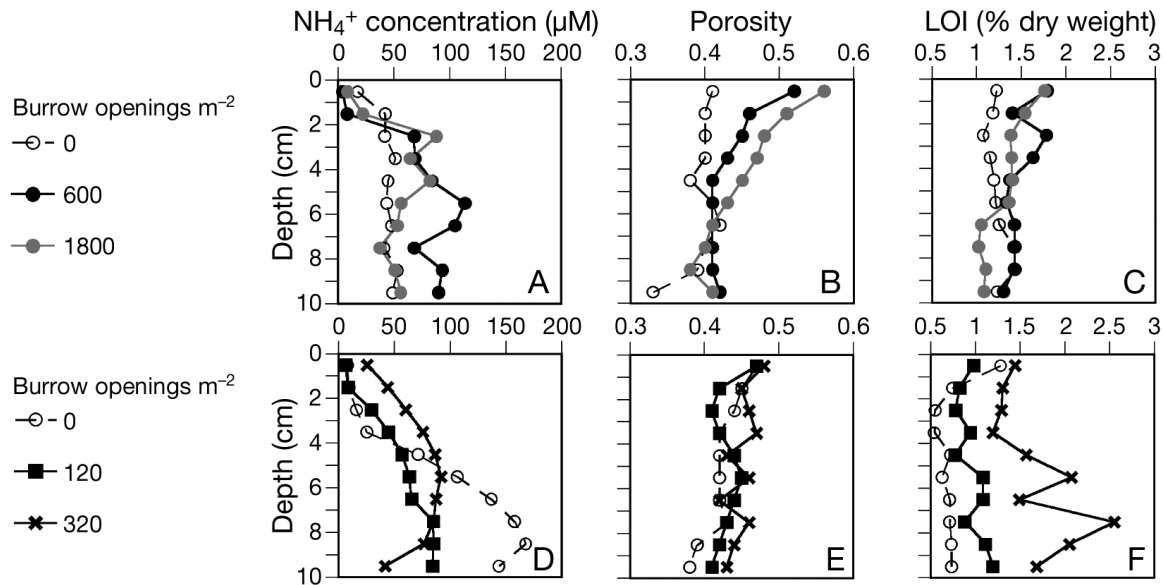


Fig. 3. Catalina Harbor sediment characteristics. (A,D) Ammonium (NH<sub>4</sub><sup>+</sup>) concentrations, (B,E) porosity, and (C,F) carbon lost on ignition (LOI) were measured at 3 different sampling locations in 2007 (upper row) and 2008 (lower row)

determination of NA because of the small sample volume (~5 cm<sup>3</sup> of sediment). However, several micro-niches of the same type were sampled for these incubations. Each 5 cm<sup>3</sup> sample was placed in a 35 ml serum vial that was flushed with N<sub>2</sub>. Each vial was then injected with 3 ml of C<sub>2</sub>H<sub>2</sub>, incubated in the dark and at *in situ* temperature, and assayed for an increase in C<sub>2</sub>H<sub>4</sub> over a 48 h period (Capone 1993).

## RESULTS

### Sediment characteristics

NH<sub>4</sub><sup>+</sup> concentrations in the top 10 cm increased with depth at all sites in both years but generally remained below 100 μM, except in 2008 when the 0(08)-BOM area reached slightly higher concentrations (up to ~170 μM) at depth (Fig. 3A,D). Porosity varied little across sampling sites, with values typically ranging between 0.4 and 0.5 (Fig. 3B,E). Porosity was highest in the surface layer of the bioturbated areas in 2007, most likely due to the higher abundances of burrow openings (600-BOM and 1800-BOM). In 2007, LOI at all 3 locations ranged from 1 to 2% dry weight, with the 600-BOM area typically having the highest % at each depth (Fig. 3C). In contrast, in 2008, LOI at each depth increased as burrow abundance increased (Fig. 3F). Additionally, in 2008, the 0(08)-BOM and 120-BOM areas generally had average LOI values below 1% dry weight whereas the 320-BOM area had increased values of 1.2 to 2.6% dry weight.

### Microbial activity

For the present study, our main focus was on the sub-surface NA at each sampling location. When the top 1 cm of surface mat was excluded from the NA assessment of sediment cores taken in 2007, NA was greatest at depths below 5 cm, with the highest rates (up to 5 nmol C<sub>2</sub>H<sub>4</sub> cm<sup>-3</sup> h<sup>-1</sup>) found in the 600-BOM area (Fig. 4A). Activity values between the 3 stations (based on a 1-way ANOVA) were significantly different at sediment depths 5 (p < 0.001), 7 (p = 0.013), and 10 cm (p = 0.008). Based on the Tukey post hoc test, at 5 cm depth, the 0(07)-BOM area was significantly different from both the 600-BOM (p < 0.001) and 1800-BOM (p < 0.001) areas. At the 7 and 10 cm depths, the 600-BOM area was significantly different than both the 0(07)-BOM (7 cm: p = 0.023; 10 cm: p = 0.011) and 1800-BOM (7 cm: p = 0.014; 10 cm: p = 0.011) areas. SRRs were also higher in the deeper sediment (5 to 10 cm) when compared to the surface layer (0 to 5 cm) at all 3 locations (Fig. 5). Notably, SRRs were highest in the 600-BOM area compared to rates measured at the 0(07)-BOM and 1800-BOM areas. Nonetheless, SRRs from each of the 3 stations across the same depth horizon were not significantly different from one another (p > 0.05), most likely because of the large variations in SRR at each location.

Although the present study did not focus on the sediment surface activity of N<sub>2</sub> fixation, we measured the highest rates of NA in 2007 within the top 1 cm of sediment (35.1 ± 16.9 nmol C<sub>2</sub>H<sub>4</sub> cm<sup>-3</sup> h<sup>-1</sup>) where a visible photosynthetic mat was present (Figs. 4A & 5).

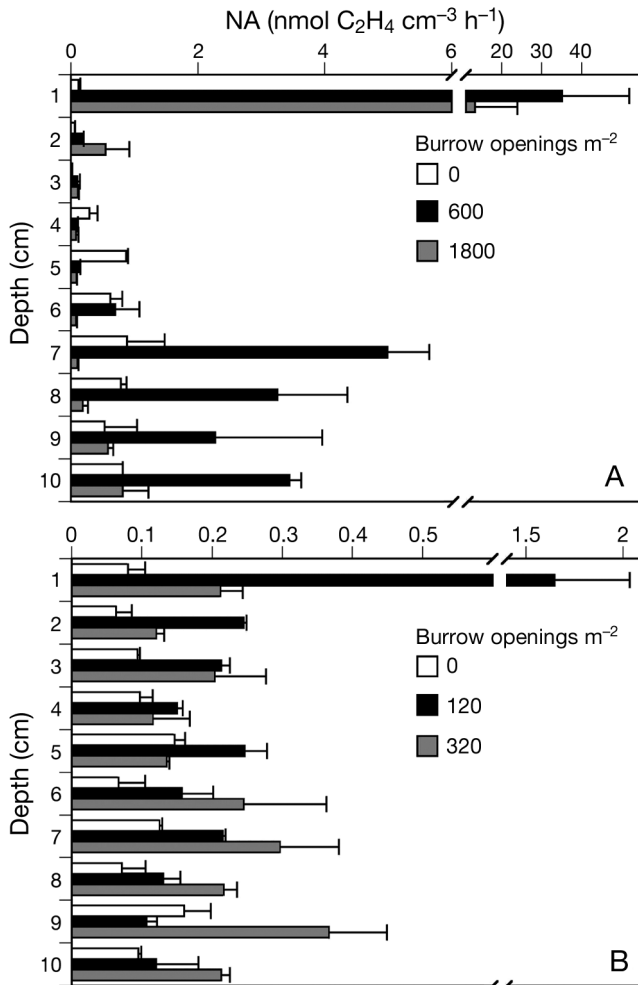


Fig. 4. Nitrogenase activity (NA) of Catalina Harbor sediments. NA was measured in 1 cm core slices from the areas of different bioturbation intensity in (A) 2007 and (B) 2008. Error bars represent SE

These rates are similar to rates seen in other studies that focused on photosynthetic mats (Capone 1983, 1988, Howarth et al. 1988). When the top 1 cm of sediment from each station was analyzed for the effect of light (light/dark incubations), there was no statistically significant ( $p > 0.05$ ) difference in averaged values of NA when comparing samples kept in the light versus in the dark (Fig. 6). However, this lack of significant difference is probably due to high environmental heterogeneity associated with this type of environment. The 0(07)-BOM area showed no change in NA under light conditions, whereas in the 600-BOM area, NA decreased from  $35.1 \pm 16.9$  to  $7.48 \pm 1.21$  nmol C<sub>2</sub>H<sub>4</sub> cm<sup>-3</sup> h<sup>-1</sup>. In contrast, the 1800-BOM area showed an increase in NA from  $13.2 \pm 10.7$  to  $23.1 \pm 5.24$  nmol C<sub>2</sub>H<sub>4</sub> cm<sup>-3</sup> h<sup>-1</sup> in the light. These findings suggest the presence of diverse microbial communities of contrary responses to light in the respective areas. These pre-

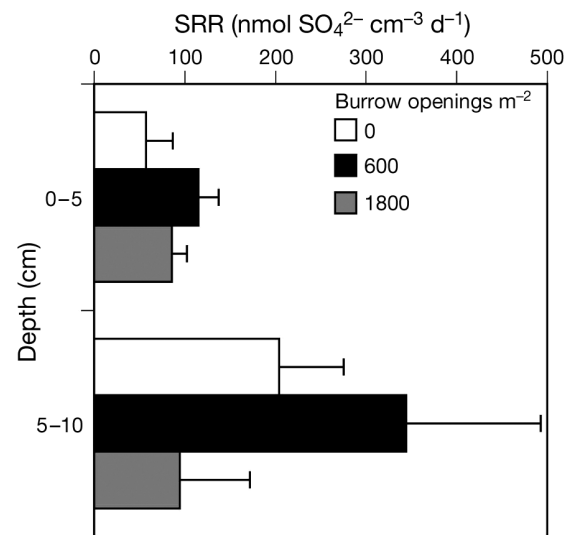


Fig. 5. Sulfate reduction rates (SRR) from non-inhibited slurry samples. Sulfate reduction was measured in slurries of sediment from the 0–5 and 5–10 cm horizon from the 0, 600, and 1800 burrow openings m<sup>-2</sup> locations in 2007. Error bars represent SE

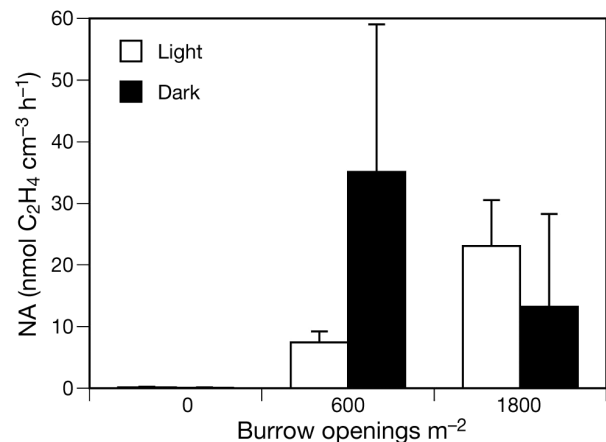


Fig. 6. Effect of light on microbial mat nitrogenase activity (NA). The top 1 cm from the 0 (2007), 600, and 1800 burrow openings m<sup>-2</sup> zones was assayed for NA when kept in the dark and when kept in the light during the day. Error bars represent SE

liminary results on the photosynthetic N<sub>2</sub>-fixing communities on the sediment surface are similar to findings by Severin & Stal (2008). Their investigations showed that photosynthetic communities that were located in higher regions of the littoral benthos were composed of different diazotrophs compared to mats along the same littoral gradient that were located closer to the low-water mark. In addition, these 2 communities displayed very different daily cycles of NA.

In 2008, NA was once again highest ( $1.64 \pm 0.39$  nmol C<sub>2</sub>H<sub>4</sub> cm<sup>-3</sup> h<sup>-1</sup>) at the surface where a photosynthetic

microbial mat was present (Fig. 4B). Overall surface NA was less than measured the previous year, possibly due to the fact that the mat was less developed in 2008, or because measurements were done slightly earlier in the year. Observations over the entire summer revealed that the microbial mat did not develop any further in 2008. When the top 1 cm was excluded, the highest NA was measured below 5 cm depth, with the 320-BOM area displaying the highest rates at these depths and the 120-BOM area displaying the highest rates at shallower depths. Based on the 1-way ANOVA, NA between the 3 stations differed significantly at depths 5 (p = 0.015), 8 (p = 0.017), and 9 cm (p = 0.03). At 5 cm depth, NA in the 120-BOM area was significantly different from both the 0(08)-BOM (p = 0.042) and 320-BOM (p = 0.016) areas, using the Tukey post hoc test. At 8 cm depth, NA in the 0(08)-BOM area was significantly different from the 320-BOM area (p = 0.014), and at 9 cm depth NA in the 120-BOM area was significantly different from the 320-BOM area (p = 0.032).

### Inhibition experiment

Using  $\text{Na}_2\text{MoO}_4$  as a specific inhibitor of sulfate reduction, we measured the inhibition of SRR and NA in 2007 in sediment slurry incubations to quantify the amount of  $\text{N}_2$  fixation associated with sulfate reduction (Table 1). SRR at all 3 locations and at both 0–5 and 5–10 cm depths was successfully inhibited ( $99.4 \pm 1.5\%$ ) when compared to controls. Inhibited SRR from the 0 to 5 cm horizon of the 0(07)-BOM area and the 5 to 10 cm horizon of the 1800-BOM area were not significantly less than the uninhibited rates (p > 0.05), despite the fact that in both cases we were unable to

Table 1. Sulfate reduction inhibition experiment. BOM: burrow openings  $\text{m}^{-2}$ ; SRR: sulfate reduction rate; NA: nitrogenase activity. Rates are shown with standard error

Sampling area	Depth (cm)	Uninhibited	Inhibited	p
<b>SRR (<math>\text{nmol SO}_4^{2-} \text{cm}^{-3} \text{d}^{-1}</math>)</b>				
0 BOM	0–5	$57.1 \pm 29.3$	0	0.062
	5–10	$204 \pm 71.4$	0	0.023
600 BOM	0–5	$115.1 \pm 22.4$	0	0.003
	5–10	$344 \pm 148.7$	$12.3 \pm 12.1$	0.045
1800 BOM	0–5	$85.5 \pm 216.7$	0	0.003
	5–10	$94.6 \pm 77.4$	0	0.144
<b>NA (<math>\text{nmol C}_2\text{H}_4 \text{cm}^{-3} \text{d}^{-1}</math>)</b>				
0 BOM	0–5	$0.29 \pm 0.04$	$0.03 \pm 0.03$	0.003
	5–10	$0.41 \pm 0.02$	$0.006 \pm 0.006$	<0.001
600 BOM	0–5	$0.64 \pm 0.32$	$0.02 \pm 0.02$	0.096
	5–10	$0.39 \pm 0.05$	$0.02 \pm 0.008$	0.009
1800 BOM	0–5	$1.86 \pm 0.98$	$0.38 \pm 0.26$	0.109
	5–10	$0.52 \pm 0.03$	$0.13 \pm 0.03$	<0.001

detect sulfate reduction activity in the inhibited samples. This lack of significant difference is probably due to the high variation in SRR for the uninhibited samples. Simultaneously, NA was decreased by  $86.4 \pm 12.7\%$  in the 0 to 5 cm interval (p = 0.003) and by  $98.5 \pm 1.5\%$  within the 5 to 10 cm section (p < 0.001) of the 0(07)-BOM area, suggesting a direct coupling between SRR and NA as sulfate reduction increased with depth. At the 600-BOM area, NA was reduced ~96%, throughout the sediment column, suggesting that almost all NA at this location was linked to SRR. At the 1800-BOM area, NA was reduced by ~77% throughout the sediment column, indicating that some of the observed NA may be linked to other processes. Similar to what was seen with the SRR, inhibited NA from the 0 to 5 cm horizons of the 600-BOM and 1800-BOM areas were not significantly less than the uninhibited rates, despite a large decrease in NA. Again, this lack of significant difference is probably due to the high variation in NA for the uninhibited samples.

### *nifH* gene analysis

To determine the identity and diversity of potential  $\text{N}_2$  fixers, we assessed the presence of a gene involved in  $\text{N}_2$  fixation (*nifH*) in our 3 contrasting habitats from 2007 (Fig. 7). As expected, we found cyanobacterial *nifH* genes within the phototrophic microbial mat, consistent with the high rates observed. Within the surface sample, as well as in the 2 deep samples, we detected *nifH* genes most closely related to those from various SRB, including *Desulfovibrio* spp. and *Desulfobacter* spp., 2 SRB that have been shown to fix  $\text{N}_2$  (Sisler & ZoBell 1951, Widdel 1987, Postgate et al. 1988), corroborating our findings that NA below the surface sediment layer may be largely carried out by SRB.

### Aquarium NA and $\text{NH}_4^+$ concentrations

Further investigations were done to determine  $\text{N}_2$ -fixation activity and  $\text{NH}_4^+$  concentrations in specific micro-environments associated with bioturbation activity (Fig. 1, Table 2). Pore water  $\text{NH}_4^+$  concentrations throughout the burrow system were ~10  $\mu\text{M}$  or lower. This low amount of  $\text{NH}_4^+$  is most likely due to the irrigation behavior of the shrimp, which would remove such waste products from the burrow and flush it into the overlying water. Using sediment collected from 2 narrow aquaria, each inhabited by a single ghost shrimp, we found that NA



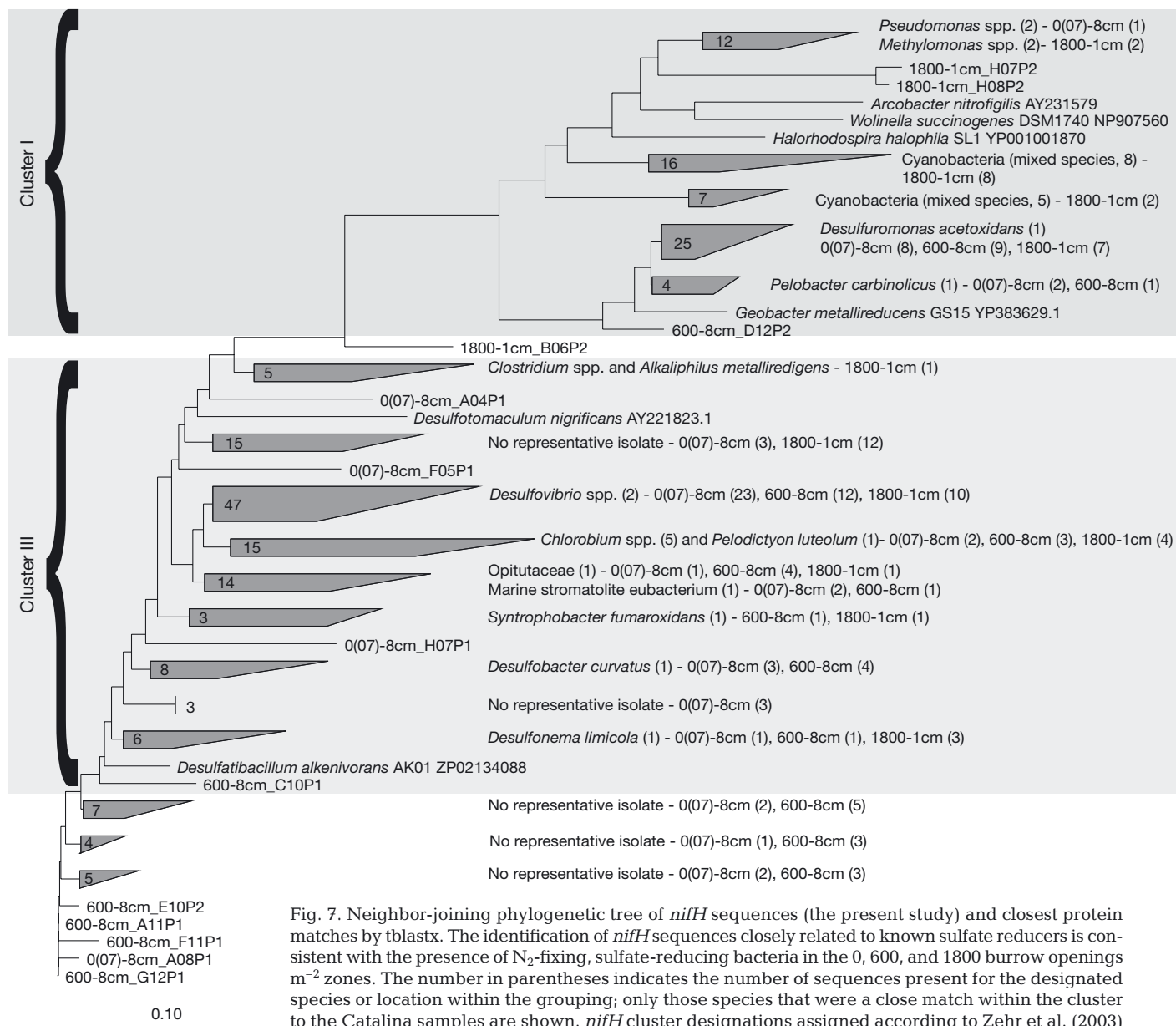


Fig. 7. Neighbor-joining phylogenetic tree of *nifH* sequences (the present study) and closest protein matches by tblastx. The identification of *nifH* sequences closely related to known sulfate reducers is consistent with the presence of  $N_2$ -fixing, sulfate-reducing bacteria in the 0, 600, and 1800 burrow openings  $m^{-2}$  zones. The number in parentheses indicates the number of sequences present for the designated species or location within the grouping; only those species that were a close match within the cluster to the Catalina samples are shown. *nifH* cluster designations assigned according to Zehr et al. (2003)

was highest ( $>1 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$ ) in different niches associated with the burrow: in deep (10 to 13 cm) burrow chambers and burrow shafts found from 2.5 to 6 cm, as well as in dark (reduced) zones at 9 to 10.5 cm. Lower rates were seen ( $<1 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$ ) throughout the rest of the aquarium, with NA still detected ( $0.86 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$ ) at 35 cm depth. Overall, the range in NA from the aquarium sampled in August (0 to  $4.96 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$ ) was greater than that in the aquarium sampled in November (0 to  $1.87 \text{ nmol C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$ ), suggesting that NA may vary temporally. During times of high pelagic primary production (spring and summer), the aquaria were presumably subjected to a greater supply of organic matter, possibly leading to the difference in

aquaria NA measured in the summer and in the fall. Water temperature also varies over this time period and may be a contributing factor to differences between the 2 aquaria. Interestingly, in both aquaria, burrow chambers at 3 cm did not show any NA. It is unclear what the cause of this lack of activity was at this depth.

## DISCUSSION

### NA of Catalina Harbor sediments

While coastal bioturbated sediments are typically thought of as being areas of increased nitrification/

Table 2. *Neotrypaea californiensis* burrow system nitrogenase activity (NA) and pore water ammonium ( $\text{NH}_4^+$ ) concentrations ( $\pm$ SE). NA measured in specific areas associated with several ghost shrimp burrow systems in August and November 2007. Pore water was also collected from several of these structures in August and analyzed for  $\text{NH}_4^+$  concentrations. Data for locations sampled in November are in *italics*. Examples of sampling locations are depicted in Fig. 1. nd: not determined

Sampling location	Depth (cm)	NA (nmol $\text{C}_2\text{H}_4 \text{ cm}^{-3} \text{ d}^{-1}$ )	$\text{NH}_4^+$ ( $\mu\text{M}$ )
Surface sediment	0	0.00	nd
	0	0.00	nd
Burrow opening	0	0.60	$4.7 \pm 1.0$
	0	0.84	$3.7 \pm 0.4$
Burrow shaft	1	0.72	$7.3 \pm 0.3$
	2.5	1.25	$5.2 \pm 0.006$
	4.5	1.87	nd
Abandoned shaft	6	1.63	nd
Burrow chamber	3	0.00	nd
	3	0.00	$4.0 \pm 0.5$
	8	0.50	nd
	10	1.65	$4.4 \pm 0.004$
	10.5	0.67	nd
	11	4.96	$10.7 \pm 0.8$
	13	4.33	$9.5 \pm 1.0$
16	0.90	$5.2 \pm 0.2$	
Black sediment	9	0.86	nd
	9	2.94	nd
	9	1.33	nd
	10.5	4.31	nd
	35	0.86	nd

denitrification (Gilbert et al. 1995, 1998, Herbert 1999), our results from Catalina Harbor show that these areas may also support increased  $\text{N}_2$  fixation (Fig. 4). Coastal burrows have previously been shown to increase sedimentary organic matter availability during formation (Aller & Aller 1986, Branch & Pringle 1987). Bioturbating organisms can accomplish this increase in organics by using mucus to stabilize burrow walls (Aller & Aller 1986, de Vaugelas & Buscaill 1990), through release of fecal pellets (Jørgensen 1977), and through irrigation techniques that bring organic particles from the overlying water into the burrow system. In a previous study, Hartwig & Stanley (1978) proposed that a lack of benthic NA is most likely due to a lack of organic matter availability. In our Catalina Harbor *Neotrypaea californiensis*-bioturbated sediments, high organic matter availability could have supported the high NA that was detected.

Interestingly, the highest subsurface NA also occurred in the presence of  $\text{NH}_4^+$  concentrations  $>50 \mu\text{M}$  (Fig. 3). It is still not clear why bacteria would carry out  $\text{N}_2$  fixation in the presence of appreciable  $\text{NH}_4^+$ , especially because  $\text{N}_2$  fixation is an energy intensive process relative to  $\text{NH}_4^+$  assimilation. However, it should be noted that Catalina Harbor  $\text{NH}_4^+$  concentrations are

equal to or lower than those found in other coastal sediments (e.g. Nishio et al. 1983, Jenkins & Kemp 1984), with  $\text{NH}_4^+$  concentrations of coastal sediments typically  $\geq 800 \mu\text{M}$  (Canfield 1993), especially in the absence of larger bioturbators.  $\text{N}_2$  fixation has also been detected under higher  $\text{NH}_4^+$  concentrations than that observed in the Catalina Harbor sediments (Capone 1988, McGlathery et al. 1998). Additionally, experiments with diazotrophic cyanobacterial isolates show that  $\text{NO}_3^-$  and  $\text{NH}_4^+$  additions in high concentrations do not completely shut down  $\text{N}_2$  fixation (Ramos et al. 1985, Holl & Montoya 2005). Our work, along with the aforementioned studies, suggests that perhaps prevailing ideas about the regulation of  $\text{N}_2$  fixation by fixed N should be reevaluated. Two possibilities that have been discussed for why this process can still continue under high  $\text{NH}_4^+$  concentrations are that  $\text{N}_2$  fixation can serve as a sink for excess electrons (Tichi & Tabita 2000) or that natural organic compounds can decouple nitrogenase from  $\text{NH}_4^+$  inhibition, as occurs with the inhibitor methionine sulfoximine (Yoch & Whiting 1986). In addition, the formation of oxidized zones surrounding the burrows might enhance N loss through nitrification, denitrification, and potentially anammox, and correspond to localized zones of N limitation and thus favorable microniches for  $\text{N}_2$  fixation. Taken together, this information raises questions about our limited knowledge on the inventory of microorganisms capable of fixing  $\text{N}_2$ , the regulation and controls on  $\text{N}_2$  fixation, and the N cycle in benthic environments.

One observation that remains unclear is why there was such a change in *Neotrypaea californiensis* bioturbation intensity and in NA between the 2 years, 2007 and 2008. One possible explanation for this change could be the negative sea surface temperature (SST) anomalies the California region experienced from December 2007 through May 2008 (NOAA, <http://coastwatch.pfel.noaa.gov/>). The colder-than-normal SST could have influenced larval recruitment to Catalina Harbor, resulting in a lower overall abundance of *N. californiensis*, which could in turn decrease overall NA. Aside from the decrease in recruitment of shrimp to Catalina Harbor, the thick microbial mat that was seen in 2007 did not fully develop in 2008. Additionally, the cooler temperatures could have independently decreased NA. This possibility is supported by a previous study that found that salt marsh sediments displayed highest NA during the warm parts of the year (Teal et al. 1979).

Results from the  $\text{Na}_2\text{MoO}_4$  inhibition experiment indicated that SRB carried out the majority of the NA that was detected in Catalina Harbor (Table 1). In all 3 locations, when sulfate reduction was inhibited, NA was decreased by at least  $74.7 \pm 6.0\%$ . This finding

was further supported by the *nifH* gene survey (Fig. 7), which showed that there were many different SRB with the genetic capability to perform N<sub>2</sub> fixation present in Catalina Harbor. As more strains of SRB are studied, it appears that many are genetically capable of N<sub>2</sub> fixation and do in fact carry out N<sub>2</sub> fixation (Sisler & ZoBell 1951, Riederer-Henderson & Wilson 1970, Widdel 1987, Postgate et al. 1988, Zehr et al. 1995). Specifically, other studies of coastal sediments have also found that SRB account for various levels of total benthic N<sub>2</sub> fixation, especially in those areas with high organic matter loading (Gandy & Yoch 1988, Welsh et al. 1996a,b, Nielsen et al. 2001, Burns et al. 2002, Steppe & Paerl 2002). It is therefore not surprising that an area such as Catalina Harbor, with a dense microbial mat and high organic matter loading due to bioturbation, should display high rates of sulfate reduction linked to N<sub>2</sub> fixation.

Detailed laboratory experiments (narrow aquaria) demonstrated that the NA occurs mainly in microniches associated with *Neotrypaea californiensis* burrow chambers and shafts, as well as in highly reduced microniches at depth (Table 2). Bioturbation frequently results in the formation of complex sediment heterogeneity where both oxic and anoxic microniches (or microenvironments) can form (e.g. Glud et al. 1996, Aller 2001, Nielsen et al. 2004, Wenzhöfer & Glud 2004, Zorn et al. 2006, Pischedda et al. 2008). These oxic and anoxic microniches have been suggested as areas of increased microbial activity within bioturbated sediments (Aller & Aller 1986, Aller 1994, Kristensen 2000). Further studies have shown that highest sedimentary SRR often occur directly below the oxic–anoxic boundary (Jørgensen & Bak 1991, Minz et al. 1999), which in our case would occur along the burrow wall. It is therefore not surprising that if SRB are responsible for the majority of the N<sub>2</sub> fixation found in Catalina Harbor sediments, then the highest NA would occur in various areas of the burrow system, more specifically burrow chambers, where organic matter can collect and fuel these microbial processes. Although the observed experimental rates

were lower than in the field, they indicate that *N. californiensis* bioturbation activity induced N<sub>2</sub> fixation. We conclude that laboratory rates were lower because they showed the effect of 1 individual shrimp, whereas subsurface microniches in the field are the result of a whole community of shrimp.

### Comparison with different benthic habitats

Areal integrated N<sub>2</sub>-fixation rates in the investigated *Neotrypaea californiensis*-bioturbated sediments from both years were 1 to 2 orders of magnitude higher than previous studies of bare (without vegetation or microbial mats) estuarine sediments (Table 3). Highest

Table 3. Integrated N<sub>2</sub>-fixation rates in Catalina Harbor sediments (BOM: burrow openings m<sup>-2</sup>) compared to other studies. Values integrated over the 0 to 10 cm horizon include the cyanobacterial mat, while those integrated over 1 to 10 cm exclude it. Examples of rates from recent studies and average (±SD) rates for different types of sediments are shown for comparison

Environment	Depth of integration (cm)	N <sub>2</sub> fixation (mmol N m <sup>-2</sup> d <sup>-1</sup> )	Study
<b>Catalina Harbor</b>			
0 BOM (2007)	0–10	0.8	Present study
	1–10	0.78	Present study
0 BOM (2008)	0–10	0.16	Present study
	1–10	0.15	Present study
120 BOM (2008)	0–10	0.52	Present study
	1–10	0.25	Present study
320 BOM (2008)	0–10	0.34	Present study
	1–10	0.31	Present study
600 BOM (2007)	0–10	8.05	Present study
	1–10	2.43	Present study
1800 BOM (2007)	0–10	2.54	Present study
	1–10	0.42	Present study
<b>Other recent studies</b>			
Lagoon sediment without visible mat	0–2	0.03	Charpy-Roubaud et al. (2001)
Lagoon microbial mat	Mat	1.17	Charpy et al. (2007)
Intertidal microbial mat	Mat	1.63 ± 1.15	Steppe & Paerl (2005)
<i>Zostera noltii</i> -colonized sediment	0–5	0.25	Welsh et al. (1996b)
Fringe to dwarf mangrove mat	0–1	0.15	Lee & Joye (2006)
<b>Averages by environment</b>			
Lake sediment			
Heterotrophic	–	0.02 ± 0.03	Howarth et al. (1988)
Phototrophic	–	0.03 ± 0.02	Howarth et al. (1988)
Atlantic Ocean (2800 m)	–	0.00008	Howarth et al. (1988)
<200 m sediments	–	0.02 ± 0.01	Capone (1983)
Bare estuarine sediments	–	0.08 ± 0.03	Capone (1983)
<i>Zostera</i> spp. estuarine sediments	–	0.39	Capone (1983)
Coral reef sediments	–	6.09 ± 5.62	Capone (1983)
Mangrove			
Rhizosphere	–	0.56	Capone (1983)
Mats	–	1.66	Capone (1983)
Saltmarsh			
Rhizosphere	–	5.27 ± 3.64	Capone (1983)
Surface sediment	–	0.38 ± 0.41	Capone (1983)

integrated rates were seen in the 600-BOM area ( $8.05 \text{ mmol N m}^{-2} \text{ d}^{-1}$ ), supporting the hypothesis that bioturbation by a deep-burrowing decapod can lead to an increase in  $\text{N}_2$  fixation in coastal sediments. This areal rate is on the same order of magnitude as other environments typically thought to be high in  $\text{N}_2$  fixation such as coral reef sediments and salt marsh rhizospheres (Capone 1983), and greater than all other environments presented. Even the area with the lowest bioturbation (120-BOM) had an areal integrated  $\text{N}_2$ -fixation rate ( $0.52 \text{ mmol N m}^{-2} \text{ d}^{-1}$ ) higher than the majority of the other studies reported. The depth of integration used in the present study may be part of the explanation for such high areal rates compared to other studies, highlighting the importance of accounting for  $\text{N}_2$  fixation at depth.

### Bioturbation and N cycling

In current global N budgets, most of the denitrification takes place in the seafloor, resulting in an overall loss of N from sediments (Middelburg et al. 1996). Additionally, anaerobic ammonium oxidation (anammox) could potentially contribute to an overall  $\text{N}_2$  loss from benthic systems (Thamdrup & Dalsgaard 2002). However, a recent study demonstrated that shallow estuarine sediments are capable of taking up  $\text{N}_2$  gas, indicating net  $\text{N}_2$  fixation (Fulweiler et al. 2007). In non-bioturbated coastal sediments, the vertical depletion of electron acceptors leads to a layered system with a very thin (<5 mm) oxic zone at the sediment surface, followed by zones of nitrification, denitrification, and sulfate reduction (Gilbert et al. 1995). Bioturbation has been shown to introduce oxygen deeper into the sediment, thus extending the zone of nitrification

(Gilbert et al. 1995). As a consequence, the coupling of nitrification-denitrification was believed to increase the overall  $\text{N}_2$  loss from sediments. For this reason, many studies pertaining to the influence of bioturbation on coastal N cycling have focused on denitrification and nitrification rates (e.g. Grundmanis & Murray 1977, Gilbert et al. 1995, 1997, 1998).

In contrast, our results support the hypothesis that bioturbation can lead to a 3-dimensional chemical zonation pattern in the sediment, where subsurface  $\text{N}_2$  fixation is carried out by SRB primarily in oxidized zones (anoxic but with low  $\text{NH}_4^+$  concentrations) associated with macrofauna burrows, and contributes to a renewal of N within the benthic system that may help balance the N lost through denitrification (Fig. 8). While we did not measure denitrification rates, the present study shows that  $\text{N}_2$  fixation should be considered in sedimentary N budgets of bioturbated environments. Rates of benthic denitrification have been found to range from  $0.02$  to  $43.1 \text{ mmol N m}^{-2} \text{ d}^{-1}$  (Hattori 1983), with one study finding that bioturbated sediments had a rate of  $5.64 \text{ mmol N m}^{-2} \text{ d}^{-1}$  (Gilbert et al. 1998). Based on our areal rates of subsurface  $\text{N}_2$  fixation (up to  $8.05 \text{ mmol N m}^{-2} \text{ d}^{-1}$ , Table 3), possibly some, though not necessarily all, sedimentary N lost through denitrification could have originally been fixed in the sediments. Further studies are needed to directly determine the relative importance of sedimentary  $\text{N}_2$  fixation with respect to denitrification.

Beginning with this example of a deep-burrowing ghost shrimp, we suggest that more detailed studies will most likely reveal the importance of subsurface  $\text{N}_2$  fixation. Bioturbation-enhanced  $\text{N}_2$  fixation should be considered in future concepts regarding the benthic N cycle, possibly with more detailed studies elucidating the role of  $\text{N}_2$  fixation for benthic systems on a larger

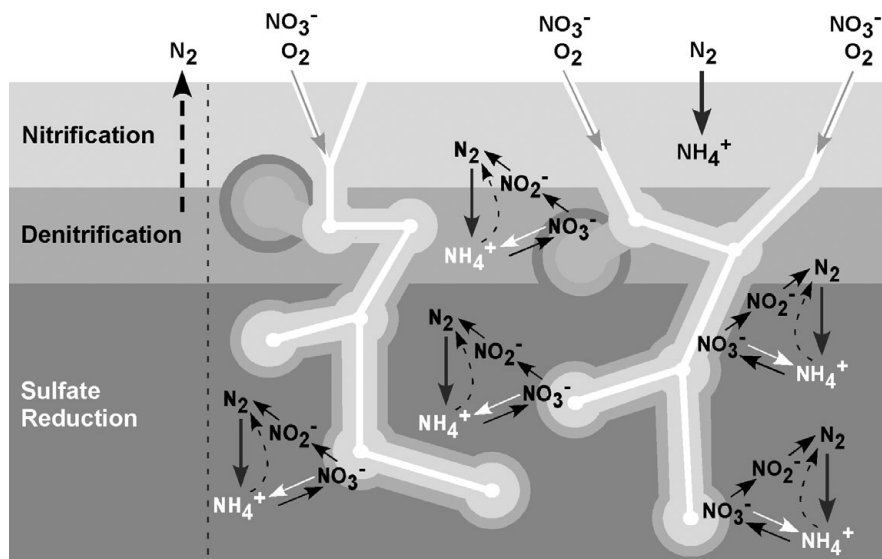


Fig. 8. Conceptual illustration of N cycling and fluxes in a highly bioturbated coastal sediments. (Left) Vertical depletion of electron acceptors in non-bioturbated coastal sediments. Bioturbation has been shown to introduce oxygen deeper into the sediment, thus extending the zone of nitrification, increasing the coupling of nitrification-denitrification, and leading to overall  $\text{N}_2$  loss from sediments (Gilbert et al. 1995). However, we hypothesize that bioturbation (right) leads to a 3-dimensional chemical zonation pattern that enhances zones of nitrification and denitrification as well as creating microniches of subsurface  $\text{N}_2$  fixation carried out by sulfate-reducing bacteria

scale. While the present study was carried out in a selected intertidal mudflat, *Neotrypaea californiensis* is known to inhabit similar intertidal and subtidal environments along the west coast of North America from Alaska to Baja California (MacGinitie 1934). To get a better idea of the possible role of *N. californiensis* in benthic N cycling throughout this habitat range, we attempted to estimate the amount of N that could be fixed in relation to this bioturbating behavior. A rough estimation of the areal extent of estuarine habitat along the North American coast, from Washington state to California (a fraction of this shrimp's range), is 4641 km<sup>2</sup> (Emmett et al. 2000). We estimated that up to  $4.6 \times 10^6$  mol N d<sup>-1</sup> could be fixed in bioturbated sediments inhabited with *N. californiensis*, assuming that (1) 41 % of the estuarine area represents mudflat possibly inhabited by *N. californiensis* (Van Dyke & Wasson 2005), (2) N<sub>2</sub> fixation occurs down to 10 cm, and (3) the average bioturbation intensity is similar to our 600-BOM area (Griffis & Suchanek 1991). This calculation only uses our sub-1 cm values of N<sub>2</sub> fixation and therefore does not take into account the potential fixation rates of any photosynthetic microbial mat associated with the sediment surface. Using values from the 320-BOM area reduces this areal estimate by an order of magnitude; however, this is still a substantial rate.

A recent study estimated that there are >20 700 km<sup>3</sup> bioturbated sediments worldwide (Teal et al. 2008). Types of bioturbation by animals, microbes, or plants can range from scouring the sediment surface, to pushing through sediment layers, to forming simple or complex burrow structures (Kristensen et al. 2005 and references therein). Not all forms of bioturbation may lead to increased N<sub>2</sub>-fixation activity; however, it is reasonable to expect a subset to occur in environments similar to Catalina Harbor that provide an ideal habitat for N<sub>2</sub> fixation. When bioirrigation, such as that performed by *Neotrypaea californiensis*, occurs in such environments, a decrease in NH<sub>4</sub><sup>+</sup> due to this flushing activity may promote N<sub>2</sub> fixation throughout the burrow system. Because there are many types and sizes of bioturbating organisms, some capable of creating deep and elaborate burrows like the cosmopolitan Thalassinidean shrimp, it is likely that bioturbation-enhanced N<sub>2</sub> fixation is an important but overlooked process influencing N cycling in coastal and shelf sediments.

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