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ORIGINAL ARTICLE

Nitrogen fixation in distinct microbial niches within a chemoautotrophy-driven cave ecosystem

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Microbial sulfur and carbon cycles in ecosystems driven by chemoautotrophy—present at deep-sea hydrothermal vents, cold seeps and sulfidic caves—have been studied to some extent, yet little is known about nitrogen fixation in these systems. Using a comprehensive approach comprising of $^{15}N_2$ isotope labeling, acetylene reduction assay and nitrogenase gene expression analyses, we investigated nitrogen fixation in the sulfide-rich, chemoautotrophy-based Frasassi cave ecosystem (Italy). Nitrogen fixation was examined in three different microbial niches within the cave waters: (1) symbiotic bacterial community of *Niphargus* amphipods, (2) *Beggiatoa*-dominated biofilms, which occur at the sulfide–oxygen interface, and (3) sulfidic sediment. We found evidence for nitrogen fixation in all the three niches, and the nitrogenase gene (homologs of *nifH*) expression data clearly show niche differentiation of diazotrophic Proteobacteria within the water streams. The *nifH* transcript originated from the symbiotic community of *Niphargus* amphipods might belong to the *Thiothrix* ectosymbionts. Two abundantly expressed *nifH* genes in the *Beggiatoa*-dominated biofilms are closely related to those from *Beggiatoa*- and *Desulfovibrio*-related bacteria. These two diazotrophs were consistently found in *Beggiatoa*-dominated biofilms collected at various time points, thus illustrating species-specific associations of the diazotrophs in biofilm formation, and micron-scale niche partitioning of sulfur-oxidizing and sulfate-reducing bacteria driven by steep redox gradients within the biofilm. Finally, putative heterotrophs (*Geobacter*, *Azoarcus* and *Desulfovibrio* related) were the active diazotrophs in the sulfidic sediment. Our study is the first to shed light on nitrogen fixation in permanently dark caves and suggests that diazotrophy may be widespread in chemosynthetic communities.
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Introduction

Chemoautotrophs—including bacteria that use reduced sulfur compounds, methane and hydrogen to fix inorganic carbon—occupy a variety of marine habitats, including deep-sea hydrothermal vents and cold seeps, and are the basis of the food web in these aphotic ecosystems (Stewart *et al.*, 2005; Petersen *et al.*, 2011). Many of these diverse bacteria are symbionts of a range of invertebrates (Dubilier *et al.*, 2008). Chemoautotrophy is also found in non-marine habitats, such as sulfidic caves (Sarbu *et al.*, 1996). Although microbial sulfur and carbon cycles in all such ecosystems have been studied to a certain degree, knowledge about the nitrogen cycling remains scarce.

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Nitrogen fixation could substantially contribute to the nitrogen demand of chemoautotrophs (Rau, 1981; Brooks *et al.*, 1987; Sarbu *et al.*, 1996). The process involves conversion of atmospheric nitrogen gas into ammonia by the enzyme nitrogenase and is mediated by certain groups of bacteria/archaea (diazotrophs) existing in various nitrogen-deficient environments, from open oceans (Zehr *et al.*, 2001) to termite guts (Desai and Brune, 2012). Obligate requirements for nitrogen have led several eukaryotes to establish stable symbiotic associations with phylogenetically diverse diazotrophs (Kneip *et al.*, 2007).

Several studies have indicated that nitrogen fixation occurs in marine chemosynthetic communities: Nitrogen fixation by deep-sea anaerobic methane-oxidizing archaea was previously demonstrated (Dekas *et al.*, 2009), and a methanogenic archaeon isolated from deep-sea hydrothermal vent fluid was found to be diazotrophic (Mehta and Baross, 2006). Using transcription and diversity analyses of the nitrogenase genes, genetic potential for nitrogen fixation was recognized in anoxic methane seeps (Miyazaki *et al.*, 2009). Finally,

diverse nitrogenase genes (homologs of *nifH*) were found in deep-sea and hydrothermal vent environments (Mehta *et al.*, 2003) and deep-sea methane hydrate reservoirs (Dang *et al.*, 2009; Dang *et al.*, 2013).

We set out to study nitrogen fixation in a terrestrial chemoautotrophic environment, Frasassi caves (Italy)—a classical example of a cave formed by sulfuric acid speleogenesis (Sarbu *et al.*, 2000; Galdenzi *et al.*, 2008). The 23-km cave passages formed in pure limestone harbor a microbial community dominated by sulfur-oxidizing biofilms composed of β-, γ- δ- and ε-Proteobacteria (Macalady *et al.*, 2006). These bacteria display niche separation driven by different flow regimes as well as spatio-temporal variations of sulfide and oxygen concentrations in the water bodies (Macalady *et al.*, 2008). Microbial biofilms formed by filamentous *Beggiatoa* (also containing δ-Proteobacteria) and *Thiothrix* are common in Frasassi cave waters (Macalady *et al.*, 2006), and some other *Thiothrix* spp. are ectosymbionts of *Niphargus* amphipods (Dattagupta *et al.*, 2009; Bauermeister *et al.*, 2012).

Although nitrogen fixation by cyanobacteria located at the entrance of a limestone cave has been previously shown (Griffiths *et al.*, 1987), the process has not been demonstrated from the permanently dark areas within caves. However, the relatively depleted $\delta^{15}N$ values of the microbial mats (-9.11 per mil) in the chemoautotrophic Movile cave ecosystem (Romania; Sarbu *et al.*, 1996), and the biovermiculations (sample PC06-106; -26.5 per mil) and *Beggiatoa* biofilms (sample GS06-15; -9.9 per mil) found in the Frasassi caves (Jones *et al.*, 2008) suggest diazotrophic activity. Ammonium (30–175 μM) has been consistently measured in Frasassi cave streams (Macalady *et al.*, 2008), despite the nitrogen demand exerted by the growth of abundant biofilms. The source of the bioavailable nitrogen within Frasassi is yet unknown. The caves receive almost no aboveground organic input (Galdenzi *et al.*, 2008), but some ammonium could presumably be from the mineralized aquifer originating from deeply buried anhydrite rocks underlying the Frasassi cave network (Sarbu *et al.*, 2000). Alternatively, the bioavailable nitrogen might be produced by diazotrophs autochthonously.

We investigated whether nitrogen fixation occurs in the Frasassi caves using a multifaceted approach consisting of acetylene reduction assays that utilize the ability of the nitrogenase enzyme to reduce acetylene to ethylene (Stewart *et al.*, 1967), analyses of the incorporation of heavy nitrogen isotope ($^{15}N_2$; Montoya *et al.*, 1996) and expression and phylogenetic analyses of the *nifH* genes (Zehr *et al.*, 2003b). As nitrogen fixation can be carried out by some *Thiothrix* spp. (Chernousova *et al.*, 2009) and *Beggiatoa* spp. (Nelson *et al.*, 1982) and is present in several types of sediments (Herbert, 1999; Dekas *et al.*, 2009; Hamilton *et al.*, 2011), we selected to examine the following niches in the cave waters: (1) symbiotic bacterial communities of *Niphargus* amphipods, including host-specific *Thiothrix* ectosymbionts attached to their legs (Bauermeister *et al.*, 2012); (2) *Beggiatoa*-dominated biofilms (Macalady *et al.*, 2008), which generally occur at the interface between oxygen and sulfide availability (Jørgensen, 1977; Nelson *et al.*, 1986); and (3) sulfidic sediment.

Materials and methods

Sample collection and geochemical measurements

Table 1 shows all the samples utilized in this study and the corresponding sampling times. Cave water parameters, namely pH, conductivity, sulfide and oxygen concentrations, were measured in the overlying water while collecting most of the samples at the Grotta Grande del Vento-Grotta del Fiume (Frasassi), Italy (43.4306° N, 12.9358° E); for site map, see Bauermeister *et al.* (2012). Samples were collected within 15 min after the geochemical analyses were made. Sulfide was measured using the Methylene Blue method (HACH LANGE, GmbH, Germany), and oxygen, pH and conductivity were measured using electrodes LDO101, PHC101 and CDC401, respectively, connected to an HQ40d multimeter (all acquired from HACH LANGE, GmbH, Germany). Ammonia measurements were made with water samples taken from the above *Beggiatoa*-dominated biofilms, which were collected shortly thereafter (for details of sampling of *Beggiatoa* biofilm, see below). To determine ammonium concentrations, water samples were filled into falcon tubes without any air bubble and transported to the nearby field station, Osservatorio Geologico di Coldigioco. Ammonium concentrations were measured within 6 h after collection using the AmVer high range ammonium measurement kit (HACH LANGE, GmbH, Germany), according to the manufacturer's instructions.

Specimens of *Niphargus ictus*, *Niphargus frasassianus* and *Niphargus montanarius* were collected at various locations (see Table 1 for names of the locations); for identification and sampling details, see Flot *et al.* (2010) and Bauermeister *et al.* (2012). Samples were rapidly preserved in RNA*later* (Ambion/Applied Biosystems, Foster City, CA, USA), transported to Osservatorio Geologico di Coldigioco on cool packs (∼subzero temperatures) and stored at $-20\,°C$ within 4 h after collection.

The *Beggiatoa*-dominated biofilms (referred to as *Beggiatoa* biofilms hereafter) were identified based on their conspicuous cottony morphology and growth on the silty sediment in slowly flowing waters (Table 1; Macalady *et al.*, 2006). *Beggiatoa* biofilms were usually present as a continuous patch spread over ∼4–10 m^2. The biofilm was collected over a length of several meters and pooled

Table 1 Sampling details of *Niphargus* spp. (*Niphargus ictus*, *Niphargus frasassianus* and *Niphargus montanarius*), *Beggiatoa* and ε-Proteobacteria biofilms and sediment in the Frasassi caves

Sample	*Sampling time*	*Experiments*
Niphargus spp.		
N. frasassianus (ST)	June 2009	RNA extraction from the whole animals, reverse transcriptase (RT)-PCR amplification of *nifH* homologs
N. ictus (PC)	July 2010	
N. frasassianus (ST)[a]	May 2009	*nifH* amplification from DNA extracted from the symbiotic microbial community associated with separated legs
N. frasassianus (RS)[a]	May 2009	
N. montanarius A (BG)[a]	January 2008	
N. montanarius B (BG)[a]	January 2008	
Beggiatoa-dominated biofilms		
PC-BeggA	July 2010	Total RNA extraction, and RT-PCR amplification of 16S rRNA and *nifH*
PC-BeggB	February 2011	Acetylene reduction assay
PC-BeggC	May 2011	Acetylene reduction assay, Isotope Ratio Mass Spectrometry (IRMS) and RT-PCR amplification of *nifH* homologs
ε-Proteobacteria biofilm		
PC-EpsilA	July 2010	IRMS
Sulfidic sediment		
PC-SedA	February 2011	Acetylene reduction assay
PC-SedB	March 2011	$^{15}N_2$ isotope labeling and IRMS
PC-SedC	May 2011	Acetylene reduction assay and IRMS
PC-SedD	July 2011	Total RNA extraction and RT-PCR amplification of *nifH* homologs

Abbreviations: BG, Il Bugianardo; PC, Pozzo dei Cristalli; RS, Ramo Sulfureo; ST, Sorgente del Tunnel.
The experiments performed with the samples are mentioned in brief (for details, refer to Materials and methods). Collection locations within the Frasassi cave complex are provided in parentheses (see Bauermeister *et al*., 2012, for a cave map). In March and July 2011, no *Beggiatoa* biofilms were present at PC; moreover, water levels were elevated in the cave by ~1 m as compared with the levels observed during other collections.
[a]These samples were collected as a part of other studies and the same DNA preparations were used here (Flot *et al*., 2010; Bauermeister *et al*., 2012).

together, which was finally treated as one sample. The pooling was necessary to obtain enough material for our analyses. The samples were collected in 50 ml falcon tubes with sterile Pasteur pipettes; utmost care was taken to avoid mixing of the underlying sediment. The samples were overlaid with 10–15 ml of cave water and an air headspace of 10–15 ml and transported and stored at the *in situ* temperature (13 °C) in the dark. It was impossible to collect the *Beggiatoa* biofilms without collecting minor amounts of sediment particles. However, the *Beggiatoa* biofilms completely rearranged inside the falcon tubes within ~8 h after collection. The almost pure biofilm was then carefully pipetted from several falcon tubes, pooled together, immediately preserved in RNA*later* and processed similar to the *Niphargus* samples (see above). The remaining portion was transported to Germany for the nitrogenase activity assay (see below). The biofilm remained alive (as seen by aggregation behavior) in the falcon tubes for 2–3 weeks. The ε-Proteobacteria biofilm was identified based on morphology and niche characteristics described by Macalady *et al.* (2008) and preserved in RNA*later*.

Sediment samples were collected 0–15 cm under the sediment–water interface. Samples were collected over a length of 5 m along the water stream and were subsequently pooled to get a homogenous representative sample. The samples for nitrogenase activity assays and $^{15}N_2$ isotope analysis were overlaid with cave water and transported and stored at the *in situ* cave temperature (13 °C) in the dark. The sample collected for RNA extraction was immediately preserved in LifeGuard Soil Preservation Solution, following the manufacturer's instructions (MO BIO Laboratories, Carlsbad, CA, USA), and transported and stored similar to the *Niphargus* samples. The killed controls for the nitrogenase assays were prepared by adding $HgCl_2$ (1 mM final concentration) within 6 h of collection to sediment and *Beggiatoa* biofilm samples (Hamilton *et al*., 2011).

$^{15}N_2$ uptake analyses

The isotope labelling experiments with $^{15}N_2$ (98 atom % $^{15}N_2$ gas, Sigma Aldrich, Munich, Germany) were performed with the sediment samples within 10 h of collection (Table 1). As it was necessary to exclude ammonium from the labelling medium, we did not use the Frasassi cave water in our labelling experiments. Instead, we prepared a basic salt solution (BSS), including all the major ions found in the cave waters, that is, $CaSO_4$ (2 mM), KCl (0.8 mM), $MgCl_2.6H_2O$ (0.8 mM), $MgSO_4.H_2O$ (0.1 mM) and NaCl (17.6 mM), with pH 7.2 and conductivity 3.27 mS cm^{-1}.

As the $^{15}N_2$ bubble method can underestimate the rates of nitrogen fixation, we used the $^{15}N_2$ saturation method (Mohr *et al*., 2010; Großkopf *et al*., 2012). For $^{15}N_2$ labelling, 1 liter serum bottles were completely filled with BSS without any air bubble, and the bottles were closed with butyl rubber septa. In order to avoid mixing of atmospheric gas with the $^{15}N_2$ gas, the headspace of the $^{15}N_2$ gas cylinder pressure gauge was flushed 3–4 times with helium using a gas-tight syringe. The same syringe was immediately used to inject 6.0 ml of $^{15}N_2$ gas at the atmospheric pressure into the bottle with BSS. The

BSS with the $^{15}N_2$ gas bubble was stirred overnight (>18 h) at room temperature. The final calculated $^{15}N_2$ label in the medium was ~36.8 atom %. The bottles were stored at 13 °C until further analysis.

Thick slurries of the sediment samples (50:50 sediment-to-water ratio) were prepared with the cave water collected at the same time when the sediment samples were collected. Ten milliliters of slurry was taken in 50-ml serum bottles and was allowed to settle in the bottle. In the mean time, the 1-liter serum bottles containing the BSS-$^{15}N_2$ label were opened. This was immediately followed by the addition of $NaHCO_3$ into the BSS-$^{15}N_2$ at a final concentration of 0.4 mg ml^{-1} and sulfide (Na_2S) at a concentration of 125–150 μM. A range of sulfide concentrations is provided as some sulfide got oxidized, and therefore, it was not possible to get a stable value. Later, the pH of the labelling medium was adjusted to 7.2. To reduce mixing of atmospheric nitrogen with the $^{15}N_2$-saturated medium, all the additions after opening the BSS-$^{15}N_2$ bottle were performed as quickly as possible. The 'no $^{15}N_2$ isotope' label control was prepared similarly except that 6.0 ml air was injected into the bottle instead of the $^{15}N_2$ gas. The labelling medium was gently transferred to all the serum bottles, including the control bottles. The bottles were carefully closed with butyl septa without introducing any air bubbles. Neglecting the minor loss of the $^{15}N_2$ label while handling, the final calculated label in the incubation bottle would be ~31 atom %. The incubations were carried out at the *in situ* temperature (13 °C) in dark. Incubations were performed for a total of seven time points: 0, 6, 12, 24, 36, 48 and 120 h (each in duplicate). The bottles were opened, and the contents were processed after each time point (the bottles were discarded); for the 0 h time point, the bottles were closed and opened immediately. The supernatant was partially removed without disturbing the sediment slurry, and the settled sediment samples (1 ml each) were transferred to 2-ml glass vials and immediately frozen at −20 °C. In addition to the no-$^{15}N_2$ control, a Na_2MoO_4 (molybdate)-amended control (final concentration 20 mM) was carried out to inhibit sulfate reduction by molybdate (Oremland and Capone, 1988) and thereby check whether sulfate reducers are dominant diazotrophs in this system.

Isotope Ratio Mass Spectrometry (IRMS)

All the frozen sediment samples from the $^{15}N_2$ uptake experiment and the samples preserved in RNA*later* (including the ε-Proteobacteria biofilm) were subjected to IRMS analyses (see Table 1 for sample names). The samples were freeze-dried overnight using a Christ Alpha I-5 dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The freeze-dried materials were homogenized manually with a small metal rod to fine powders. The powdered materials were weighed in tin capsules (4–10 mg each), packed and subjected to IRMS at the Centre for Stable Isotope Research and Analysis, Göttingen, Germany (Werner *et al.*, 1999).

Acetylene reduction assay

Nitrogenase activity in *Beggiatoa* and sediment samples was determined within 7 days of collection. The almost-pure *Beggiatoa* biofilms isolated in the second round and the sediment slurry (both prepared as mentioned above) were subjected to the acetylene reduction assay. The sediment slurry was prepared a day before the experiments; 10 ml slurry was distributed in 50-ml serum bottles and was allowed to settle overnight; the control with molybdate (final concentration 20 mM) was also prepared at the same time. The second round of biofilm purification was performed an hour before injecting acetylene, and 1 ml biofilm was taken into 10-ml serum bottles, except that the control with molybdate was prepared a day earlier. It should be noted that the biofilms actively aggregated in the serum bottles, confirming that they were alive. The samples, including the $HgCl_2$-killed controls and filter-sterilized cave water, were sealed with rubber stoppers, and 15% pure acetylene at atmospheric pressure was injected with a gas-tight syringe. The incubations were carried out at 13 °C in dark for 16 h. The headspace (100–250 μl) samples were examined for ethylene by gas chromatography (GC 14b; Shimadzu, Griesheim, Germany). The gas chromatograph comprised of a flame ionization detector and a stainless-steel column filled with Porapak R (Macherey-Nagel, Darmstadt, Germany, 80/100 mesh, 6′ × 1/8″). Nitrogen was used as a carrier gas (isothermal conditions, 40 °C). Later, the samples were dried at 80 °C for 48 h and weighed.

Nucleic acid extraction

DNA extraction procedure from the legs of *Niphargus* spp. is mentioned previously (Bauermeister *et al.*, 2012). Total RNA extraction from the RNA*later*-preserved *Niphargus* and *Beggiatoa* biofilm samples were performed using TRIzol Reagent (Invitrogen, Karlsruhe, Germany) with the following modifications: *Niphargus* individuals (3–5) and *Beggiatoa* biofilm (1 ml) were centrifuged (6000 *g*, 5 min, 4 °C). Excessive RNA*later* was removed and to remove the loosely attached bacteria, the samples were washed three times with phosphate-buffered saline (0.13 M NaCl, 10 mM Na-phosphate; pH 7.4). Bead beating was performed at 5700 r.p.m. for two cycles of 45 s, with a pause of 20 s. As these RNA*later*-preserved samples had high amounts of salt, we determined that treating the samples two times with TRIzol, with half the TRIzol amount for the second time, led to optimal RNA purity and yield (NanoDrop measurements: 260/280 and 260/230 ratios close to 2.0). Therefore, after the

first TRIzol addition (1 ml), bead beating and centrifugation, the upper phase was removed and half the volume of TRIzol (that is, 0.5 ml) was added to the upper phase. The samples were then vigorously mixed and were treated according to the manufacturer's instructions (Invitrogen). The RNA samples were dissolved in nuclease-free water and stored at $-20\,^{\circ}$C.

RNA was extracted from sediment samples using the RNA PowerSoil Total RNA Isolation Kit (MO BIO Laboratories), following the manufacturer's instructions. RNA samples were resuspended in RNase/DNase-free water (Solution SR7) provided with the MO BIO RNA PowerSoil Kit; the RNA samples were stored at $-20\,^{\circ}$C. RNA extractions with RNA*later*-preserved sediment samples were never successful (details not shown). This might be owing to the high salt concentration in the samples and RNA*later*. However, when we preserved the samples in LifeGuard Soil Preservation Solution (MO BIO Laboratories), good quality RNA extracts were obtained (NanoDrop measurements: 260/280 and 260/230 were nearly 2.0). Integrity of all the RNA samples (that is, *Niphargus* spp., *Beggiatoa* biofilms and sediment) was confirmed by standard agarose gel (2%) electrophoresis.

PCR, reverse transcriptase (RT)-PCR and cloning of nifH homologs
We tested multiple sets of previously published *nifH* primer sets on the DNA extracted from the microbial community associated with the legs of *Niphargus*, *Beggiatoa* biofilms and the sediment samples (details not shown; Ohkuma *et al*., 1999; Zehr *et al*., 2003a; Hongoh *et al*., 2008; Hamilton *et al*., 2011). Among all the primer sets, the nested-PCR approach of Zehr *et al.* (2003a) (outer primers, *nifH3* and *nifH4*; inner primers, *nifH1* and *nifH2*) provided optimum results, with sharp bands of expected size ($\sim$360 bp) and no non-specific amplification. Thirty-five cycles were used for the both primer sets, and the optimum $MgCl_2$ concentration was 2.5 mM. Addition of bovine serum albumin (1 µl of 0.4 mg ml^{-1} in 50 µl PCR mixture) enhanced the PCR product quality and was therefore routinely added in all the reactions.

The primer sets of Zehr *et al.* (2003a) were used to amplify the *nifH* genes from the ectosymbiotic microbial community associated with the legs of *Niphargus* and *nifH* transcripts from the RNA extracted from the symbiotic community associated with the whole *Niphargus* animals, the *Beggiatoa* biofilms and the sediment (Table 1). Single-tube, one-step, two-enzyme RT-PCR (Access RT-PCR System kit, Promega, Mannheim, Germany) was performed with RNA extracts digested with RNase-free DNase (RQ1, Promega), according to the manufacturer's instructions. One microliter of the cDNA template obtained from the RT-PCR was used for the nested PCR. Quality of the PCR products was checked by standard agarose gel (1%) electrophoresis. Simultaneous assays without reverse transcriptase did not produce any products, confirming the absence of DNA template in the RNA extracts. Moreover, the DNase-digested RNA extracts were subjected to amplification of the 16S rRNA genes, and no PCR products were obtained, corroborating the absence of DNA templates. Assays without RNA templates did not produce any products, confirming purity of the RT-PCR and PCR chemicals.

All the amplicons were cloned using TOPO TA cloning kit, following the manufacturer's instructions (Invitrogen). Inserts were sequenced using T3 and T7 primers at the Göttingen Genomics Laboratory (Göttingen, Germany). The *nifH* gene sequences have been submitted to the EMBL/GenBank database under accession numbers HF565511–HF565570 (Table 2).

Phylogenetic analysis
For phylogenetic analysis of *nifH* homologs, we constructed a complete database of cultured representatives of all the standard *nifH* lineages (Zehr *et al*., 2003b), using the ARB software suite (http://www.arb-home.de). The translated amino-acid sequences derived from the sequences obtained in this study were subjected to BLASTp, and the closest *nifH* sequences (originating from both cultured and uncultured bacteria) were imported in the database. Phylogenetic tree was calculated from the translated amino-acid sequences using the maximum-likelihood algorithm provided in ARB (PhyML with Dayhoff PAM, 100 bootstraps); tree topology was validated with maximum-parsimony analysis (PHYLIP Protein parsimony; 1000 bootstraps).

Results

Geochemical parameters
Samples corresponding to the geochemical parameters reported here are mentioned in Table 1. Conductivity and pH values were in the same range as reported earlier (Macalady *et al*., 2006). All the values of sulfide and ammonium reported here are the averages of three measurements. Sulfide concentrations in the location Pozzo dei Cristalli (PC) where most of the samples were collected were 419 µM (June 2009), 459 µM (July 2010), 267 µM (March 2011) and 356 µM (May 2011). Oxygen values in PC varied between 12 µM (June 2009), 30 µM (July 2010), 28 µM (March 2011) and 26 µM (May 2011). Sulfide was always non-detectable and oxygen $\sim$150 µM in six sampling trips (2007–2011) to the location BG where *N. montanarius* was collected. In locations where *N. frasassianus* individuals were collected in May–June 2009, sulfide was 136 µM (ST) and 109 µM (RS), and oxygen was 31 µM (ST) and 10 µM (RS). Sulfide and oxygen values for samples collected in May–June 2009 were previously reported in Flot *et al.* (2010) and were not determined for samples

Table 2 Phylotypes of *nifH* homologs obtained from different samples (*Niphargus frasassianus*, *Niphargus montanarius*, *Beggiatoa*-dominated biofilms and sulfidic sediment) collected at different sites in the Frasassi cave complex

Sample	*Phylotypes*	*Phylogenetic group*	*cDNA or DNA clones/ library*	*Genotypes*[a]
Niphargus spp.				
N. frasassianus (ST) total RNA	Thio1a	γ-Proteobacteria (I)	14/15	HF565531–38
	Thio1b	γ-Proteobacteria (I)	1/15	HF565539
N. frasassianus (ST) legs DNA	Thio1a	γ-Proteobacteria (I)	2/8	HF565540
	Thio1d	γ-Proteobacteria (I)	3/8	HF565541–43
	Thio1e	γ-Proteobacteria (I)	3/8	HF565544–46
N. frasassianus (RS) legs DNA	Thio1a	γ-Proteobacteria (I)	3/8	HF565555–57
	Thio1c	γ-Proteobacteria (I)	1/8	HF565558
	Thio1d	γ-Proteobacteria (I)	1/8	HF565560
	Thio1e	γ-Proteobacteria (I)	2/8	HF565559, HF565561
	Geob	*Geobacter* (I)	1/8	HF565569
N. montanarius A (BG) legs DNA	Thio1a	γ-Proteobacteria (I)	6/9	HF565547–51
	Thio1c	γ-Proteobacteria (I)	2/9	HF565552–53
	Thio1d	γ-Proteobacteria (I)	1/9	HF565554
N. montanarius B (BG) legs DNA	Thio1a	γ-Proteobacteria (I)	3/7	HF565562
	Thio1c	γ-Proteobacteria (I)	3/7	HF565563–65
	Thio1d	γ-Proteobacteria (I)	1/7	HF565566
Beggiatoa-dominated biofilm				
PC-BeggA total RNA	Desulfo1a	*Desulfovibrio* (III)	15/22	HF565524–27
	Desulfo1b	*Desulfovibrio* (III)	1/22	HF565511
	Desulfo1d	*Desulfovibrio* (III)	1/22	HF565529
	Begg	*Beggiatoa* (I)	3/22	HF565530
	Geob	*Geobacter* (I)	2/22	HF565569
PC-BeggC total RNA	Desulfo1a	*Desulfovibrio* (III)	22/28	HF565513–23
	Desulfo1c	*Desulfovibrio* (III)	1/28	HF565528
	Desulfo2	*Desulfovibrio* (III)	1/28	HF565512
	Begg	*Beggiatoa* (I)	4/28	HF565530
Sulfidic sediment				
PC-SedD total RNA	Thio2	γ-Proteobacteria (I)	10/30	HF565567
	Azoarc	*Azoarcus* (I)	4/30	HF565568
	Geob	*Geobacter* (I)	12/30	HF565569
	Desulfo3	*Desulfovibrio* (III)	4/30	HF565570

The abundance of each phylotype (derived from cDNA or DNA) and the GenBank accession numbers of the corresponding genotypes are provided. Abbreviations of collection locations within the Frasassi caves are provided in parentheses; see Table 1 for full names. For phylogenetic positions of individual phylotypes, see Figure 2. In parentheses are the phylogenetic assignments (I or III) to the existing *nifH* groups (Zehr *et al*., 2003b).
[a]For sequences showing the same amino-acid signature, representative sequences were submitted to GenBank. When nearly identical genotypes were obtained from two different samples, representatives from both libraries were submitted—such genotypes usually had >99% DNA sequence similarity.

collected in January 2008, February 2011 and July 2011. Ammonium concentrations in PC were 142 μM (July 2010) and 105 μM (May 2011); note that the ammonium measurement method used in this study only provides approximate concentrations.

Nitrogenase activity assay
Both samples each of *Beggiatoa* biofilms and sulfidic sediment investigated in this study showed formation of ethylene in the acetylene reduction assay, demonstrating nitrogenase activity in these samples (Table 3). In the *Beggiatoa* biofilms, the rates of ethylene production were nearly twofold higher for the biofilm collected in May as compared with the biofilm collected in February (Tables 1 and 3). However, the ethylene production rates for the sediment samples collected in February and May did not show considerable difference (Table 3). The molybdate-amended *Beggiatoa* biofilms (PC-BeggC) showed an order of magnitude decrease in the ethylene production. It should be noted that the molybdate-amended biofilms did not show aggregation behavior and their color had changed from white to light red. On the contrary, ethylene production by the molybdate-amended sediment samples (PC-SedC) showed no difference. Even after 16 h of incubation with acetylene, no ethylene production was detected in $HgCl_2$-killed (carried out only for samples collected in May 2011) and filter-sterilized cave water controls, as well as for unfiltered cave water controls (data not shown), confirming that the nitrogenase activity originated from the biofilm and sediment samples. Although we

also performed the acetylene reduction assay with live amphipods (10–15 individuals of *N. frasassianus*) collected from the cave site PC, no ethylene was detected (details not shown); the ethylene signal might be below the detection limit of the gas chromatograph (3.5 pmol), owing to an insufficient number of animals used for the assay.

$\delta^{15}N$ natural abundance and $^{15}N_2$ uptake analyses

Stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) for several samples were as follows (shown as $\delta^{13}C$ and $\delta^{15}N$ in per mil, respectively): *Beggiatoa*-dominated biofilm (PCBeggC; $n=4$), -35.347 ± 2.254 and -12.984 ± 0.194; ε-Proteobacteria biofilm (PC-EpsilA; $n=4$), -29.894 ± 0.230 and -1.041 ± 0.240; and sulfidic sediment (PC-SedB; $n=4$), -23.709 ± 0.624 and -1.389 ± 0.285. All four replicates for each sample were derived from the same starting sample; see Table 1 for sample names. Similar results were found for the sediment sample collected in May 2011 (PC-SedC; data not shown). The $\delta^{15}N$ of the *Beggiatoa* biofilm (PCBeggC) was substantially depleted in comparison with the ε-Proteobacteria biofilm and sediment. The $^{15}N_2$ labelling experiment with one sediment sample (PC-SedB) showed a clear increase in $\delta^{15}N$, except for the last time point (120 h) where the uptake seemed to stabilize (Figure 1). A control without $^{15}N_2$ isotope label did not exhibit any incorporation, corroborating that the sediment actively fixes nitrogen. The molybdate-supplemented sediment showed no significant change in the uptake of $^{15}N_2$ isotope label, thus providing similar results to the nitrogenase activity assays (see above).

Based on the amount of $^{15}N_2$ label incorporated in the sediment (PC-SedB), rates of nitrogen fixation were calculated (Capone and Montoya, 2001)—the rates (expressed in nmol N_2 fixed $g^{-1} h^{-1}$) for the individual time points in the $^{15}N_2$ uptake experiment were 0.266 ± 0.112 (6 h), 0.387 ± 0.128 (12 h), 0.176 ± 0.032 (24 h), 0.159 ± 0.025 (36 h) and 0.151 ± 0.026 (48 h) ($n=2$ for all time points). The rate of fixation for the final time point (120 h) is not mentioned, because the isotope uptake did not increase for this time point (Figure 1).

Table 3 Rates of acetylene reduction to ethylene by *Beggiatoa*-dominated biofilms and sulfidic sediment collected at the cave site Pozzo dei Cristalli (PC) within the Frasassi caves

Sample	*Ethylene (nmol g^{-1} h^{-1})*[a]	
	February 2011[b]	*May 2011*[b]
Beggiatoa-dominated biofilm	PC-BeggB	PC-BeggC
Only biofilm	3.94 ± 0.24	7.18 ± 0.61
+Molybdate	NM	0.72 ± 0.03
Sulfidic sediment	PC-SedA	PC-SedC
Only sediment	3.27 ± 0.11	4.40 ± 0.37
+Molybdate	NM	4.64 ± 0.34

Abbreviation: NM, not measured.
[a]Values over an interval of 1 h and 2 h were considered for biofilm and sediment, respectively.
[b]Analyzed with two and three replicates for samples collected in February and May, respectively.

Diversity of nifH transcripts associated with distinct cave niches

The results of RT-PCR for *nifH* genes (~360 bp) were positive for all the investigated microbial niches associated with the cave waters (except *N. ictus*; see below), confirming the genetic potential for nitrogen fixation. *nifH* genes of the same size were also obtained from the DNA extracts of the microbial community associated with the legs of *Niphargus* spp. (Table 2). Cloning and phylogenetic analysis showed that all the *nifH* genes obtained in this study were novel (at least 2.5% amino-acid sequence divergence with the sequences in GenBank, except for one sequence (Thio1d, Figure 2) that showed a divergence of 0.9%; 120 amino-acids were considered). The amino-acid alignments of translated *nifH* clones manifested the four cysteine and one arginine residues that are conserved in all nitrogenase iron proteins, corroborating the function of these genes in diazotrophy (Dean and Jacobson, 1992). The *nifH* clones showed distinct amino-acid signature patterns, which were used as a criterion to define a phylotype—the *nifH* clones possessing the same amino-acid signature pattern were treated as one phylotype. Based on the phylogenetic positions of the phylotypes, they all belonged to Proteobacteria and could be putatively assigned to distinct bacterial groups (Table 2; Figure 2).

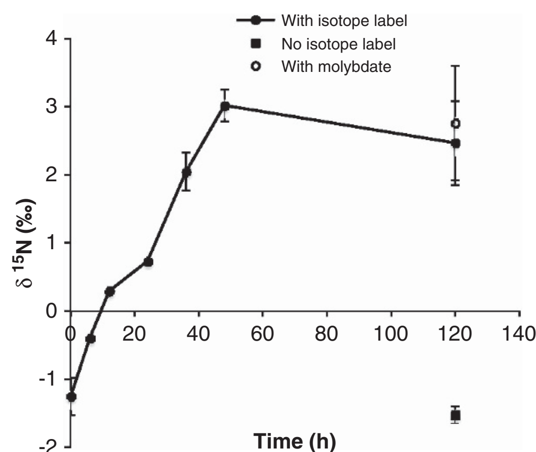

Figure 1 Bacterial uptake of $^{15}N_2$ gas into the sulfidic sediment (PC-SedB; Table 1) collected at the cave site PC; error bars represent s.d. ($n=2$). Uptake of the isotope label by sediment sample amended with molybdate (performed only for the final time point, 120 h) is shown. The parallel experiment with the control without isotope label (also for the final time point, 120 h) displays no significant change in the $\delta^{15}N$ value.

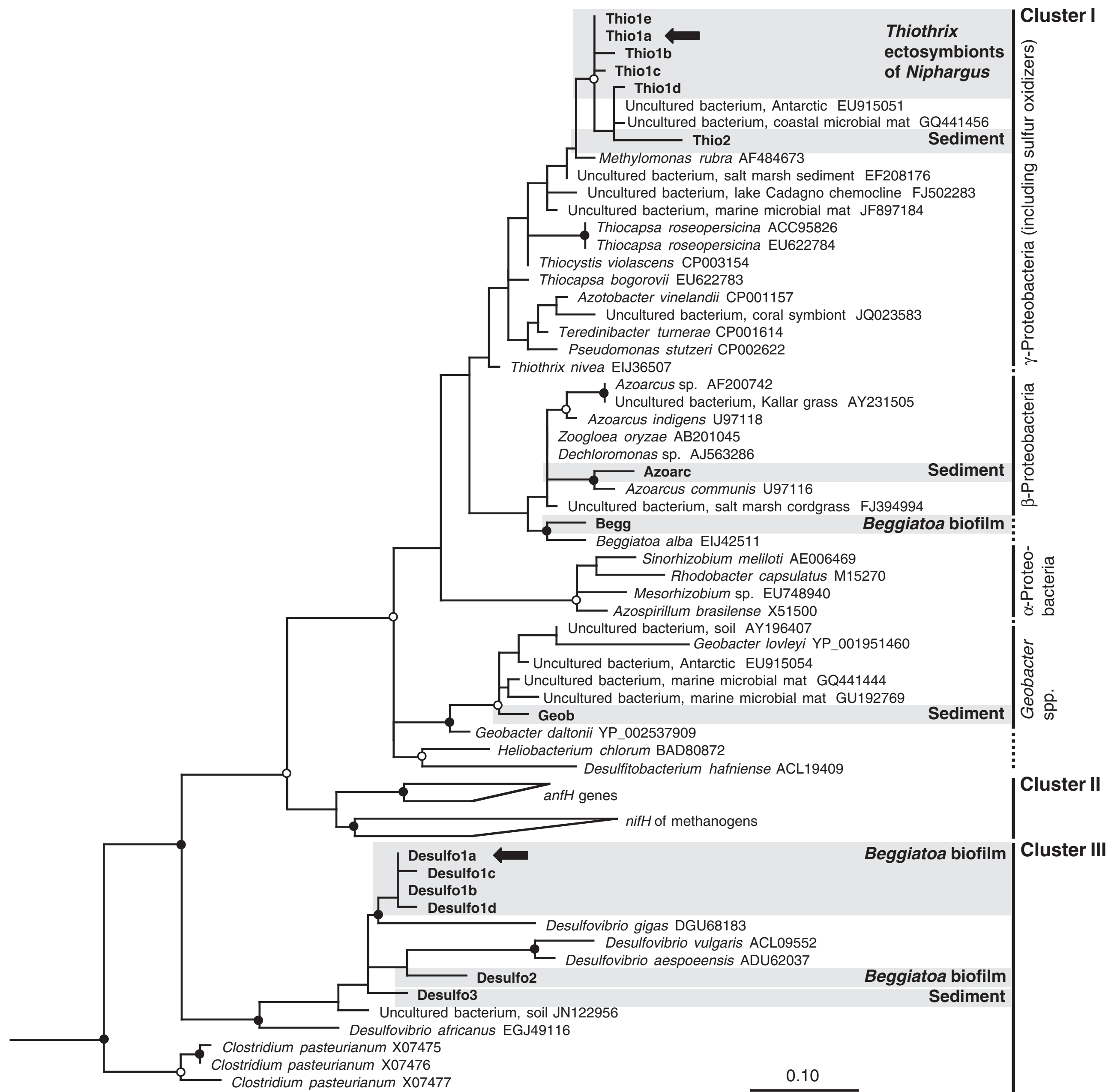

Figure 2 Maximum-likelihood tree exhibiting the phylogenetic relationship of *nifH* transcripts and genes derived from *Niphargus* spp., *Beggiatoa*-dominated biofilms and sulfidic sediment within the Frasassi caves with closely related and prototypical isolates and sequences obtained from other habitats. One hundred and three unambiguously aligned amino-acid positions were considered. Sequences from this study are shown in bold; when numerous closely related phylotypes were obtained from a particular sample, the preferentially expressed phylotype is shown with arrows (see Table 2 for details). Cluster nomenclature is based on Zehr *et al.* (2003b); vertical lines denote distinct *nifH* Clusters, and for Cluster I, different groups are connected with dotted lines. The tree was rooted using *nifH* gene of *Methanosarcina barkeri* (GenBank accession no. AB019139), which falls in Cluster IV. Tree topology was validated by maximum-parsimony analysis; nodes with strong bootstrap values are marked (○ >50%; ● >70%). For tentative assignment of different phylotypes to specific bacterial groups, see Discussion.

Out of the two *Niphargus* spp. (*N. ictus* and *N. frasassianus*; Table 1) that were analyzed with RT-PCR for the *nifH* transcripts of their symbiotic bacterial communities, only the samples of *N. frasassianus* showed transcripts of the expected size. The clone library prepared from the RT-PCR product consisted of two closely related phylotypes (Thio1a and Thio1b; amino-acid identity >98.3%). However, only one phylotype (Thio1a) was preferentially expressed (Table 2; Figure 2). The phylotypes belonged to *nifH* Cluster I and fell into the γ-Proteobacteria *nifH* group comprising of *nifH* genes from various sulfur-oxidizing bacteria, with an amino-acid identity of 97.5%, 96.5%, 95.5% and

92.5% to *Thiocystis violascens*, *Methylomonas rubra*, *Thiocapsa roseopersicina* and *Thiothrix nivea*, respectively (percentage calculated with Thio1a). As *Thiothrix* ectosymbionts are abundant on the legs of these amphipods (Bauermeister *et al*., 2012), to locate the origin of expressed *nifH* phylotype on the amphipod body, we also carried out *nifH* clone libraries with the DNA extracted from the symbiotic microbial community associated with the legs of *Niphargus* spp. (Table 1). PCR products of the similar size were obtained from the legs' bacterial community of *N. frasassianus*; some individuals were collected at the same location during the same sampling trip, as of *N. frasassianus* used to examine *nifH* transcripts (Table 2). We also obtained *nifH* genes from the microbial community associated with the legs of a third species, *N. montanarius* (Table 2). Analyses of the *nifH* genes obtained from the microbial community of legs of all the *Niphargus* samples showed only phylotypes related to the γ-Proteobacteria group, except for one clone affiliated to the *Geobacter* group (phylotype: Geob; Table 2). The only expressed phylotype in the *N. frasassianus* transcript library, Thio1a, was always present in all the four leg-associated clone libraries (Table 2). The other phylotypes (Thio1b, c, d and e) had $>98.3\%$ amino-acid sequence identity with Thio1a, strongly indicating that the bacteria carrying these *nifH* genes belong to the same genus.

We performed the *nifH* transcript analyses of the *Beggiatoa* biofilms collected during two sampling trips (July 2010 and May 2011) at the cave site PC (Table 1). It should be noted that when we collected the sediment samples in March 2011, the water levels in the stream had risen and no *Beggiatoa* biofilms were observed at PC. Thus, the biofilms were formed newly when the water levels decreased in May 2011. The *nifH* transcript analyses of both the biofilms displayed the existence of identical transcripts (Table 2; Figure 2). Although the amino-acid signature patterns of the phylotypes showed that both libraries comprised of several closely related *Desulfovibrio*-related phylotypes ($>98.3\%$ amino-acid sequence identity among several phylotypes), only one phylotype (Desulfo1a) was preferentially expressed in both the biofilms (Table 2). The second dominant phylotype (Begg), expressed in both the biofilms, belonged to the Cluster I and clustered together with the *nifH* gene of *Beggiatoa alba*, with an amino-acid identity of 93.5%. The biofilm collected in July 2010 also contained the *nifH* phylotype (Geob) related to the *Geobacter* group that was found with the microbial community from the legs of *N. frasassianus* (Table 2).

nifH transcript analysis of the sulfidic sediment yielded four *nifH* phylotypes that were different from those found from *Niphargus* bacterial communities and the *Beggiatoa* biofilms, except for the phylotype Geob (see above). The phylotypes belonged to either Cluster I or Cluster III (Figure 2). The most abundant phylotype (Geob), obtained in low abundance in the *Niphargus* symbiotic bacterial community and the *Beggiatoa* biofilm, clustered together with the *nifH* genes of *Geobacter* spp. (96% amino-acid identity with *Geobacter lovleyi* and *Geobacter daltonii*). The second dominant phylotype (Thio2) fell into the γ-Proteobacteria group and was closely related to the phylotypes derived from the symbiotic bacterial community of *Niphargus* spp. (Figure 2). The remaining two transcripts were present in equal proportion and clustered together with *Desulfovibrio* and *Azoarcus* spp. (Table 2, Figure 2; 95% amino-acid identity to *Azoarcus communis*).

Discussion

Our report of nitrogen fixation from the sulfide-driven Frasassi caves (Italy) is the first demonstration of this ecologically important process from a permanently dark, chemoautotrophy-based cave ecosystem. The results of the acetylene reduction assay, $^{15}N_2$ isotope studies and nitrogenase gene (*nifH*) expression analyses collectively document nitrogen fixation in various microbial niches in the cave waters. The nitrogen-fixing bacteria (diazotrophs) include ectosymbionts of *Niphargus* amphipods, occur within the chemoautotrophic *Beggiatoa*-dominated biofilms (*Beggiatoa* and *Desulfovibrio* related) and exist mostly as heterotrophs in the sulfidic sediment (*Geobacter*, *Azoarcus* and *Desulfovibrio* related). These findings display niche segregation of diazotrophic Proteobacteria within the cave water streams. As the Frasassi caves harbor an autochthonous ecosystem, diazotrophy may have an important role in sustaining both the microbial and macrofaunal diversity within the cave ecosystem (Sarbu *et al*., 2000). Our results suggest that nitrogen fixation may be prevalent in chemoautotrophy-based sulfidic caves in the other parts of the world (Sarbu *et al*., 1996; Chen *et al*., 2009; Gray and Engel, 2013) and may be widespread in both marine and terrestrial chemosynthetic ecosystems.

Three species of amphipods—*N. ictus, N. frasassianus and N. montanarius*—are prevalent in the streams and lakes of Frasassi caves (Flot *et al*., 2010; Karaman *et al*., 2010). All the three species carry host-specific filamentous ectosymbionts (on their legs and antennae) that belong to *Thiothrix* Clades T1, T2 and T3 in the 16S rRNA gene phylogeny (Dattagupta *et al*., 2009; Bauermeister *et al*., 2012). Based on the phylogenetic position of the *nifH* transcripts obtained from the microbial community associated with *N. frasassianus* and recovery of identical *nifH* genes from the ectosymbiotic bacterial community attached on the legs of several *Niphargus* specimens (Table 2), the expressed genes could be tentatively assigned to *Thiothrix* ectosymbionts (Figure 2). As *N. frasassianus* and *N. montanarius* share only *Thiothrix* Clade T3 ectosymbionts, a lineage that is distantly related to

the other two lineages (T1 and T2), the T3 ectosymbionts probably possess and express the *nifH* genes (Table 2; Figure 2). This is further corroborated by the fact that we did not obtain any *nifH* transcripts from the symbiotic community of *N. ictus*, which either completely lacks or sparsely possess the Clade T3 ectosymbionts (Bauermeister *et al.*, 2012). It is possible that transintegumental transfer of nitrogenous compounds from the ectosymbionts to the *Niphargus* hosts occurs, similar to the transfer of fixed inorganic carbon that was recently shown for the ectosymbionts of the shrimp *Rimicaris exoculata* (Ponsard *et al.*, 2013).

Another substantial source of nitrogen to *Niphargus* and other Frasassi-dwelling macroinvertebrates could be the chemoautotrophic biofilms growing in the cave waters. Among the numerous biofilm types, *Beggiatoa* biofilms offer an ideal niche for diazotrophic activity, as these gliding filamentous bacteria occur in a low oxygen niche (Preisler *et al.*, 2007; Macalady *et al.*, 2008). Although cultures of marine and freshwater *Beggiatoa* strains were shown to possess the ability to fix nitrogen (Nelson *et al.*, 1982), nitrogenase activity and gene expression were not yet demonstrated in *Beggiatoa* biofilms, which are also abundant at deep-sea hydrothermal vents and reduced sediment habitats (Nelson *et al.*, 1989; Preisler *et al.*, 2007). The isotopically lighter $\delta^{15}N$ values of the *Beggiatoa* biofilm, nitrogenase activity (Table 3) and active expression of *nifH* genes (Table 2; Figure 2) undoubtedly demonstrate diazotrophic activity in these biofilms. The rates of ethylene production found in the acetylene reduction assay may be minimum estimates, as the biofilm was exposed to higher amounts of oxygen during collection and treatment as compared with its natural habitat, which could have reduced the efficacy of the oxygen-sensitive nitrogenase enzyme (Dixon and Kahn, 2004). The phylogenetic analysis of the *nifH* transcripts displays the identities of the diazotrophs as *Desulfovibrio* related and *Beggiatoa* spp. (Figure 2). Intriguingly, identical *nifH* genes were obtained in *Beggiatoa* biofilms collected at different time points, which may be owing to cell-to-cell signalling of these sulfur-cycling bacteria that can reform the biofilms when water levels in the caves are reduced.

Although syntrophic associations of sulfate-reducing and sulfur-oxidizing (and carbon-fixing) bacteria are known (Dubilier *et al.*, 2001), diazotrophy has not been found in such associations. Our study displays an example of nutritional availability and metabolic co-operativity in a biofilm, which are among the reasons why bacteria form biofilms (Davey and O'toole, 2000). As *Desulfovibrio* are strictly anaerobic and *Beggiatoa* bridges the gap between sulfidic and oxic microenvironments, our results suggest niche partitioning of the two distinct diazotrophs driven by steep redox conditions within the biofilm. Although the *Desulfovibrio*-related *nifH* transcripts are more abundant than those of *Beggiatoa* (Table 2), our small subunit rRNA transcript analysis of the biofilm PCBeggA demonstrated nearly 70% *Beggiatoa* and only 5% δ-Proteobacteria (details not shown; these sequences affiliated with *Beggiatoa* and δ-Proteobacteria were identical to the 16S rRNA gene sequences with GenBank accession numbers DQ415807 and DQ133916, respectively; Macalady *et al.*, 2008). An order of magnitude decrease in the rates of ethylene formation in the molybdate-supplemented biofilm (PC-BeggC; Table 3) further supports that the sulfate-reducing bacteria are the major diazotrophs in these biofilms. As the *Beggiatoa* biofilms failed to aggregate when molybdate was added, the sulfate-reducing bacteria may also be important in the formation of these biofilms. Alternatively, molybdate may have a direct inhibitory effect on the formation of these biofilms.

The diazotrophs in the underlying sediment, however, do not seem to contain the *Beggiatoa* biofilm-related *nifH* transcripts (Table 2; Figure 2). Our results of acetylene reduction assay, $^{15}N_2$ isotope incorporation and *nifH* transcript analysis show consistent diazotrophic activity of mostly heterotrophic bacteria in the sulfidic sediment. The rates of ethylene formation in the acetylene reduction assay are in the same range as for bioturbated marine sediment (V. Bertics, personal communication) (Bertics *et al.*, 2010), 4–6-fold higher than those of anoxic coastal sediments (Hartwig and Stanley, 1978), nearly 50-fold higher than the acidic high-temperature geothermal springs in Yellowstone National Park (YNP) (Hamilton *et al.*, 2011) and about 25-fold greater than a lake sediment from Canada (Knowles, 1979). The $\delta^{15}N$ values of the Frasassi sediment are in the same range as reported for the sediment from the YNP, which also has diazotrophic activity (Hamilton *et al.*, 2011; Loiacono *et al.*, 2012). Incorporation of $^{15}N_2$ gas by the cave sediment further corroborates autochthonous nitrogen fixation (Figure 1).

As the rates of nitrogen fixation obtained in the acetylene reduction assay and $^{15}N_2$ isotope labelling did not change in the molybdate-amended sediment samples, it appears that the dominant diazotrophs in the sediment are not sulfate reducers. Instead, the most abundant *nifH* transcripts putatively belong to *Geobacter*, members of which are common heterotrophic diazotrophs in subsurface sediments (Holmes *et al.*, 2004). It is striking to find *Azoarcus*-related *nifH* genes in the cave sediment—these bacteria are known to fix nitrogen only as symbionts within plant roots (Reinhold-Hurek and Hurek, 1997). As nitrogen fixation was observed in the sediment in different months (in the presence and absence of biofilms), it appears that the sediment is a consistent source of nitrogen for the cave ecosystem.

Considering that several locations within the Frasassi caves are seemingly connected with water

channels (Galdenzi *et al.*, 2008) and contain *Beggiatoa* biofilms and sediments, it is possible that the diazotrophs discovered in the cave site PC are spread in other locations within the cave system. Moreover, the *Niphargus* spp. possessing *Thiothrix* T3 ectosymbionts occur at numerous cave locations (Bauermeister *et al.*, 2012). The low $\delta^{15}N$ values measured on the walls of Frasassi caves suggest ammonia degassing (Jones *et al.*, 2008); thus, the fixed nitrogen may also be available for the microbial communities on the cave walls, for example, acidic snottites (Jones *et al.*, 2012). Factors that might drive the level of diazotrophic activity and niche separation within Frasassi may include sulfide and oxygen concentrations in waters at distinct cave locations (Macalady *et al.*, 2008), because high concentrations of both can completely shut down nitrogen fixation (Tam *et al.*, 1982; Dixon and Kahn, 2004). Nitrate can also inhibit diazotrophy (Streeter, 1985), but the nitrate concentrations found in distinct water streams in Frasassi caves are negligible (Macalady *et al.*, 2008). Nitrogen fixation within Frasassi occurs even in the presence of ammonium in the water streams. Despite the energetically expensive and highly regulated nature of the process of nitrogen fixation (Postgate, 1972), the presence of ammonium does not inhibit diazotrophy in other habitats also (Capone, 1988; McGlathery *et al.*, 1998; Holl and Montoya, 2005; Bertics *et al.*, 2010; Hamilton *et al.*, 2011), the possible reasons for which have been discussed previously (Bertics *et al.*, 2010).

Conflict of Interest

The authors declare no conflict of interest.

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