**Biogeosciences
Discussions**

Activity and abundance of denitrifying bacteria in the subsurface biosphere of diffuse hydrothermal vents of the Juan de Fuca Ridge

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Activity and abundance of denitrifying bacteria

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Abstract

Little is known about nitrogen (N) transformations in general, and the elimination of N in particular, at diffuse vents where anoxic hydrothermal fluids have mixed with oxygenated crustal seawater prior to discharge. Oceanic N sinks that remove bio-available N ultimately affect chemosynthetic primary productivity in these ecosystems. Using ^{15}N paired isotope techniques, we determined potential rates of fixed N-loss pathways (denitrification, anammox) and dissimilative nitrate reduction to ammonium (DNRA) in sulfidic hydrothermal vent fluids discharging from the subsurface at several sites at Axial Volcano and the Endeavour Segment on the Juan de Fuca Ridge. We also measured physico-chemical parameters (i.e. temperature, pH, nutrients, H_2S and N_2O concentrations) as well as the biodiversity and abundance of chemolithotrophic nitrate-reducing, sulfur-oxidizing γ-proteobacteria (SUP05 cluster) using sequence analysis of amplified small subunit ribosomal RNA (16S rRNA) genes in combination with taxon-specific quantitative polymerase chain reaction (qPCR) assays. Denitrification was the dominant N-loss pathway in the subsurface biosphere of the Juan de Fuca Ridge, with rates of up to ~ 1000 nmol N l^{-1} day^{-1}. In comparison, anammox rates were always < 5 nmol N l^{-1} day^{-1} and below the detection limit at most of the sites. DNRA rates were up to 152 nmol N l^{-1} day^{-1}. These results suggest that bacterial denitrification out-competes anammox in sulfidic hydrothermal vent waters. Taxon-specific qPCR revealed that γ-proteobacteria of the SUP05 cluster sometimes dominated the microbial community (SUP05/total bacteria up to 38 %). Significant correlation existed between fixed N-loss (i.e., denitrification, anammox) rates and in-situ nitrate and dissolved inorganic nitrogen (DIN) deficits in the fluids, indicating that DIN availability may ultimately regulate N-loss in the subsurface. Based on our rate measurements, and on published data on hydrothermal fluid fluxes and residence times, we estimated that, on average, ~ 10 Tg N yr^{-1} could globally be removed in the subsurface biosphere of hydrothermal vents systems, and could thus represent a small, but significant, fraction of the total marine N loss (240–400 Tg N yr^{-1}).

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1 Introduction

Nitrogen (N) is an essential macronutrient for all organisms, and oceanic N sinks that remove biologically available N via denitrification and anaerobic ammonium oxidation (anammox) ultimately limit ocean primary productivity. Denitrification, the stepwise re-duction of nitrate to N_2 gas, is mediated by both heterotrophic and chemolithotrophic bacteria, and occurs in the anoxic and suboxic oceanic water-column (Codispoti et al., 2001; Lavik et al., 2009; Ward et al., 2009; Grote et al., 2012) and sediments (e.g., Christensen et al., 1987). For the past decade, anammox, i.e the conversion of ammo-nium and nitrite to N_2 gas by autotrophic anaerobic bacteria, has also been reported to account for a significant part of the N loss in oceanic anoxic zones (Dalsgaard et al., 2003; Kuypers et al., 2003, 2005; Lam et al., 2009; Jensen et al., 2011). While stud-ies of N cycling in marine ecosystems have concentrated on oxygen-deficient waters in coastal and open ocean oxygen minimum zones and sediments (see references above), little is known about metabolic processes and bacterially-mediated N-cycle dy-namics that occur in the subsurface biosphere of hydrothermal vent systems.

Hydrothermal fluids originate from seawater that circulates through the upper oceanic crust at mid-ocean ridges where it is heated and chemically altered in the subsurface, and rises back to the seafloor. The fluids are either discharged directly through high-temperature vents (up to ~400 °C) or through diffuse, low temperature (low-T) vents (typically <50 °C) following subsurface mixing with crustal seawater. These ecosystems are sustained by biomass production through chemolithotrophic bacteria that gain their metabolic energy primarily from the oxidation of reduced sulfur species and molecular hydrogen (H_2) present in vent fluids.

Observations of abundant and diverse microbial populations at low-T vents (e.g., Huber et al., 2002, 2007; Alain et al., 2004), as well as nitrate removal in the sub-seafloor mixing zone (Butterfield et al., 2004), and stable isotope measurements of dissolved N species in hydrothermal fluids of the Juan de Fuca Ridge (Bourbonnais et al., 2012a) provide insight into microbial N processes in the subsurface biosphere.

There is also more conclusive evidence for both denitrification and anammox activity in hydrothermal vent systems. For example, Wang et al. (2009) detected an almost complete spectrum of the functional genes required for denitrification, i.e., *nar* (nitrate reductase), *nir* (nitrite reductase), *norB* (nitric oxide reductase) and *nosZ* (nitrous oxide reductase), in DNA extracted from hydrothermal vent chimneys of the Juan de Fuca Ridge using functional gene arrays (GeoChip). Byrne et al. (2009) documented, for the first time, the presence and activity of anammox bacteria in hydrothermal vents of the Mid-Atlantic Ridge. However, their reported anammox rate, measured from an aqueous mixture of chimney samples, were low (up to 60 nmol N l^{-1} day^{-1}). Major metabolic pathways for fixed N-loss have never directly been quantified in discharging fluids, and environmental controls on the activity and abundance of microbial populations that are responsible for fixed N-loss processes in the hydrothermal vent fluids remain to be determined.

Information on the specific microbial agents mediating N loss processes in the subsurface biosphere of hydrothermal vent systems is still emerging. Culture-independent molecular surveys of microbial diversity in these systems have identified 16S rRNA gene sequences affiliated with known autotrophic denitrifiers (e.g., Lopez-Garcia et al., 2002; Hodges and Olson, 2009). ε-proteobacteria are generally the dominant group of chemolithotrophs in hydrothermal vent fluids (Lopez-Garcia et al., 2003; Alain et al., 2004; Nakagawa et al., 2005a; Huber et al., 2007, 2010). Cultivation studies have confirmed that many of these ε-proteobacteria retain the capacity to oxidize reduced sulfur species and H_2, while reducing nitrate or other electron acceptors (e.g. O_2, S^0 and $S_2O_3^-$) (Nakagawa et al., 2005a; Takai et al., 2006).

In addition, 16S rRNA gene sequences affiliated with SUP05 γ-proteobacteria related to the gill symbionts of deep-sea clams and mussels have also been identified as major constituents of redox-active hydrothermal vent plumes (Sunamura et al., 2004; German et al., 2010). SUP05 was first described in the Suiyo Seamount hydrothermal plume by Sunamura et al. (2004) and later associated with oxygen-deficient marine waters in the Eastern Tropical South Pacific (ETSP) (Stevens and Ulloa, 2008), the

Namibian upwelling system (Lavik et al., 2009) and the Northeastern subarctic Pacific (Zaikova et al., 2010; Walsh et al., 2009). Ecophysiological studies, metagenome assembly and gene expression profiling indicate that SUP05 couples the oxidation of reduced sulfur species with nitrate reduction (Lavik et al., 2009; Walsh et al., 2009; Stewart et al., 2012). Consistent with this, qPCR analyses reveals that SUP05 populations thrive under conditions of sulfide and nitrate depletion (Zaikova et al., 2010). Based on these observations, SUP05 populations are expected to inhabit subsurface conduits and mixing zones that supply diffuse hydrothermal vents. In these ramified interstitial spaces, sulfide concentrations are high with continuous re-supply of nitrate from entrained crustal seawater.

In the present study, we examined the relative importance of potential rates of denitrification, anammox and dissimilative nitrate reduction to ammonium (DNRA) in relation to specific microbial agents in diffuse hydrothermal vent fluids of the Juan de Fuca ridge using a combination of ^{15}N paired isotope labelling, 16S rRNA gene clone library sequencing and quantitative polymerase chain reaction (qPCR) methods. The resulting data sets were used to assess the diversity and abundance of potential sulfur-oxidizing denitrifiers and examine chemical and physical constraints on the activity and abundance of microbial communities that mediate biological N-loss in the subsurface of hydrothermal vents. These observations were used, in turn, to evaluate the role of the subsurface hydrothermal biosphere in modulating geochemical N fluxes in the deep ocean.

2 Material and methods

2.1 Site description and sample collection

Vent fluids were sampled at different sites on Axial Volcano and Endeavour Segments, both located on the Juan de Fuca Ridge, in the Northeast Pacific Ocean (Fig. 1). Sampling took place during 4 summer/autumn cruises in August 2007, June and August–

September 2008 and June 2009 using submersibles supported by the R/V John P. Tully and R/V Atlantis during the New Millennium Observatory (NEMO) and Geomicrobiology and Endeavour-Axial Geochemistry and Ecology Research (EAGER) Projects.

The latitude/longitude coordinates of sampled vents are given in Table 1. Diffuse vents were sampled at the Main Endeavour (ME), High Rise (HR) and Mothra (M) vent fields at depths of 2100–2300 m. Sediments that are now buried by ridge basalts are thought to be the origin of anomalously high methane and ammonium concentrations observed in vent fluids at this site (Lilley et al., 1993). Axial Volcano is the most volcanically active site on the Juan de Fuca Ridge and rises 700 m above the ridge crest. It has a 3-sided rectangular shaped caldera (3 × 8 km) that lies between two rift zones, with vents located near the caldera fault and along the rift zones, at depth of 1500 to 1550 m. The diffuse vents were sampled in the ASHES, CASM and South Rift Zone vent fields. A January–February 1998 seafloor volcanic eruption covered most of the South Rift Zone vent field and resulted in the formation of new vents (Embley et al., 1999). A more recent eruption was detected in July 2011 (Chadwick et al., 2012). During the June 2008 cruise, low and high temperature fluids were collected using a suction sampling device, that pumped water into 2 l acrylic jars mounted on the Remotely Operated Vehicle (ROV) ROPOS. The jars were flushed several times prior to sample collection. During all other cruises, samples were collected using the Hydrothermal Fluid and Particle Sampler (HFPS) mounted to the ROV JASON or the Deep Submergence Vehicle (DSV) ALVIN. HFPS samples were collected either in collapsible Tedlar® bags with valves (of up to ∼600 ml volume each), or in PVC piston samplers with Teflon spring seals. The average inlet temperature during sample collection was calculated from in-situ temperature recorded at 1 Hz.

During the June 2008 cruise, samples for denitrification rate measurements were transferred without contact with the atmosphere to 500 ml amber bottles, capped with silicone stoppers containing two lengths of 1/16 nylon tubing with valves, and connected to the suction jar by 1/8- and 5/16-inch Tygon tubes, as described in Devol et al. (2006). During the August–September 2008 cruise, ^{15}N experiments for rate determi-

nations were directly conducted in the Tedlar[®] collapsible bags. Samples were stored in a cool room after each dive until further processing. Subsamples were taken from the collapsible bags using a syringe, and transferred into acid-washed and DI-rinsed 60-ml HDPE brown bottles for nutrient concentration determination. All nutrients subsamples were purged with N_2 gas for at least 10 min in order to remove H_2S, which potentially interferes with colorimetric nutrient analyses. The purged nutrient samples were kept frozen at $-20\,°C$ until analysis.

2.2 Physico-chemical properties

Chemical analyses of fluid samples were conducted on board within less than ~8 h after sampling. pH was analyzed potentiometrically with a standard deviation of 0.01 pH unit. Hydrogen sulfide (H_2S), dissolved silica (Si^{4+}) and ammonium (NH_4^+) concentrations were analyzed using standard colorimetric methods (methylene blue, molybdenum blue and indophenol blue methods, respectively), with standard precisions of 4 %, 1 % and 7 %. NO_x (nitrate and nitrite) was measured by reduction to nitric oxide (NO) in a heated solution of acidic vanadium (III) and subsequent detection of NO (Braman and Hendrix, 1989), with an analytical precision for replicate analyses of $\pm 0.2\,\mu mol\,l^{-1}$. Nitrate (NO_3^-), nitrite (NO_2^-), and phosphate (PO_4^{3-}) were also analyzed on shore using a colorimetric nutrient analyzer. Dissolved NO_x concentrations measured colorimetrically and by chemiluminesce were generally in good agreement. Nitrous oxide (N_2O) concentrations were measured onboard immediately after sample collection. Replicates of 25 ml fluid samples and blanks (DI water equilibrated with air onboard the R/V) were anaerobically transferred into 60 ml syringes prefilled with 12 ml of ultra-pure He, and shaken for 10 min to ensure complete equilibration, following which the gas phase was injected into 12 ml evacuated exetainers and analyzed on a Varian CP-3800 GC equiped with an electron capture detector (ECD). Dissolved [N_2O] was calculated using the Weiss and Price (1980) parametric equation for the solubility of N_2O in seawater, accounting for any blank contribution. One to five sub-samples were collected at each

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sampling site (for all physico-chemical parameters except N_2O) during each dive and, where applicable, only the average and standard deviation for all sub-samples at a given site is reported.

2.3 Denitrification, anammox and DNRA rates

During the 2008 cruises, isotopically-labeled incubations were conducted at the measured in-situ sample temperatures in 500 ml amber glass bottles or Tedlar® bags. We followed the Devol et al. (2006) protocol, with some modifications: for the June 2008 samples, the bottles were pre-incubated for 12 h before the start of the incubations to remove any oxygen from seawater entrained during sampling by the suction pump. Anoxia in the samples was verified by a resazurin oxidation indicator. Added $^{15}N-NO_3^-$ ($\sim 10\,\mu M$) never represented more than 46 % of the ambient NO_3^- concentration (Table 1). At 3 different time points (between 0 and ~ 48 h) ~ 125 ml of sample were transferred, without contact with the atmosphere, into 250 ml evacuated glass bottles, poisoned with 100 µl of saturated mercuric chloride ($HgCl_2$) and equiped with gas tight 9 mm bore Louwers-Hapert O-ring valves (Emerson et al., 1999). In the shore-based laboratory, the flasks were weighed and the dissolved gases in the water samples were equilibrated with the headspace in a constant temperature water bath overnight. The liquid was then removed using a vaccum pump, leaving most (> 95 %) of the gas behind. The remaining gas was then transferred to a stainless steel tube immersed in liquid He. Water and CO_2 were cryogenically trapped during the gas extraction process and oxygen was removed using a hot copper furnace, to avoid isobaric interference during the analysis of $^{30}N_2$. A known amount of ^{36}Ar was also added during this step to determine the absolute concentrations of Ar in the samples. Gas samples were then analyzed for mass ratios 28 : 40, 29 : 28, 30 : 28 and 36 : 40, relative to in-house standards with known gas ratios, on a Finnigan Delta XL mass spectrometer at the University of Washington. Average precision based on duplicate measurements was 0.6 % for the N_2/Ar ratio, 0.01 ‰ for $\delta^{29}N_2$ and 2 ‰ for $\delta^{30}N_2$ (where

$\delta^{30}N_2 = [(R^{30}_{sample}/R^{30}_{standard}) - 1] \times 1000$ and $\delta^{29}N_2 = [(R^{29}_{sample}/R^{29}_{standard}) - 1] \times 1000$; R represents the molar ratio of mass 29 (or mass 30) and mass 28 N_2 in a sample or in the standard, respectively). Denitrification rates were calculated according to Devol et al. (2006) and Ward et al. (2009).

Denitrification, anammox and DNRA rates were also measured during the 2009 cruise following a slightly different protocol. ~20 μM ^{15}N-labelled NO_3^-, NO_2^- or NH_4^+ were added to the hydrothermal vent fluids. Sample vials were then purged with helium (He), and sample aliquots were anaerobically transferred to 12 ml exetainers and incubated at the measured in-situ temperature without headspace (see Kuypers et al., 2005) for up to ~53 h. About 10 μM natural-abundance NH_4^+ was also added in the ^{15}N-nitrite experiments. At specific time points, 1 ml of water was removed and replaced with He, and saturated $HgCl_2$ was added to stop biological activity. The production of $^{29}N_2$ and $^{30}N_2$ for all labelled experiments was determined in the headspace through IRMS measurements (VG Optima Micromass) using air as a standard. Denitrification and anammox rates were calculated according to Nielsen (1992) and Thamdrup et al. (2006), considering only the linear portions of the curves. The errors on the slopes of the regression lines were calculated (see Figs. 2 and 3).

For DNRA rate measurements, 6 ml exetainers were filled with fluid samples (from the 12 ml exetainers above, after incubating with ^{15}N-NO_3^- or NO_2^- and measuring the $^{29}N_2$ and $^{30}N_2$ in the headspace for denitrification rates). About 5 μM $^{14}NH_4^+$ was added, to increase the background ammonium concentration, and the headspace was flushed with 5 ml He gas using a Hamilton gas-tight syringe to remove labelled $^{15}N_2$ product from the previous experiment. 1 ml of water was removed and replaced with He and 200 μl of a freshly made sodium hypobromide solution was then added to the exetainer to convert ammonium to N_2. The solution was mixed using a vortex mixer for at least 30 s. In addition, three replicates of a mixture made from 10 μM of Na$^{15}NO_3$ and 10 μM $^{14}(NH_4)_2SO_4$ were used as abiotic controls and were treated as samples. The exetainers were turned upside-down to prevent any gas loss and samples were incu-

bated overnight at room temperature. The production of $^{29}N_2$ and $^{30}N_2$ for all labelled experiments was then determined in the headspace through IRMS measurements as described above, and DNRA rates were calculated (from the production of $^{29}N_2$ only). The calculated conversion factor, i.e., the fraction of NO_3^- converted to NH_4^+ from the abiotic controls, was close to 90 % and data were corrected accordingly.

2.4 Environmental DNA extraction and diversity profiling

2.4.1 DNA extraction

Environmental DNA (eDNA) was extracted from 0.2 µM Sterivex filters (Millipore) following the protocol described in Huber et al. (2002) with some modifications. For instance, 20 µl of proteinase K (10 mg ml^{-1}) was added with the sodium dodecyl sulfate (SDS; 20 %) before incubation at 65 °C for 2 h, instead of at the beginning of the extraction together with extraction buffer. 200 µl lysozyme (50 mg ml^{-1}) was also added at this step. Finally, The eDNA was resuspended in 95 µl of TE buffer (10 mM Tris,1 mM EDTA, pH 8.0) and was stored at −80 °C.

2.4.2 PCR, cloning and sequencing

Following dilutions of the original eDNA obtained from Cloud (2007), Bag City (2008), Hulk (2008), Godzilla and Phang, near complete 16S rRNA genes were amplified using primers targeting the bacterial domain: B8F (5′-AGAGTTTGATCCTGGCTGAG-3′) and B1492R (5′-GGTTACCTTGTTACGACTT-3′) under the following PCR conditions: 2 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 2 min, and a final extension of 10 min at 72 °C. Each 20 µl reaction contained 1 µl of template DNA, 1 µl of a 5 µM forward and reverse primer, respectively, 2 µl of 8 µM deoxynucleotide solution, 1.25 µl of a 50 µM MgCl$_2$ solution, 2.75 µl of a 1× Invitrogen PCR Buffer, and 1 U of Taq DNA polymerase (Bio-Rad). The PCR amplification products were visualized by UV excitation following electrophoresis on ∼1 % (wt/vol) agarose gels stained with SYBR

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safe (Invitrogen), reconditioned to eliminate heteroduplexes (Thompson et al., 2002) and purified using the QIAquick PCR Purification Kit according to the manufacturer's instructions. DNA was ligated into a pCR® 2.1 TOPO® vector (Invitrogen) and trans-formed into chemically competent TOP10F′ One Shot® *E. coli.* cells following the man-

5　ufacturer's instructions. Transformants (white colonies) were randomly picked, grown in LB + Ampicillin, and cloned inserts were amplified with the vector primers M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) in a 50 µl re-action mixture described in Forget et al. (2010). Inserts (218 in total, ∼30 to 60 at each site) were sequenced at the High-Throughput Genomics Unit (University of Washing-

10　ton) and at the University of Victoria using the PCR primers B8F, B1492R, B515F (5′-GTGCCAAGCMGC CGCGGTAA-3′) and B907R (5′-CCGTCAATTCMTTTRAGTTT-3′).

2.4.3　Phylogenetic analysis

All sequences were edited manually using Sequencher v.4.7 (Gene Codes Cor-poration). The open source Bellerophon application (http://comp-bio.anu.edu.au/

15　bellerophon/bellerophon.pl) (Huber et al., 2004) and PinTail (Ashelford et al., 2006) were used to detect chimeric sequences. Chimeras were excluded from further anal-ysis. Nucleotide sequences (213 in total) were aligned using the ClustalW Application (Thompson et al., 1994) in BioEdit (version 7.0.5.3) and manually checked. Closest rel-atives were identified for each sequenced clone using the BLAST (Altschul et al., 1990).

20　Sequence were classified for phylogenetic analysis using the Ribosomal Database Project (RDP) Classifier tool (Wang et al., 2007). Maximum likelihood trees were in-ferred by PhyML (version 3.0 for Window) (Guindon et al., 2010) using an HKY85 model of nucleotide evolution with 100 bootstrap replicates.

2.4.4　Richness and diversity analysis

25　Rarefaction curves were calculated using mothur version 1.22.2 (Shloss et al., 2009). Sequences with ≥ 97 % similarity were treated as distinct operational taxonomical units

(OTU). OTU richness was calculated using the Chao-1 estimator (Chao, 1984). Diversity in the small subunit (SSU) rRNA clone libraries were generated using the Shannon-Wiener diversity index (H) (Krebs, 1989). The coverage for each clone library was calculated as in Ravenschlag et al. (1999) according to the formula:

$$C = [1 - (n_1/N) \times 100] \tag{1}$$

where n_1 is the number of different OTUs within the library and N is the total number of clones sequenced. See Table 2 for 16S rDNA clone libraries information and OTUs richness (Chao-1) and diversity (Shannon-Wiener) estimates.

2.4.5 Sequences accession numbers

Sequences were deposited to GenBank under the accession numbers JQ712372-JQ712487.

2.5 Total bacterial cells and abundance of SUP05 and anammox bacteria

Total cell abundances in diffuse hydrothermal fluids collected in 2008 and 2009 were determined by epifluorescence microscopy following DAPI staining in the laboratories of J. Holden (University of Massachusetts) and J. Baross (University of Washington). Total bacterial and SUP05 group specific SSU rRNA gene copy numbers were determined by qPCR using an Opticon® 2 DNA Engine Real-Time PCR detection system (Bio-Rad) according to Zaikova et al. (2009). The following primer pairs were used for the quantification of Bacteria: 27F, (5′-AGA GTT TGA TCC TGG CTC AG); DW519R (5′-GNT TTA CCG CGG CKG CTG) and SUP05: Ba519F (5′-CAG CMG CCG CGG TAA NWC-3′) and 1048R, 5′-CCA TCT CTG GAA AGT TCC GTC T-3′).

We used the same standard as Zaikova et al. (2009) to quantify bacteria and SUP05 with initial concentrations of 2.8×10^{10} copies μl^{-1} and 8.5×10^{8} copies μl^{-1}, respectively, as determined from PicoGreen assays using the Quant-iT PicoGreen® dsDNA

kit (Invitrogen). For all qPCR assays, calibration curves were constructed with six 10-fold dilutions of the standards. The limit of detection, set above the Ct values of the no-template controls, was generally less than ∼50 copies µl^{-1} for total bacteria and SUP05 qPCR assays for all triplicate samples. The amplification efficiency for each run was estimated by the slope of the standard curve according to the equation: $E = (10^{-1/\text{slope}})$ and was in all cases above 95 %.

We also assessed anammox bacterial abundances using a qPCR approach following the method described in Humbert (2011). Standard curves were prepared by serial dilution of a plasmid preparation of an anammox clone from Lake Lugano (Wenk et al., 2012). All samples and standards were run in triplicates on a Rotor-gene™ 3000 Real-Time PCR detection system (Corbett Research, Sydney, Australia). No anammox bacteria were detected in any of the samples from this study, while anammox-bacteria amended control samples yielded positive results (data not shown).

3   Results

3.1   Physico-chemical properties

Temperature, pH, and Mg^{2+}, N_2O, H_2S, PO_4^{3-}, NO_3^-, and NH_4^+ concentrations are summarized in Table 1 for all low-T vent fluids sampled at Axial Volcano and Endeavour Segment. Diffuse fluid temperatures ranged from 7 °C (Cloud) to 37 °C (Hermosa) and pH varied between ∼5.5 and 7.0. Mg^{2+} concentrations ranged between 42 and 53 mmol/kg, and were generally close to the [Mg^{2+}] for background seawater (53 mmol/kg). Mg^{2+} is almost completely removed from the aqueous solution during high-T hydrothermal circulation through various water-rock reactions, and resupplied during mixing with crustal seawater at lower temperatures (Edmond et al., 1979). As a result, a clear linear relationship exists between temperature and [Mg^{2+}] (Butterfield et al., 2004; Bourbonnais et al., 2012a), and [Mg^{2+}] can thus be used as a proxy for the degree of mixing in the hydrothermal vent fluids. Nitrous oxide (N_2O) concentrations

ranged between ~0 (not detectable after blank correction) and 347 nmol l^{-1}, which is similar to what was observed in previous work at Axial Volcano (Butterfield et al., 2004). Hydrogen sulfide concentrations were generally high (up to 1300 µM at Marker 113, AV). Phosphate concentrations varied from 1.5 to 4 µM (compared to ~3 µM in back-ground seawater). Nitrate concentrations ranged from 1.6 µM (Marker 113, AV, 2007) to 41.7 µM (Hermosa, AV) and were generally lower than background deep-sea water (~40–42 µM). [NO$_2^-$] was low, accounting for generally less than 2 % (data not shown in Table 1) of total NO$_x$.

Ammonium concentrations were generally low (≤ 3 to 13 µM) at Axial Volcano, com-pared to Endeavour Segment, where [NH$_4^+$] were between 15 µM and 56 µM. Lower ammonium concentrations (1 to 4 µM) were observed in the ROPOS suction samples from the June 2008 cruise, when fluid samples were collected using the high-volume suction pump instead of the dedicated fluid sampler, and are likely the result of dilution by entrainement of surrounding seawater during sampling rather than reflecting actual in-situ concentrations. In an attempt to estimate the net consumption or production of fixed N (i.e., nitrate and ammonium) in diffuse HV fluids, we calculated nitrate, am-monium and total fixed N deficits, according to the following equations that take water mixing into account:

$$NO_{3\,\text{deficit}}^- = ([Mg^{2+}]_{\text{meas}}/[Mg^{2+}]_{\text{sw}}) \times [NO_3^-]_{\text{sw}} - [NO_3^-]_{\text{meas}} \tag{2}$$

$$NH_{4\,\text{deficit}}^+ = [(1 - [Mg^{2+}]_{\text{meas}}/[Mg^{2+}]_{\text{sw}}) \times [NH_4^+]_{\text{high-T}} - [NH_4^+]_{\text{meas}} \tag{3}$$

$$DIN\ \text{deficit} = NO_{3\,\text{deficit}}^- + NH_{4\,\text{deficit}}^+ \tag{4}$$

where [NO$_3^-$]$_{\text{meas}}$, [NH$_4^+$]$_{\text{meas}}$, and [Mg^{2+}]$_{\text{meas}}$ are nitrate, ammonium and magnesium concentrations measured in diffuse fluids, respectively, [NO$_3^-$]$_{\text{sw}}$ and [Mg^{2+}]$_{\text{sw}}$ are ni-trate and magnesium concentrations in background seawater collected near AV and ES, and [NH$_4^+$]$_{\text{high-T}}$ is the ammonium concentration in the high-T end-member. [NO$_3^-$]$_{\text{sw}}$ is constant at ~40 µmol l^{-1} and the average ammonium concentrations in high-T vent fluids varies between vent fields: 14.0 µmol l^{-1} (AV), 396.0 µmol l^{-1} (ES, Mothra),

410.0 μmol l^{-1} (ES, MEF), 863.0 μmol l^{-1} (ES, High-Rise) (data from Bourbonnais et al., 2012a). Nitrate, ammonium and dissolved inorganic nitrogen (DIN) deficits ranged from 33 μM (Marker 113, AV, 2009) to −1 μM (Hermosa, AV), from 16 μM (Fairy Castle, ES) to −12 μM (Marker 113, AV, 2007) and 30 μM (Bag City, AV, 2008) to −3 μM (Hermosa, VA), respectively (Table 1) (with negative values indicating a surplus).

3.2 Potential rates of denitrification, anammox and DNRA

Production of $^{29}N_2$ and $^{30}N_2$ during ex-situ incubations generally showed linear behavior during the incubation period (Fig. 2). Nitrate concentration changes in the incubations (not shown) were generally qualitatively consistent with observed N_2 production trends. Parallel incubations of 2009 samples with addition of either ^{15}N-labelled NO_3^- or NO_2^- generally yielded similar denitrification and DNRA rates, usually with less than 30 % variation between the two series of incubations. Average rates for both series of incubations (2009 samples) are indicated in Fig. 3 when production of $^{29}N_2$ and $^{30}N_2$ was linear.

Denitrification rates in diffuse vent fluids varied strongly between sites, ranging from 0.5 nmol N l^{-1} day^{-1} (Easter Island, ES, 2008) to 977 nmol N l^{-1} day^{-1} (Hermosa, AV) (Fig. 3). Some temporal variability was observed at sites sampled multiple times. For example, measured denitrification rates ranged from 2 nmol N l^{-1} day^{-1} (June 2008) to 577 nmol N l^{-1} day^{-1} (June 2009) at Hulk (ES) and from 83 nmol N l^{-1} day^{-1} (August–September 2008) to 251 nmol N l^{-1} day^{-1} (June 2009) at Marker 113 (AV). However, denitrification rates were always relatively low at Easter Island (1 nmol N l^{-1} day^{-1} (June 2008) and 5.1 nmol N l^{-1} day^{-1} (August–September 2008)). No significant differences were found between denitrification rates at Axial Volcano and Endeavour Segment for all samples collected during the 2008 and 2009 cruises or during individual cruises (Mann-Whitney test for non-parametric data, 95 % confidence limit).

Anammox rates in vent fluids were below 5 nmol N l^{-1} day^{-1} at Hermosa, Marker 113 and Phang and below the detection limit for the two other measured sites (Marker 113

and Fairy Castle). DNRA rates ranged from $\sim 0\,nmol\,N\,l^{-1}\,day^{-1}$ (below detection limit) at Marker 33 (AV) and Fairy Castle (ES) to $152\,nmol\,N\,l^{-1}\,day^{-1}$ (Hulk) (Fig. 3). There was no significant difference in DNRA rates between Axial Volcano and Endeavour Segment (Mann-Whitney test, 95 % confidence limit).

3.3 16S rRNA gene clone libraries composition

Bacterial diversity in diffuse vent fluids was generally high (Shannon-Wiener index ≥ 2.9) except at Hulk (Shannon-Wiener index of 1.7) (Table 2). 16S rRNA gene clone libraries derived from Cloud, Bag City, Godzilla and Phang were dominated by ε-proteobacteria (up to $\sim 80\,\%$ of total clones sequenced at Bag City). The dominant genera of ε-proteobacteria were, in order of importance, *Sulfurovum* (up to 56 % of total clones at Godzilla), *Sulfurimonas* (up to 19 % of total clones at Bag City), *Arcobacter* (up to 23 % of total clones at Bag City) and *Nitratifractor* (up to 7 % of total clones at Phang) (Fig. 4). See Fig. S1 for a partial 16S rDNA ε-proteobacteria tree showing phylogenetic relationships for all 16S rDNA sequences in these four major ε-proteobacteria genera.

In contrast, 16S rRNA clone library derived from the Hulk site showed a dominance of γ-proteobacteria, accounting for up to 77 % of the sequences (Fig. 4). Sequences affiliated with SUP05 were generally the most prevalent γ-proteobacteria, accounting for up to 68 % of the total 16S rDNA clone library at Hulk (Fig. 4). At Cloud, Bag City and Hulk, γ-proteobacteria of the SUP05 sequences were most similar to hydrothermal vent bacteria from an iron chimney-like structure on Volcano 19 in the South Pacific Ocean (99 %) (Forget et al., 2010), and bacteria from the Saanich Inlet water column, e.g. clone FGYC49P14 and clone SHBH489 (Walsh et al., 2009), corresponding to phylotype SI-1. γ-proteobacteria from the Arctic96BD-19 clade (see Bano and Hollibaugh, 2002) were not detected in our samples (Fig. 5).

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3.4 Relation between denitrifying bacteria activity and abundance, as well as environmental factors

Relationships between potential activities and abundance of denitrifying, anammox and DNRA bacteria (ex-situ rates) as well as environmental physico-chemical factors (e.g., temperature, pH, phosphate, hydrogen sulfide and nitrous oxide concentrations, cell counts and %SUP05 bacteria (relative to total bacteria)) were evaluated using multiple Pearson's correlations. Since denitrification rates were not significantly different between the two sampled ridge segments (i.e., Axial Volcano and Endeavour Segment), we pooled data from all vent fields/years for our analysis. Significant linear (negative) relationships were observed between denitrification rate and the in-situ nitrate deficit ($r^2 = 0.40$, p-value $= 0.020$, $n = 13$), and between the anammox rate and the total DIN deficit ($r^2 = 0.89$, p-value $= 0.017$, $n = 5$) (Fig. 7), although sample size was limited for the latter dataset. It should be noted that since we added the same amount of ^{15}N-labelled NO_3^- in all incubations performed during the same year, the degree of correlation (and significance) observed between total nitrate (including labelled ^{15}N-labelled NO_3^- added before the incubations) and denitrification rates were basically the same ($r^2 = 0.44$, p-value $= 0.014$, $n = 13$). A positive linear relationship was observed between the DNRA/denitrification ratio and nitrate deficit ($r^2 = 0.62$, p-value $= 0.035$, $n = 7$, not shown). Weak, but non-significant linear relationships were also observed between denitrification rate and total N deficit (negative, $r^2 = 0.27$, p-value $= 0.07$, $n = 13$) and N_2O concentration and SUP05 bacteria relative abundance (positive, $r^2 = 0.50$, p-value $= 0.08$, $n = 7$).

4 Discussion

4.1 Denitrification as dominant N sink in low-T HV fluids

Our results suggest that denitrification is by far the dominant N loss process in the subsurface hydrothermal biosphere of the Juan de Fuca Ridge (Fig. 3). Denitrification rates in discharging hydrothermal fluids were 10 to 200 times higher than anammox rates and generally ∼5 to 60 times higher than DNRA rates. The latter were higher (by 30 %) than denitrification rates only at one site (Marker 113, AV, 2009). It is interesting to note that unusually high NH_4^+ concentrations (up to ∼13 μM), possibly produced during DNRA, were observed at Marker 113 in 2007, 2008 and 2009. The rates presented here are potential ex-situ rates (measured at in-situ temperatures), without accounting for possible pressure effects. Devol et al. (2006) reported no significant difference in denitrification rates measured in-situ and ex-situ in the Arabian Sea oxygen deficient zone, at a depth range of 150 to 300 m. While barophilic bacteria from great depths in the ocean are known to have pressure-dependent metabolic and growth rates (reviewed by Yayanos, 1995), there is currently no evidence to suggest that bacteria from the depths encountered here (1500 to 2200 m) should have different metabolisms at sea surface pressures.

At some sites, N_2 production by denitrification was significantly higher than what is generally observed for other open ocean and coastal oxygen-deficient zones. For example, denitrification rates ranged from 9.1 (open ocean) to 33.2 (coastal) $nmol\,N\,kg^{-1}\,day^{-1}$ in the Arabian Sea (Devol et al., 2006), and were up to 22 $nmol\,N\,kg^{-1}\,day^{-1}$ in the Black Sea (Fuchsman et al., 2008; Jensen et al., 2008). Our higher rates were similar to rates reported by Lavik et al. (2009) for chemolithotrophic denitrification coupled to sulfide oxidation in the Namibian shelf waters (i.e., ∼600 $nmol\,N\,kg^{-1}\,day^{-1}$) and by Manning et al. (2010) in Saanich Inlet, a production British Columbia fjord (86 to 206 $nmol\,N\,kg^{-1}\,day^{-1}$). Bowles and Joye (2011) recently reported heterotrophic denitrification rates of up to 32 $μmol\,N\,kg^{-1}\,day^{-1}$, in surficial cold seep sediments with *Beggiatoa* sp. mats in the Gulf of Mexico. From

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a hydrochemical point of view, cold seeps are environments that are very similar to hydrothermal vents, with cold seep fluids containing substantial reducing substances such as hydrogen sulfide and methane, albeit at lower discharge rates. This suggests that N loss via denitrifying bacteria (whether autotrophic or heterotrophic), can be an important process in these reducing seafloor and subseafloor environments. Although complete denitrification is almost certainly dominant, we note that estimates of total NO_3^- conversion to gaseous products reported here may be conservative underestimates as we only measured $N_2(g)$ and not N_2O production. Measured $[N_2O]$ in sampled fluids was as high as $347\,nmol\,l^{-1}$ (Fairy Castle, ES). Some denitrifiers, including sulfur-oxidizing bacteria, lack the gene coding for nitrous oxide (N_2O) reductase (*nosZ*), so that the final product of denitrification is $N_2O(g)$. In particular, the *nosZ* gene was not found in the metagenome of the SUP05 bacteria (Walsh et al., 2009), suggesting that their energy metabolism can result in net N_2O production. Incomplete denitrification could, in fact, explain the near-significant correlation (*p*-value= 0.08) observed in this study between N_2O concentrations and SUP05 relative abundances. N_2O is also produced during nitrification (Betlach and Tiedje, 1981), which can occur in subsurface hydrotheral conduit systems as a result of the discontinuous mixing of anoxic hydrothermal fluids and oxic seawater. Indeed, Wang et al. (2009) reported the presence of bacterial and archaeal *amoA* (ammonia monooxygenase) genes required for nitrification in HV vent chimneys of the Juan de Fuca Ridge. This additional process confounds any attempt to estimate the relative proportion of N_2O produced during denitrification only. Also, N_2 production at the microbial community level should be seen as the definitive end product of N loss, whereas N_2O produced by distinct groups of denitrifiers could be further reduced to N_2 by other groups of bacteria.

Potential anammox rates were relatively low ($< 5\,nmol\,N\,l^{-1}\,day^{-1}$, Hermosa) in HV fluids, compared to denitrification. In fact, we rarely observed any ammonium deficits in HV fluids, but rather a surplus of ammonium with respect to NH_4^+ concentrations expected for conservative mixing (Table 1). This, in fact, supports net ammonium production, rather than consumption by anammox. For comparision, Byrne et al. (2009)

measured potential anammox rates of up to $60\,\mathrm{nmol\,N\,l^{-1}\,day^{-1}}$ in an aqueous mixture of hydrothermal vent chimneys from the Mid-Atlantic Ridge. Their rates are higher than ours possibly because of the increased microbial population density of their aqueous mixture compared to diffuse hydrothermal fluids. Our low to undetectable anammox rates agree with our microbiological results (see Sect. 4.2).

As observed in previous studies in other sulfidic marine environments (e.g., Lavik et al., 2009), our results indicate that during N_2 production, fast growing denitrifying bacteria (generation times of 1.5–2 h for sulfide-oxidizing autotrophic denitrifiers, Sievert et al., 2008) generally out-compete generally slow-growing anammox bacteria (generation times of 8–10 days, Strous et al., 1999) for NO_x in diffuse fluids. It has previously been suggested that fluctuating oxic-anoxic conditions could favor the more metabolically versatile denitrifying bacteria over anammox bacteria. Indeed, many denitrifiers can survive for extended periods of time in the absence of NO_x (Risgaard-Petersen et al., 2005) and perform aerobic respiration or use other electron acceptors (e.g., Fe), and this may confer them a competitive advantage over anammox bacteria in variable redox regimes.

4.2 Denitrifier community in the subsurface biosphere

Denitrifying bacteria have been reported to belong to diverse phylogenetic groups (Zumft, 1997). The capacity for autotrophic denitrification has been documented for α, β, γ and ε-proteobacteria in a wide range of environments (e.g., hydrothermal vents, deep-sea redox transition zones, sediments, soils, and inland soda lakes, review by Shao et al., 2010). Denitrification capacity has also been identified in extremophile Archaea (see review by Cabello et al., 2004). Archaea only constituted a small component of the total microbial community associated with hydrothermal vent fluids in this study (qPCR archaeal abundance up to 6 % at Cauldron, ES – data not shown) and Mariana Arc seamounts (typically less than 3 %; Huber et al., 2010). Therefore, archaeal denitrification was not further addressed.

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Chemolithotrophic ε-proteobacteria have been identified as the dominant species in different hydrothermal vent habitats such as vent plumes and fluids and living in symbioses with animals, using both culture and molecular approaches (Lopez-Garcia et al., 2003; Alain et al., 2004; Nakagawa et al., 2005a; Huber et al., 2010). These bacteria are versatile and can use different electron donors, mostly sulfur compounds (S^0, $S_2O_3^{2-}$), hydrogen, organic matter like formate and fumarate, and electron acceptors, e.g. NO_3^-, O_2, S^0 and $S_2O_3^{2-}$ (Shao et al., 2010). Culture experiments by Nakagawa et al. (2005a) showed that ε-proteobacteria strains isolated from HV fluids, colonization devices and annelid polychaete tubes at the Iheya North hydrothermal field in the Okinawa Trough were able to couple hydrogen, $S_2O_3^{2-}$ and S^0 oxidation with nitrate reduction, illustrating the versatility of bacterial metabolisms in these environments. In addition, these authors showed that some isolates preferentially used nitrate as an electron acceptor during sulfide oxidation, even in the presence of oxygen. These autotrophic denitrifying ε-proteobacteria included members of the genera *Sulfurimonas* (comprising *Sulfurimonas paralvinellae* sp., Takai et al., 2006), *Sulfurovum* (comprising *Sulfurovum* sp. NBC37-1, Inagaki et al., 2004) and *Nitratifractor* (i.e., *Nitratifractor Salsuginis*, Nakagawa et al., 2005b). Additionally, Sievert et al. (2008) and Grote et al. (2012) reported that other ε-proteobacteria of the genus *Sulfurimonas* (*Sulfurimonas denitrificans* str. DMS1251 and *Sulfurimonas gotlandica* str. GD1) isolated from marine environments can perform both chemolithotrophic denitrification (coupled to sulfur oxidation) and heterotrophic denitrification. All of the above mentioned ε-proteobacteria genera, plus the genus *Arcobacter*, were generally present in HV fluids in this study (Fig. 4) and represented up to 77 % (Bag City, 2008) of the 16S rDNA clone librairies. ε-proteobacteria of the genus *Arcobacter* have also been shown to perform sulfur oxidation with denitrification in pure cultures (e.g., Gevertz et al., 2000) and in the environment (Lavik et al., 2009) (Fig. 4 and Fig. S1).

Our qPCR results indicate that γ-proteobacteria, mostly belonging to the SUP05 cluster, were particularly abundant in specific hydrothermal vent fluid samples (Hulk, ES up to 38 % and Cloud, AV up to 27 %, Fig. 6). This finding was qualitatively

supported by sequence abundances in 16S rDNA gene clone libraries. SUP05 is a chemolitoautotrophic γ-proteobacteria capable of oxidizing reduced sulfur compounds using NO_3^- as terminal electron acceptor (Lavik et al., 2009; Walsh al., 2009). A BLAST search revealed that SUP05 sequences were generally present in samples from hydrothermal vent systems, but had not been specifically identified as such in previous studies. For example, SUP05 sequences in this study shared a 99%-similarity with a bacterial sequence from an iron oxide chimney-like structure on Volcano 19, in the South Tonga Arc, (acc. #: FJ905679; Forget et al., 2010) and 98%-similarity relative to sequences from the Suiyo Seamount hydrothermal plume (e.g., Acc. #: AB112459, AB112455 and AB112451) (Fig. 4). Moreover, bacteria of the SUP05 clade, which seem to only possess the *nirK* form of nitrite reductase (Walsh et al., 2009) were generally more abundant than *nirS* genes based on qPCR assays, which represented up to $\sim$8% of the total bacterial abundance in hydrothermal vent fluids of the Juan de Fuca Ridge (Bourbonnais et al., 2012b). This suggests that SUP05 bacteria can represent an important fraction of the total denitrifying population in the subsurface biosphere of HV systems. However, it should be noted that because denitrifiers are highly diverse, it is virtually impossible to confirm the capacity of an organism to perform denitrification based solely on phylogenetic affiliation. Studies of the genes involved in the denitrification process, e.g. nitrite reductase (*nirS* and *nirK*), and nitrous oxide reductase (*nosZ*) could represent a more direct approach to describing denitrifying bacterial communities from a functional perspective.

4.3 Environmental controls on denitrifier (and anammox) abundance

Potential environmental controls on denitrifier activity and abundance in the subsurface biosphere of the Juan de Fuca hydrothermal vents can be evaluated by comparing measured microbiological attributes with physical, chemical and biological fluid properties.

NO_2^- and NO_3^- supply have been shown to stimulate denitrification and influence the distribution of denitrifying genes in marine, estuarine (e.g., Jayakumar et al., 2004;

Dong et al., 2009) and terrestrial (e.g., Bradley et al., 1992; Smith et al., 2006; Opdyke et al., 2007; Zhong et al., 2009) environments. The ratio of DNRA/denitrification in sediments has also been shown to be modulated by the availability of nitrate along an estuarine gradient (e.g., Dong et al., 2009). The significant negative linear relationship observed between denitrification rates and in-situ nitrate deficit ($r^2 = 0.4$, p-value = 0.02) suggests that nitrate supplied by mixing between NO_3^--poor hydrothermal fluids and NO_3^--rich crustal seawater may ultimately regulate denitrification in the subsurface biosphere of hydrothermal vents. Although our DNRA rate dataset is limited, the positive linear correlation ($r^2 = 0.7$, p-value = 0.04) observed between the DNRA/denitrification ratio and the in-situ nitrate deficit, also observed by Dong et al. (2009), suggests that DNRA is favored over denitrification as the nitrate deficit increases in hydrothermal vent fluids.

We also found a significant relationship between anammox rates and the DIN deficit. However, this relationship should be interpreted with caution because of the limited number of measurements available for comparison (Fig. 7b). Previous studies have found that anammox was stimulated by ammonium, nitrite and/or nitrate additions (e.g., Trimmer et al., 2005). No correlation was found between annamox rate and ammonium deficit in this study. In fact, net ammonium production seemed to occur, as indicated by a general ammonium surplus at most sites, probably through DNRA or organic matter decomposition. Our results suggest that, if anything, NO_x rather than NH_4^+ modulates anammox rates in the vent fluids. Additionally, it should be noted that, in agreement with most recent observations from an eutrophic Lake in Switzerland (Wenk et al., 2012) we were able to measure low anammox rates, despite the high hydrogen sulfide concentration of up to 1000 µM in the fluids, suggesting that anammox growth is not completely inhibited by H_2S, as previously suggested by Jensen et al. (2008).

Our molecular results suggest that sulfide-oxidizing denitrifiers represent a relatively large fraction of the total bacterial community at some subsurface hydrothermal environments. Sulfur driven autotrophic denitrification has been reported to be a significant process in other hydrothermal systems (Lopez-Garcia et al., 2003 and reference

therein). However, the lack of a significant relationship observed between denitrification rates and [H_2S] suggests that hydrogen sulfide concentrations are not limiting denitrification. Furthermore, we cannot exclude that a portion of the total denitrification in hydrothermal vent fluids is heterotrophic, as high heterotrophic denitrification rates have been measured in a similar (cold seep) environment (Bowle and Joye, 2011).

The fact that no correlation was observed between total microbial abundance (measured by DAPI counts and qPCR) and denitrification rates suggests that, overall, denitrifiers represent only a minor fraction of the total microbial community. Indeed, in most samples, the relative abundance of SUP05 bacteria was less than 10 % (except for Cloud, AV, 2007 (SUP05 = 27 %) and Hulk, ES, 2008 (SUP05 = 38 %) Fig. 6). The lack of significant correlation between denitrification rate and SUP05 abundances is also not surprising, because SUP05 bacteria probably only represent a part of the total denitrifying community. Other potential candidates in our fluids include members of the ε-proteobacteria, as discussed in Sect. 4.2. We also only analyzed the DNA fraction of the microbial community, but not gene expression. However, even if we had quantified mRNA transcripts, there are generally delays observed between enzyme synthesis, expression and formation of denitrification products (e.g. see Baumann et al., 1996), such that it is generally difficult to directly relate gene transcriptional activity to denitrification rate measurements.

Other factors, e.g. pH and temperature, have been shown to influence N loss rates (e.g., Bradley et al., 1992; Opdyke et al., 2007; Zhong et al., 2009). However, in this study, no relationship was found between pH and temperature, and denitrification rates. Interestingly, higher temperatures did not seem to inhibit denitrification, suggesting the potential importance of mesophilic organisms in mediating N-cycle processes in the subsurface biosphere. In fact, the highest denitrification rate ($\sim$1000 nmol l^{-1} N day^{-1}) was measured at Hermosa, where the highest fluid temperature (37 °C) sampled in this study was observed.

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4.4 Total N loss in the subsurface biosphere of diffuse hydrothermal vents

Total N loss fluxes in hydrothermal vent systems are difficult to accurately quantify given the observed high variability of flow rates (e.g., Sarrazin et al., 2009) and our rudimentary knowledge of fluid residence times in the subsurface. Codispoti (2007) first estimated that ~10 Tg N is lost every year during hydrothermal denitrification, assuming a hydrothermal flow associated with ridges and ridge flanks of 2.4×10^{16} kg yr^{-1} (Schultz and Elderfield, 1997), an initial average nitrate concentration of 30 µM, and assuming that denitrification occurs along the entire flow pathway. The latter assumption in particular may not be reasonable, as it includes the high-T portion of the HV systems. Hence this is likely a gross over-estimate of total net N loss in hydrothermal systems. On the other hand, Codispoti (2007) did not consider N$_2$ fixation, which would add new nitrate to HV fluids and which could compensate in part for N loss by denitrification, thus allowing an even larger gross N loss. N$_2$ fixation has indeed been reported to occur in hydrothermal vent fluids of the Juan de Fuca Ridge (e.g., Mehta and Baross, 2006).

Given recent advances in our understanding of the important role of the subsurface biosphere of diffuse hydrothemal vents in modulating geochemical fluxes in the deep ocean (e.g., Wankel et al., 2011; Bourbonnais et al., 2012a), we attempt here to improve upon Codispoti's estimate and at least derive a possible range of values for gross global marine N loss occuring in the subsurface biosphere, based on denitrification rates measured in this study and current knowledge of hydrothermal flow and fluid residence times in the subsurface. We used the following equation to derive gross global N loss (in Tg yr^{-1}) in the subsurface biosphere of hydrothermal vents:

$$\text{N loss (Tg N yr}^{-1}) = \text{denitrification rate} \times \text{volume flux} \times \text{residence time} \qquad (5)$$

We used an average denitrification rate of 240 nmol l^{-1} day^{-1}, representative of all vent fields and years derived in this study and a hydrothermal fluid volume flux of 6.3×10^{15} to 1.6×10^{16} l^{-1} yr^{-1}, considering both axial (10 % at 350 °C and 90 % at 5 °C) and off-

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axial (5–15 °C) hydrothermal flows (Elderfield and Schultz, 1996). We based our range of values for residence times on data from Johnson et al. (2010) and Foustoukos et al. (2009). Johnson et al. (2010) calculated a crustal residence time of 2.1 yr (assuming 10 % porosity) for the across axis flow in the Endeavour Segment axial valley, Juan de Fuca Ridge, using conductive heat flow measurements. Foustoukos et al. (2009) estimated a crustal residence time of few ten's of hours for the very shallow circulation cells feeding low-T diffuse vents adjacent to the Endeavour high-temperature fields, using CO_2/CO equilibrium data. For our calculations, we assumed a range of residence times in the subsurface of 24 h to 2 yr, and assumed that only 5 % to 25 % of the subsurface hydrothermal system, including the recharge zone, is microbiologically active. Using this approach, we estimated that a range from 0.001 to 9.9 Tg N yr^{-1} could be removed globally by denitrification in hydrothermal vent systems.

Coincidently, our upper range of ~10 Tg yr^{-1} for N loss in hydrothermal systems is the same as reported by Codispoti (2007), although this previous study did not measure denitrification rates. Our upper estimate is relatively low, when compared to estimates of the global ocean fixed N sink by benthic and water-column denitrification between ~240 to more than 400 Tg N yr^{-1} for the global ocean (Gruber, 2004; Codispoti, 2007). We should note that our high-end estimate may still be conservative, as it assumes that all denitrifying organisms are in the flowing water (i.e., not attached to surfaces in the flow path; e.g., microbial mats). Our results nonetheless confirm the possible role of the subsurface biosphere in modulating geochemical fluxes in general, and fixed N fluxes, in particular.

5 Summary and final remarks

In this study we report, for the first time, potential denitrification, anammox and DNRA rates in diffuse hydrothermal vent fluids of the Juan de Fuca ridge. The activity of denitrifying bacteria in this subsurface biosphere and its prevalence over anammox and DNRA were confirmed by ^{15}N paired isotope experiments at 13 different sites at Axial

Volcano and the Endeavour Segment on the Juan de Fuca Ridge. Potential denitrification rates in subsurface hydrothermal vent fluids varied strongly in space and with time. The high denitrification potential observed at some sites (up to ~1000 nmol N l^{-1} day^{-1}, Hermosa, AV) indicates that denitrification is a significant N sink in the subsurface biosphere. Cloning and sequencing of the 16S rRNA genes revealed the dominance of ε and γ proteobacteria in hydrothermal vent fluids, with the potential to oxidize sulfide or hydrogen, using nitrate as terminal electron acceptor. Our qPCR results indicated that a single γ-proteobacteria, related to the sulfur-oxidizing, nitrate-reducing SUP05 bacteria, contributed up to 38 % of the total bacterial population at some sites (Hulk, ES, 2008), consistent with an important role for chemolithotrophic denitrification in hydrothermal vent fluids. While this study suggests that denitrification, possibly coupled to sulfide oxidation, is an important process in hydrothermal vents of the Juan de Fuca Ridge, more phylogenetic analysis of functional genes related to denitrification (e.g., *nirS*, *nirK*, *nosZ*) will be essential to better documenting and understanding the denitrifying microbial communities in the subsurface biosphere.

Our understanding of environmental controls on N-transformations in hydrothermal fluids is still very limited. In this study, denitrification, DNRA and anammox rates did not appear to be affected by most measured physico-chemical factors. Significant negative linear relationships were only observed between denitrification, anammox rate and in-situ nitrate and DIN deficits, respectively. A positive linear relationship was also observed between the DNRA/denitrification ratio and in-situ nitrate deficit, suggesting that DNRA is favored over denitrification when the nitrate deficiency increases. This implies that bioavailable inorganic N availability may ultimately regulate N loss and transformations in the subsurface biosphere of hydrothermal vents of the Juan de Fuca Ridge.

Combining potential rates from this study with published data on hydrothermal vent fluid residence times in the subsurface and annual seawater fluxes through these systems, we estimated that, up to ~10 Tg N yr^{-1} could be removed by denitrification in the subsurface of the world's hydrothermal systems, suggesting that hydrothermal vent denitrification represents a minor, but significant fraction, of total marine fixed N loss.

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Table 1a. Physico-chemical and microbiological properties of the sampled vent fluids at Axial Volcano (AV) and the Endeavour Segment (ES) on the Juan de Fuca Ridge. Values shown are means ± one standard deviation for all samples collected from a given vent on each cruise. 2008a and 2008b refers to samples collected during the June and August 2008 cruises, respectively.

Field	Vent	Year	Latitude	Longitude	Depth (m)	n	Temp. (°C)	pH	$[Mg^{2+}]$ (mmol kg^{-1})	$[N_2O]$ (nmol l^{-1})	$[H_2S]$ (µmol l^{-1})
AV	Bag City	2007	45.92	129.99	1533	3	13.7 ± 0.9	6.5 ± 0.1	50.5 ± 1.3	na	39.3 ± 19.3
AV	Cloud Pit	2007	45.93	129.98	1521	3	6.5 ± 0.2	6.9 ± 0.1	52.4 ± 0.1	na	76.5 ± 130.3
AV	Marker 113	2007	45.92	129.99	1523	3	31.2 ± 0.4	5.7 ± 0.2	49.9 ± 0.9	na	1324.6 ± 71.1
AV	Gollum	2007	45.93	130.01	1544	2	22.0 ± 0.5	5.4	50.5 ± 0.1	na	154.3 ± 24.5
AV	Cloud Pit	2008b	45.93	129.98	1522.0	4	6.8 ± 0.0	6.9 ± 0.0	52.6 ± 0.0	na	1.9 ± 0.3
AV	Marker 33	2008b	45.93	130.98	1520.0	4	18.5 ± 2.1	5.8 ± 0.1	47.8 ± 0.7	na	434.0 ± 43.9
AV	Bag City	2008b	45.92	129.99	1532.0	3	11.2 ± 0.0	6.5 ± 0.2	51.5 ± 0.1	na	55.3 ± 48.4
AV	Marker 113	2008b	45.92	129.99	1521.0	3	23.5 ± 3.5	6.0 ± 0.1	51.1 ± 0.6	na	947.0 ± 176.3
AV	Gollum	2009	45.93	130.01	1542.0	5	14.2 ± 1.2	5.7 ± 0.0	51.7 ± 0.2	256.8 ± 9.4	89.6 ± 11.6
AV	Marker 113	2009	45.92	129.99	1521.0	4	29.9 ± 5.0	6.1 ± 0.1	50.8 ± 0.4	11.3 ± 9.4	1020.0 ± 194.2
AV	Marker 33	2009	45.93	129.98	1520.0	4	34.4 ± 0.9	5.5 ± 0.0	41.9	214.4 ± 0.4	711.7 ± 19.0
AV	Hermosa	2009	45.93	129.98	1519.0	3	37.0 ± 4.1	5.6 ± 0.3	51.1 ± 0.9	78.0 ± 4.3	253.2 ± 160.3
AV	Bkgd	2009	45.90	130.00	> 1200	6	3.0 ± 0.5	7.1 ± 0.4	52.4 ± 0.3	na	0.4
ES-MEF	Easter Island	2008a	47.95	129.10	2199	2	20	na	na	na	na
ES-MEF	Hulk	2008a	47.95	129.10	2201	2	25	na	na	na	na
ES	Clam bed	2008a	47.96	129.09	2188	1	21.2 ± 4	na	na	na	na
ES-MEF	Easter Island	2008b	47.95	129.10	2197	4	16.6 ± 3.6	6.7 ± 0	50.8 ± 0.1	na	11.9 ± 3.7
ES-MEF	Hulk	2008b	47.95	129.10	2197	4	16.4 ± 1.7	6.5 ± 0.1	50.7 ± 0.6	na	92.4 ± 8.2
ES-M	Cauldron	2008b	47.93	129.11	2249	3	36.3 ± 4.7	6.4 ± 0.1	47.1 ± 0.5	na	219.6 ± 61.0
ES-HR	Godzilla	2008b	47.97	129.09	2135	3	21.7 ± 8.6	6.2 ± 0.3	49.6 ± 1.7	na	169.6 ± 126.5
ES-HR	Fairy Castle	2009	47.97	129.09	2157.0	2	23.4 ± 1.8	6.2	49.3 ± 0.2	346.5 ± 4.3	245.0 ± 35.4
ES-M	Phang	2009	47.92	129.11	2277.0	5	24.1 ± 1.7	6.5 ± 0.3	47.5 ± 2.0	107.0 ± 14.6	255.6 ± 133.6
ES-MEF	Hulk	2009	47.95	129.10	2198.0	4	29.5 ± 10.6	6.3 ± 0.3	48.3 ± 1.9	BDL	212.5 ± 132.7
ES	Bkgd	2009	48.00	129.10	> 2000	8	2.2 ± 0.3	7.3 ± 0.2	52.7 ± 0.4	na	1.0

Table 1b. Physico-chemical properties of the sampled vent fluids at Axial Volcano and the Endeavour Segment on the Juan de Fuca Ridge.

Field	Vent	Year	$[PO_4^{3-}]$ (μmol l^{-1})	$[NO_3^- + NO_2^-]$ (μmol l^{-1})	$[NO_3^-]$ deficit (μmol l^{-1})	$[NH_4^+]$ (μmol l^{-1})	$[NH_4^+]$ deficit (μmol l^{-1})	DIN deficit (μmol l^{-1})	Cell count/ ml
AV	Bag City	2007	3.5 ± 0.5	8.6 ± 2.7	31.4 ± 4.7	1.9 ± 0.7	−1.4 ± 3.0	30.0 ± 4.8	na
AV	Cloud Pit	2007	3.1 ± 0.2	18.1 ± 1.8	23.4 ± 4.2	1.4 ± 0.3	−1.4 ± 3.0	22.0 ± 4.2	na
AV	Marker 113	2007	3.5 ± 0.0	1.6 ± 0.5	37.9 ± 3.7	12.6 ± 0.4	−11.9 ± 2.9	26.0 ± 3.8	na
AV	Gollum	2007	4.2 ± 0.2	26.7 ± 0.4	13.3 ± 3.7	3.1 ± 0.9	−2.6 ± 3.0	10.7 ± 3.8	na
AV	Cloud Pit	2008b	3.2 ± 1.7	16.0 ± 0.4	24.5 ± 3.9	1.4 ± 0.2	−0.3 ± 3.0	24.2 ± 3.9	(5.3 ± 0.4) × 10^5
AV	Marker 33	2008b	3.9 ± 0.9	31.2 ± 1.6	5.6 ± 3.9	1.9 ± 0.6	0.4 ± 2.8	6.0 ± 3.9	(2.0 ± 0.5) × 10^5
AV	Bag City	2008b	2.6 ± 0.1	9.1 ± 3.0	30.6 ± 4.8	1.8 ± 0.1	−0.4 ± 3.0	30.1 ± 4.8	(1.7 ± 0.6) × 10^5
AV	Marker 113	2008b	3.5 ± 0.0	15.8 ± 4.6	23.6 ± 5.9	10.7 ± 3.3	−9.2 ± 4.4	14.4 ± 6.8	(8.0 ± 0.3) × 10^5
AV	Gollum	2009	na	14.1 ± 5.2	16.6 ± 5.2	2.6 ± 0.7	−2.5 ± 3.0	14.1 ± 5.2	(2.6 ± 1.0) × 10^4
AV	Marker 113	2009	na	7.1 ± 3.4	33.1 ± 5.0	10.2 ± 3.1	−9.7 ± 4.2	23.4 ± 5.9	(1.1 ± 0.2) × 10^6
AV	Marker 33	2009	na	15.7 ± 2.0	17.4 ± 3.6	4.4 ± 0.2	−1.6 ± 2.4	15.8 ± 3.7	(2.8 ± 0.5) × 10^5
AV	Hermosa	2009	na	41.7 ± 2.2	−1.3 ± 4.4	1.9 ± 1.1	4.1 ± 3.1	−2.9 ± 4.5	(1.3 ± 0.5) × 10^4
AV	Bkgd	2009	2.7	41.5 ± 3.8	0	0.4 ± 0.3	−0.4 ± 0.3	−0.4 ± 5.4	na
ES-ME	Easter Island	2008a	na	37.9	na	4.3	na	na	(2.3 ± 0.1) × 10^5
ES-ME	Hulk	2008a	na	36.3	na	3.3	na	na	(2.3 ± 0.1) × 10^5
ES	Clam bed	2008a	na	39.9	na	0.9	na	na	(9.2 ± 0.3) × 10^5
ES-ME	Easter Island	2008b	2.5 ± 0.6	17.8 ± 4.7	21.4 ± 5.1	15.2 ± 1.2	0.1 ± 2.6	21.5 ± 5.6	(1.3 ± 0.3) × 10^5
ES-ME	Hulk	2008b	2.4 ± 0.1	29.6 ± 1.3	9.5 ± 3.9	21.7 ± 2.5	−5.8 ± 5.5	3.7 ± 5.1	(2.8 ± 0.5) × 10^5
ES-M	Cauldron	2008b	3.2 ± 0.8	29.2 ± 3.3	7.2 ± 4.8	47.6 ± 3.2	−5.6 ± 5.2	1.6 ± 6.1	(4.7 ± 1.0) × 10^5
ES-HR	Godzilla	2008b	1.5 ± 0.2	34.1 ± 3.1	4.1 ± 5.0	56.3 ± 34.1	−4.5 ± 44.3	−0.4 ± 35.3	(2.6 ± 0.6) × 10^5
ES-HR	Fairy Castle	2009	na	28.0 ± 1.3	10.0 ± 2.3	41.4 ± 3.8	15.5 ± 6.8	25.5 ± 9.6	(6.1 ± 2.2) × 10^4
ES-M	Phang	2009	na	30.4 ± 4.1	6.2 ± 4.7	42.7 ± 20.8	−3.3 ± 25.6	2.9 ± 21.5	(4.4 ± 1.5) × 10^4
ES-ME	Hulk	2009	na	27.8 ± 2.6	9.4 ± 3.6	38.0 ± 20.5	−3.8 ± 25.6	5.6 ± 21.4	(1.0 ± 0.4) × 10^5
ES	Bkgd	2009	3.1	40.6 ± 2.0	0	1.4 ± 2.2	−1.4 ± 2.2	1.4 ± 3.7	(2.5 ± 0.9) × 10^4

Table 2. Bacterial 16S rDNA clone libraries information and OTUs richness (Chao-1) and diversity (Shannon-Wiener) estimates. Sequences with a similarity ≥ 97 % are considered the same OTU. Uncertainties for the Chao-1 estimator (in brackets) and the Shannon-Wiener index represents 95 % confidence intervals.

Vent field	Site	Year	Volume of fluid filtered (l)	Average fluid temp. (°C)	# of clones sequenced	# of OTU	Coverage (%)	Chao-1 estimator	Shannon-Wiener index (H′)
AV	Cloud	2007	3.0	6.8	60	39	50	133 (74–295)	3.47 ± 0.43
AV	Bag City	2008	1.6	11.2	43	26	63	43 (31–82)	3.09 ± 0.25
ES	Hulk	2008	4.0	24.8	60	18	77	60 (31–161)	1.72 ± 0.43
ES	Godzilla	2008	4.0	29	28	19	50	67 (37–179)	2.95 ± 0.29
ES	Phang	2009	2.7	24	27	19	56	34 (25–67)	2.94 ± 0.27

Fig. 1. Map of the hydrothermal vent fields, i.e., Axial Volcano and Endeavour Segment on the Juan de Fuca Ridge (North-East Pacific Ocean), indicated by red stars. See Table 1 for precise location of the sampled vent sites.
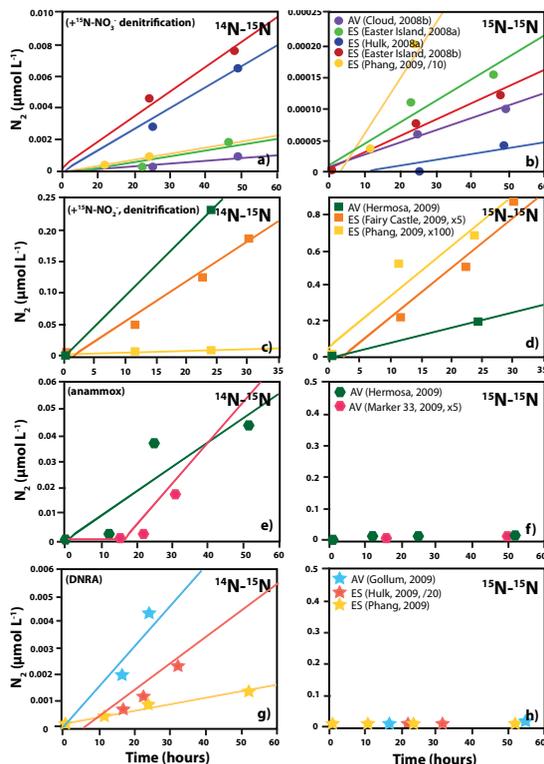
Fig. 2. Production of $^{14,15}N_2$ and $^{15,15}N_2$ over time for selected ^{15}N-labelled incubations: addition of $^{15}NO_3^-$ **(a, b)**, $^{15}NO_2^-$ **(c, d)**, $^{15}NH_4^+$ **(e,f)** and $^{15}NO_3^-$ followed by the addition of hypobromite (see text for more detail) **(g, h)** to measure potential denitrification **(a)**, anammox **(b)**, and DNRA **(c)** rates in diffuse hydrothermal vent fluids. All N_2 values were normalized to $0\ \mu mol\ l^{-1}$ at time $t = 0$. Values at some sites were multiplied or divided by 5, 10, 20, or 100 times (numbers in brackets) to fit on the same scales.
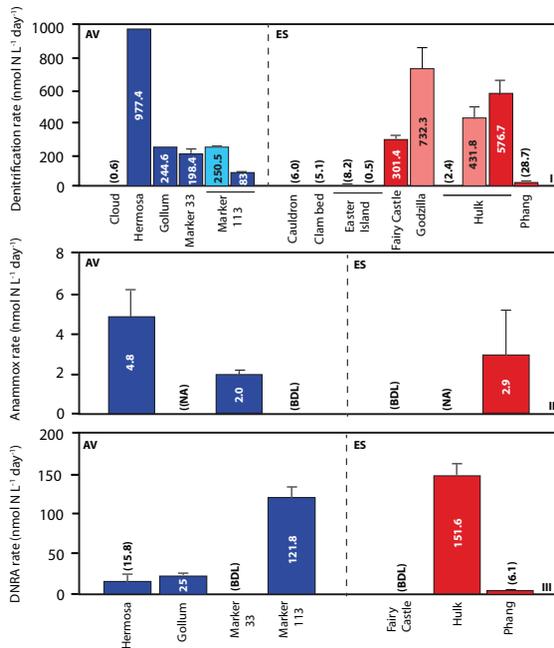
Fig. 3. Denitrification (I), anammox (II) and DNRA (III) rates in nmol N l^{-1} day^{-1} at Endeavour Segment (ES) and Axial Volcano (AV) on the Juan de Fuca Ridge. The average denitrification and DNRA rates obtained from both $^{15}NO_3^-$ and $^{15}NO_2^-$-labelled incubations during the 2009 cruise are indicated when linear relationships were obtained (i.e., at Marker 113, Marker 33, Fairy Castle, Phang and Hulk). Note the different scales for the y-axis. Numbers inside bars or in brackets are actual values. Color of the bar represents the sampled vent field (i.e., AV = blue and ES = red) and color intensity (i.e., no color, medium and dark) represents sites sampled during the June 2008 (always low rates, i.e., Clam bed, Easter Island and Hulk (first bars)), August 2008 and August 2009 cruises, respectively. Vertical lines represent the error propagation of the standard errors of the slopes of the linear relationships represented in Fig. 2 (i.e., $^{14,15}N_2$ and/or $^{15,15}N_2$ versus time).

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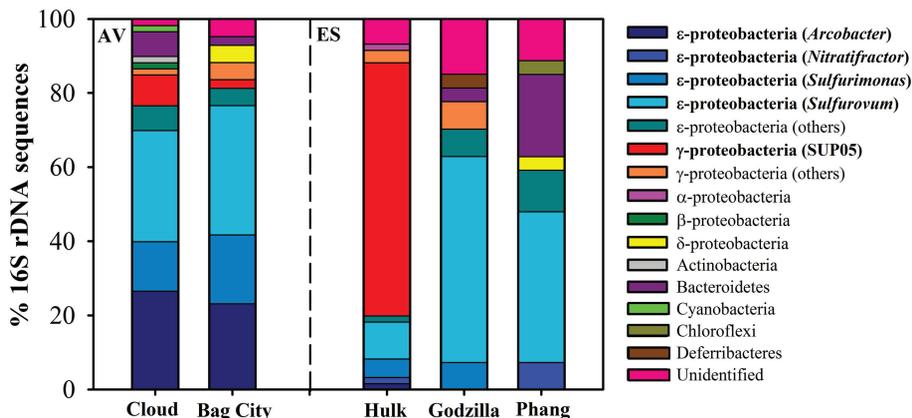
Fig. 4. Composition of the 16S rRDA clone libraries recovered from DNA extracted from 5 DNA samples from diffuse vent fluids at Axial Volcano (AV) and the Endeavour Segment (ES) on the Juan de Fuca Ridge: Cloud (AV07-CP), Bag City (AV08-BC), Hulk (ES08-H), Godzilla (ES08-G), and Phang (ES09-P) (see Table 2). SUP05 and other potential nitrate reducing bacteria are in bold. See Table 2 for more information about DNA samples and 16S rDNA clone libraries.

γ-proteobacteria

Clone AV08-BC-14 (+1 clone)
Clone ES08-H-11
54
Riftia pachyptila endosymbiont [U77478]
Clone ES08-H-4
Clone AV07-CP-11
52
Thiohalomonas denitrificans strain HLD 4 [EF117911]
Eastern North Pacific clone J8P41000_1C09 [GQ351125]
Vailulu'u Seamount (South Pacific) clone VS_CL-161 [FJ497410]
Southwest African Shelf (Namibia) clone GSO_6 [FM246509]
100
Southwest African Shelf (Namibia) clone GSO_2 [FM246516]
South Pacific Ocean (Volcano 19, Tonga Arc) [FJ905679]
Clone AV07-CP-1 (+4 clones)
Clone ES08-H-18 (+40 clones)
Clone AV08-BC-24
Sannich Inlet clone SHBH489 [GQ346053]
68
Sannich Inlet clone FGYC_49P14 [GQ351268]
100
Bathymodiolus sp. thiotrophic endosymbiont [AJ745718]
91
Suiyo Seamount hydrothermal plume clone SUP05-21 [AB112459]
100
64
Nitinat Lake clone Nit2A0626_237 [FJ628192]
Saanich Inlet clone SHAN672 [GQ346328]
53
Maorithyas hadalis gill thioautotrophic symbiont [AB042413]
Bathymodiolus thermophilus gill symbiont [M99445]
100
Bathymodiolus azoricus thioautotrophic gill symbiont [AY235676]
Candidatus Vesicomyosocius okutanii HA [NC009465]
Candidatus Ruthia magnifica str. Cm [CP000488]
100
Southwest African Shelf (Namibia) clone GSO_3 [FM246508]
100
Southwest African Shelf (Namibia) clone GSO_1 [FM246510]

SUP05

58

51

100

Antarctic clone ANT10A4 [DQ925854]
Saanich Inlet clone SHAB450 [GQ346479]
Saanich Inlet clone FPPX_45A23 [GQ369726]
97
Southwest African Shelf (Namibia) clone GSO_4 [FM246511]
Eastern North Pacific Ocean clone J8P41000_TH08 [GQ351096]
Arabian Sea clone A723905 [AY907823]
100
Arabian Sea clone A723009 [AY907822]
Monterey Bay clone EBAC080-L31E09 [AY458646]
94
Arctic clone Arctic96BD-19 [AF354606]
Rimicaris exoculata ectosymbiont clone LOG_283/7-4_c9 [FM203390]

ARCTIC96BD-19

100
Clone ES08-G-23
100
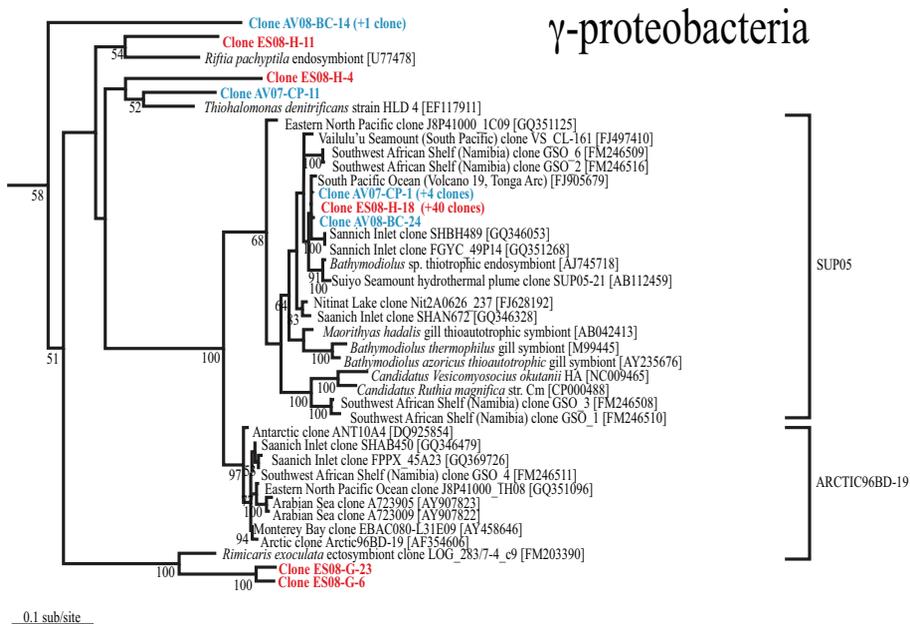Clone ES08-G-6

0.1 sub/site

Fig. 5. Partial 16S rDNA γ-proteobacteria phylogenetic tree constructed using the maximum likelihood method implemented in PHYML. Clone prefixes were assigned as follows: the first letters indicate the vent fields: AV = Axial Volcano (in blue) and ES = Endeavour Segment (in red), followed by the year (07 = 2007 and 08 = 2008) and the last letters the diffuse vent sampled: CP = Cloud Pit, BC = Bag City, H = Hulk, and G = Godzilla. The last numbers indicate clones #. The number of clones that are ≥ 97 % identical to a given hydrothermal vent clone is indicated in parentheses. GenBank accession numbers are provided (in brackets) for all other clones not sequenced in this study. The percentage of 100 bootstrap resamplings above 50 % is indicated. The scale bar indicates the number of amino acid substitutions per site.

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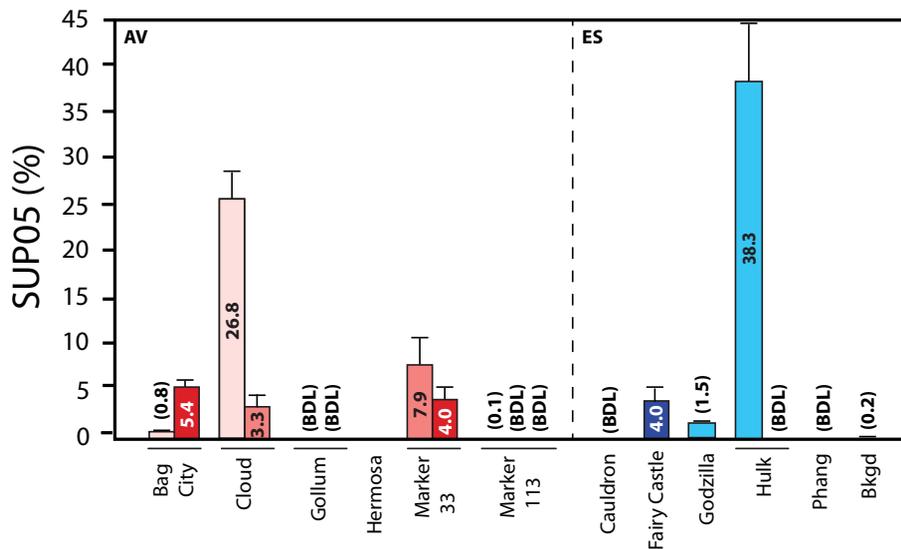
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Fig. 6. SUP05 relative abundance (in %, relative to 16S rRNA gene copy number/ml seawater) at Axial Vocano (AV) and the Endeavour Segment (ES) on the Juan de Fuca Ridge. Same color sheme as in Fig. 3 (paler colors represent samples from the 2007 cruise). Actual values are indicated inside the bars or within parentheses. Bkgd is a background sample that was collected near the Endeavour Segment in 2009. BDL means Below Detections Limit. See Table 1 for chemico-physical properties of sampled sites.
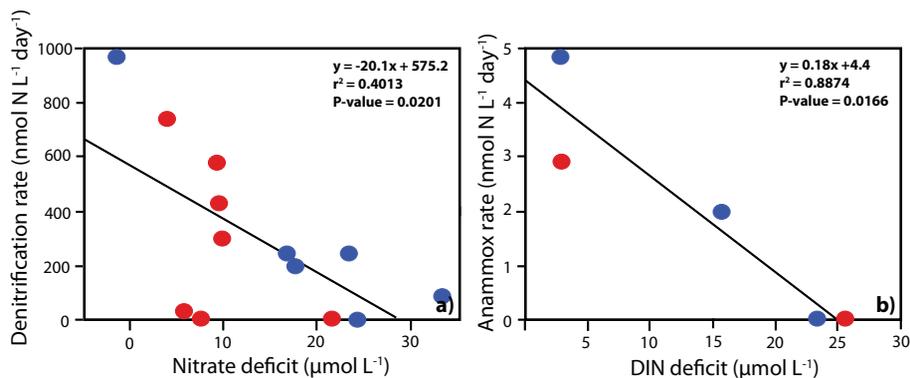
Fig. 7. Relationships between denitrification rate and nitrate deficit **(a)** and anammox rate and total DIN deficit **(b)** in hydrothermal vent fluids of the Juan de Fuca Ridge. See text for detail. Hydrothermal vent fluids sampled from Axial Volcano and the Endeavour Segment are indicated in blue and red, respectively. R^2 and p-value for the linear regression are indicated.