"Identification of active denitrifiers by DNA-Stable Isotope Probing and amplicon sequencing reveals *Betaproteobacteria* as responsible for attenuation of nitrate contamination in a low impacted aquifer"

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Abstract

Groundwater reservoirs constitute important freshwater resources. However, these ecosystems are highly vulnerable to contamination and have to rely on the resident microbiota to attenuate the impact of this contamination. Nitrate is one of the main contaminants found in groundwater and denitrification is the main process that removes the compound. In this study, the response to nutrient load on indigenous microbial communities in groundwater from a low impacted aquifer in Uruguay was evaluated. Denitrification rates were measured in groundwater samples from three different sites with nitrate, acetate and pyrite amendments. Results showed that denitrification is

feasible under *in situ* nitrate and electron donor concentrations, although the lack of readily available organic energy source would limit the attenuation of higher nitrate concentrations. DNA Stable-isotope probing (SIP), combined with amplicon sequencing of 16S rRNA, *nirS* and *nirK* genes, was used to identify the active denitrifiers. Members of the phylum *Betaproteobacteria* were the dominant denitrifiers in two of three sites, with different families being observed; members of the genus *Vogesella* (*Neisseriaceae*) were key denitrifiers at one site, while the genera *Dechloromonas* (*Rhodocyclaceae*) or *Comamonas* (*Comamonadaceae*) were the main denitrifiers detected at the other sites.

Introduction

Aquifers are key freshwater resources and their water quality represents an important environmental issue worldwide. Domestic, industrial and agricultural activities rely on the quality of groundwater, which is strongly linked to the geochemical processes modulated by the activity and diversity of indigenous microbiota (Griebler and Lueders 2009). Nitrate (NO_3^-) is one of the key contaminants that affect groundwater quality (Rivett 2008). Unlike other pollutants that usually reach the subsurface by defined, well-localized sources, nitrate load in aquifers occurs continuously and in a diffusive way, making both contamination control and removal difficult. Thus, natural attenuation will be a key process in the regulation of nitrate concentration. Denitrification is the metabolic process that consumes the largest amount of nitrate in subsurface (Rivett 2008; Weymann 2008). Despite its importance, factors that limit denitrification *in situ,* such as geochemical conditions,

remain unclear, especially for aquifers with lower pollution levels (Rivett 2008; Eschenbach 2014).

During complete denitrification, nitrate is used as a terminal electron acceptor and it is reduced to molecular nitrogen (N_2) after passing through several reduction states. The reduction of nitrite (NO_2^-) to nitric oxide (NO) is considered a key step for denitrifiers, since the nitrogen goes from a dissolved anion state to gaseous state and can be easily removed from the system (Tiedje 1988). Several geochemical factors are involved in the regulation of the process, such as electron donor composition and availability, oxygen concentration, pH, temperature, microbial acclimation, salinity, presence of inhibitory substances, although electron donor and oxygen concentration are considered the main limiting factors (Smith and Duff 1988; Santoro 2006; Rivett 2008). The variety of electron donors used for denitrification is broad and both organotrophic and lithotrophic processes occur in aquifers (Smith and Duff 1988; Rivett 2008; Weymann 2008; Torrentó 2011).

Denitrification is a metabolic trait that is widespread among bacteria, archaea and fungi (Shoun 1992; Philippot 2007). Therefore, analysis of ribosomal RNA genes alone may not be suitable for assessing denitrifier diversity. The use of nitrite-reductase encoding genes, *nirS* and *nirK,* as biomarkers in combination with ribosomal genes allow the assessment of both functional diversity and the phylogenetic affiliation of denitrifiers.

Stable Isotope Probing (SIP) of nucleic acids and fatty acids is a technique that has been used since 2000 for the identification of active populations within a microbial community in a specific metabolic process (Neufeld

2007b). It has enabled the study of a wide diversity of processes, such as syntrophic interactions for propionate oxidation (Lueders 2004), methylated compounds assimilation (Neufeld 2007a; Antony 2010) and identification of acetate-utilizing microbes (Osaka 2006; Schwarz 2007) and pollutants degraders (Singleton 2005; Sun 2012). Denitrification has also been studied using SIP technique coupled with the analysis of both ribosomal and denitrification genes, with the utilization of labelled carbon compounds such as acetate, succinate and/or methanolas substrates for nitrate reduction in coastal sediments, paddy soil and activated sludge (Maneesha 2004; Ginige 2005; Osaka 2006; Osaka 2008; Saito 2008). However, reports on the use of SIP (both DNA and SIP coupled to Fatty Acids Analysis) to study denitrification in aquifers are scarce and are more frequently linked to the degradation of pollutants such as aromatic or complex compounds (Pelz 2001; Fischer 2016).

Our study site, Raigón aquifer, is a sand and gravel groundwater system located in south-west Uruguay. Nitrate levels in this groundwater reservoir will eventually increase due to nearby agricultural and industrial activities. A previous study evaluated the potential for denitrification by groundwater microbial communities from the Raigón aquifer and characterised more than 50 denitrifiers isolated from several sampled wells (Bellini 2013). This study describes the use of DNA-SIP combined with amplicon sequencing of 16S rRNA and *nirS* and *nirK* genes to determine active indigenous denitrifiers and assess their capacity to remove nitrate from this groundwater system in a scenario of an increasing nutrients load.

Materials and Methods

Sample collection and physicochemical parameters determination

Groundwater samples were obtained in July 2012 from three water supply wells (C, D and H) at Raigón aquifer, as previously described by Bellini et al., 2013. Briefly, groundwater samples were collected from water supply wells after three bottle volumes (5 L) were pumped and a constant value of dissolved oxygen was reached. Twenty-five litres of groundwater were collected from each site. Groundwater was transported at 4 $^{\circ}$C, and was processed within 24 h after sampling for all experiments. Groundwater temperature, pH and dissolved oxygen were measured at the site. The sampling wells lie along a 50-kilometre stretch and have depths that lie between 37 and 50 m. The three selected wells cover the range of nitrate (Table 1) concentrations detected along the aquifer and they are representative of various human activities that occur on the surface of the aquifer. In the surroundings of site C, an agricultural site where diary milking and other farming activities occur, while site D is located in a potato cleaning and processing plant. Site H is located in a small town, which relies on septic tanks for sewage treatment.

Nitrate, nitrite, total iron and sulphate were analysed according to the American Public Health Association *Standard Methods for the examination of Water and Wastewater* methods (APHA 1998), with 4500-NO_3- B, 4500-NO_2- B, 3500-Fe-B Phenanthroline Method and 4500 methods, respectively. Total organic carbon was analysed with a Shimadzu TOC-VSCN analyser and the limit of quantification was 1.1 mg L^{-1}.

Denitrification potential of groundwater samples

The potential for nitrate attenuation of indigenous microbial community was tested by measuring denitrification rates in groundwater samples with different amendments and compared to endogenous rates (i.e. without amendment). Rates were measured in groundwater obtained from wells C, D and H with the acetylene blockage technique (Mahne and Tiedje 1995). Triplicate sterile 120 mL-flasks were flushed with filtered O_2-free N_2 while being filled aseptically with 40 mL of groundwater. Nitrate plus either acetate or pyrite (crushed and sieved to particles of <65 μm) were added to compare organotrophic and lithotrophic denitrification capacity, respectively. In addition, flasks with only nitrate amendment (containing between three and 12 times the nitrate concentration measured in the wells), non-amended flasks and acetate or pyrite-amended sterile controls (autoclaved) were also set up for each well. Acetate was chosen as the carbon amendment since it is a non-fermentable substrate and other parallel processes besides denitrification would be unlikely, due to incubation conditions. In addition, most of the previously isolated bacteria from the Raigón aquifer were able to use it as a carbon and electron source for denitrification (Bellini 2013). Acetate and pyrite were added according to a complete NO_3^- reduction stoichiometry. Every set of triplicate flasks contained (final concentration): phosphate buffer (1 mmol L^{-1}, pH 7.1) and either 2 mmol L^{-1} KNO_3 and 1.5 mmol L^{-1} ammonium acetate (nitrate plus acetate amended), 2 mmol L^{-1} KNO_3, 1200 mg L^{-1} pyrite and 0.5 mmol L^{-1} Na_2CO_3 (nitrate plus pyrite amended) or 2 mmol L^{-1} KNO_3 (nitrate amended). Acetylene was added (10% of the headspace) to every flask and flasks were incubated in the dark

with continuous shaking at 20 °C. Samples from the headspace of every flask were removed at several time points for N_2O measurement by gas chromatography (Shimadzu GC-2014 gas chromatograph); gas chromatography conditions and calculations of denitrification rates were done as described in Bellini et al., 2013. Nitrate and nitrite concentrations were also measured during the incubations by HPLC as described by Tarlera and Denner 2003. To test if denitrification rates from different treatments or wells were significantly different, a squared root transformation was applied to rates and then compared with an analysis of variance (ANOVA) using $p \leq 0.05$ and Tukey correction with InfoStat software (Di Rienzo 2009). All transformed data satisfied the ANOVA assumptions.

Assessment of groundwater basal communities: 16S rRNA gene amplicon sequencing

To estimate the structure of the naturally occurring bacterial communities present in the sampled groundwater, 20 L of groundwater were filtered through a 0.22 μm pore cellulose acetate membrane. DNA was then extracted from biomass using the PowerWater® DNA Isolation Kit (MoBio Laboratories, Inc., CA, USA). For amplicon libraries, the 16S rRNA gene was amplified with PCR primers 563F and 802R (Cole 2009). PCR amplicons were purified using Agencourt AMPure XP beads (Agencourt Bioscience Corporation, MA, USA) and sequenced at the INDEAR sequencing facilities (Rosario, Argentina) on a Genome Sequencer FLX (454-Roche Applied Sciences) according to the manufacturer's instructions.

Stable Isotope Probing incubations, gradient centrifugation and DNA extraction

SIP incubations were set up in 150 mL microcosms. Two replicates, named A and B, were prepared for each sampled site and treatment; 70 mL of groundwater were aseptically placed in sterile flasks containing (final concentration): phosphate buffer (1 mmol L^{-1}) and KNO_3 (2 mmol L^{-1}) while flushed with O_2-free N_2. Organotrophic treatment flasks were amended with 1.5 mmol L^{-1} (final concentration) of ^{13}C-labelled sodium acetate ($Na^{13}CH_3COO$); granulated pyrite (particle size <65 μm) and $Na_2^{13}CO_3$ were added to lithotrophic treatment flasks to a final concentration of 1200 mg L^{-1} and 0.5 mmol L^{-1}, respectively. In order to confirm isotopic enrichment of DNA in ^{13}C incubations, ^{12}C unlabelled controls were set up for every treatment. Controls with only acetate were not included because other parallel processes (fermentation, heterotrophic anaerobic respiration, lithotrophic anaerobic denitrification) are unlikely or marginal in the incubation conditions. In addition, determination of the denitrification rates and the SIP incubations showed that the amounts of acetate and nitrate consumed were stochiometric corresponding to a denitrification processes (see below). Incubations were carried out in the dark at 20 °C with continuous shaking. Nitrate and nitrite were monitored in every flask as described before. Acetate consumption at the end of the incubation was determined by HPLC, with an Aminex-87H column and 5 mmol L^{-1} sulphuric acid as the mobile phase. Runs were carried out at 35 °C and UV detection at 210 nm was used. Biomass was harvested by centrifugation when at least 90% of added nitrate and the stoichiometric amount of acetate had been consumed. Pellets were frozen at -20 °C until DNA extraction.

DNA was extracted from cell pellets using a modification of the protocol described in Neufeld 2007a. Briefly, 200 µL of SET buffer (40 mmol L^{-1} ethylenediamine tetra-acetic acid, 50 mmol L^{-1} Tris-HCl pH 9.0 and 0.75 mmol L^{-1} sucrose) and 22.5 µL of freshly prepared lysozyme solution (9 mg mL^{-1}) were added to the pellets. After incubation at 37 °C for 30 min, 25 µL of 10% (w/v) SDS and 7 µL of a fresh solution of proteinase K (20 mg mL^{-1}) were added and the tubes were incubated at 55°C for 2 h. Following the addition of 125 µL of SET buffer, the lysates were transferred to a phase-lock tube (Eppendorf, Hamburg, Germany) and two extractions with 375 µL of phenol:cholorform:isoamylalcohol (25:24:1) were performed. DNA in the aqueous phase was precipitated with 2 µL of glycogen (Roche, Basel, Switzerland), 170 µL of a 7.5 mol L^{-1} ammonium acetate solution and 700 µL of 95% ethanol, overnight at -20 °C. Tubes were centrifuged at 21 000g and the pellets were washed twice with 500 µL 80% (v/v) ethanol. After drying, DNA was re-suspended in 50 µL of sterile water. DNA was run on an agarose gel and concentration and quality were analyzed with a NanoDrop 2000 spectrophotometer. Ultracentrifugation, fractionation and precipitation of DNA were carried out according to Neufeld 2007b. Twelve or thirteen fractions, were collected from ^{13}C duplicates and from the ^{12}C control flask from each well. The buoyant density of each fraction was determined indirectly by measuring the refractive index (nD-TC) with a digital refractometer (Reichert AR200, Reichert Inc., NY, USA). Those fractions with the highest densities for which a positive 16S rRNA gene amplification was obtained (see later) were selected for further analysis and designated heavy fractions (^{13}C-DNA, densities between 1.7211 and 1.7269 g mL^{-1}). Fractions

with densities of 1.7035 to 1.7094 g mL^{-1} were designated and analysed as light fractions (^{12}C-DNA).

SIP incubations follow up: DGGE analysis of 16S rRNA genes

In order to confirm that heavy fractions from ^{13}C incubations showed a different community pattern than light fractions from ^{13}C incubations and than heavy fractions from ^{12}C incubations, denaturing gradient gel electrophoresis analysis (DGGE) of 16S rRNA genes was carried out. In addition, DGGE was performed to compare the structure of communities from replicated SIP incubations on non-fractionated DNA from each replicate and well.16S rRNA gene PCR primers for *Bacteria* were 341F-GC(5'-CGCCCGCCGCGCGCGG CGGGCGGGGCGGGGGCACGGGGGGGCCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') (Muyzer 1993); PCR reactions were carried out in a total volume of 50 µL and contained 2 mmol L^{-1} MgCl$_2$, 0.2 mmol L^{-1} dNTP, 0.2 µmol L^{-1} of each primer, 2.5 U of DreamTaq DNA Polymerase (Fermentas, Burlington, Ontario, Canada), 5 µL 10X Taq buffer, 0.07% BSA and 30 ng DNA. Cycling conditions included an initial step of 5 min at 94 °C followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, with a final extension step of 72 °C for 8 min. PCR products were checked for size and purity on agarose gels and the concentration was estimated by comparison to a 1 kb ladder (Fermentas, Burlington, Ontario, Canada). Approximately 300 ng of PCR product were loaded in each lane of an 8% (w/v) polyacrylamide gel with a gradient of denaturants (urea and deionized formamide) that ranged from 20 to 60%. Electrophoresis was performed using a D-Code System (Bio-Rad, Hercules, CA, USA) on TAE 1X buffer at 60 °C for 16 h at 80 V. The gel was stained with SYBR Gold Nucleic

Acid Gel Stain (Invitrogen, Paisley, UK) for 1 h and visualized with an imaging device. The predominant bands from heavy fractions were excised and 16S rRNA gene was amplified as previously described, with the exception that the primers used did not have a GC-clamp. Amplicons were sequenced by Sanger method at Source BioScience, Nottingham, UK. Sequences were used in a BLAST search (Altschul 1997) and deposited in GenBank with the accession numbers KY548747 to KY548762.

The potential role of archaeal communities in denitrification during SIP incubations was also tested. A nested PCR of the 16S rRNA gene specific for *Archaea* domain (Cunliffe 2008) was performed on heavy and light fractions of DNA isolated from every well and only weak amplifications were observed in heavy fractions from site H.

Amplicon sequencing from SIP fractions

DNA from heavy and light fractions from ^{13}C incubations was used as a template for triplicate PCR amplification of 16S rRNA gene with 503F (5'-GTGCCAGCMGCNGCGG-3') and 1100R (5'-GGGTTNCGNTCGTTR-3') primers. Reaction mixtures were performed in a total volume of 50 µL and contained 2 mmol L^{-1} MgCl$_2$, 0.2 mmol L^{-1} dNTP, 0.3 µmol L^{-1} of each primer, 1.25 U of DreamTaq DNA Polymerase (Fermentas, Burlington, Ontario, Canada) and 5 µL 10X Taq buffer. Cycling conditions included an initial step of 5 min at 94 °C followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, with a final extension step of 72 °C for 10 min.

DNA from heavy and light fractions from replicates A was also used as a template for triplicate PCR amplification of *nirS* and *nirK* genes. The primers

and conditions used were as follows: *nirS* cd3aF/R3Cd (Throback 2004) and *nirK* F1aCu/R3Cu (Hallin and Lindgren 1999); reactions mixtures were prepared in a final volume of 50 µL and contained 2 mmol L^{-1} MgCl$_2$, 0.2 mmol L^{-1} dNTP, 1.25 U of DreamTaq DNA Polymerase (Fermentas, Burlington, Ontario, Canada), 5 µL 10X Taq buffer, 0.2 µmol L^{-1} of each primer for *nirS* gene and 0.5 µmol L^{-1} for *nirK* and 30 ng DNA. Cycling conditions included an initial step of 94 °C for 4 min followed by 30 cycles of 30 s at 94 °C, 1 min at 57 °C and 1 min at 72 °C with a final extension step of 10 min at 72 °C. PCR products were checked for size and purity on agarose gels.

All PCR products were visualized in an agarose gel, replicates were pooled and purified with Agencourt AMPure XP beads (Agencourt Bioscience Corporation, MA, USA) and checked on an agarose gel. PCR products were sent to Mr DNA Molecular Research (Lubbock, TX, USA) for barcoded sequencing where a second 5-cycle PCR was performed to add barcodes and sequencing keys. Following PCR, all amplicons from different samples were mixed in equal concentrations and purified using Agencourt AMPure XP beads (Agencourt Bioscience Corporation, MA, USA)Samples were sequenced using a Roche 454 FLX Titanium instrument and reagents according to the manufacturer's instructions.

Bioinformatic analysis of amplicon sequences

16S rRNA data were analysed using the pipeline available through the QIIME platform, version 1.7 (Caporaso 2010b). Libraries were split using default parameters except that the number of ambiguous bases allowed was one instead of six and primer mismatches allowed was zero. Chimera checking

(*de novo* and reference-based) and OTU picking (0.97 cut-off) were performed with usearch61 (Edgar 2010) and taxonomy assignment was done using the Greengenes 13_5 database with the RDP Classifier (Wang 2007). The representative set was aligned with Pynast software (Caporaso 2010a) against the Greengenes core reference alignment (DeSantis 2006). Finally low abundance OTUs (less than 1% of the dataset) and those that failed to align were filtered from the OTU table. Similar filtering options were used for naturally occurring (basal) communities' data, except only singletons and doubletons were removed. Alpha diversity indexes were calculated within QIIME. Rarefaction analysis of the libraries was performed using the free Analytic Rarefaction 1.3 software (http://strata.uga.edu/software/index.html).

Data from sequencing of denitrification genes were initially split into libraries, followed by a *de novo* chimera checking with usearch61 (Edgar 2010) within the QIIME platform. The split and filtered libraries were then screened for frame shifts using the RDP FunGene (Fish 2013) FrameBot tool (Wang 2013) with a length cut-off of 80 amino acids and an identity cut-off of 0.4. Frame-corrected nucleotide sequences were then clustered (0.03 distance) using uclust (Edgar 2010) within QIIME and an OTU table was constructed. Low abundance OTUs (less that 1% of the dataset) were filtered from the OTU table. Rarefaction analysis of the libraries was performed as described previously. Representative sequences were found for *nirK* and *nirS* libraries and compared with the EMBL/GenBank database using the NCBI Blast algorithm (Altschul 1997). Translated sequences were aligned with MUSCLE at amino acid level. The resulting alignments were checked via comparison with aligned HMM training sequences obtained from FunGene repository

(Functional gene pipeline & repository http://fungene.cme.msu.edu/index.spr) and corrected manually when necessary. Phylogenetic trees were constructed with nucleotide sequences based on the neighbor-joining (NJ) method and maximum likelihood (ML) algorithms to confirm overall topology using MEGA6 (Tamura 2013) software. A bootstrap confidence analysis was performed with 1000 replicates. 303 informative positions were taken into account for the analysis of the *nirS* gene while 272 were used for *nirK* gene.

All datasets have been deposited at MG RAST database under Project *Nitrate contamination in a low impacted aquifer*: *Clean_X_16S_heavy/light* corresponds to 16S rRNA amplicon libraries from SIP heavy/light fraction from site X (ie C, D or H); *Clean_X_nirS/nirK_heavy* corresponds to amplicon libraries from *nirS/nirK* SIP heavy fractions from site X (ie C, D or H); *Clean_X_16S* corresponds to 16S rRNA amplicon libraries from basal groundwater communities from sites C, D or H.

Abundance of nirS *and* nirK *genes as determined in DNA-SIP incubations*

To determine the abundance of *nirS* and *nirK* genes in DNA samples from DNA-SIP incubations, qPCR was performed. PCR products used for standards curves in qPCR experiments were obtained from the isolated denitrifiers AR45 (*Pseudomonas stutzeri*) and AR11 (*Achromobacter sp.*) for *nirS* and *nirK,* respectively (Bellini 2013). PCR products were cloned using the TOPO TA cloning kit (Invitrogen®) following the manufacturer's instructions. Clones were subjected to PCR using primers T3–T7 provided with the cloning kit. Correct fragment length and identity of the PCR product were verified by electrophoresis on a 1.5% agarose gel and sequenced at the Macrogen Sequencing Service, Korea, using the T7 primer. Plasmid was

extracted using the Purelink Quick Plasmid miniPrep kit (Invitrogen®), and the size of the plasmid fragment was verified by electrophoresis in a 1.5% agarose gel. A triplicate amplification of the cloned fragments with T3 and T7 primers was performed. PCR products were pooled, diluted to 90 μL with MilliQ water and purified with MICROCON®100 columns. Following a fragment size checking in an agarose gel, the product was quantified with Qubit dsDNA HS Assay kit in a Qubit 2.0 Fluorometer (Invitrogen®) and the genes copy number was calculated based on the total fragment length (partial *nirS* or *nirK* fragment plus cloning vector fragments flanking the insertion point), the molecular weight of the amplicon and Avogadro's number. *nirS* and *nirK* quantification on DNA samples from heavy and light fractions from SIP incubations was performed in duplicate for samples and in triplicates for standard curves. One-in-ten and one-in-one hundred sample dilutions were included in the run to test for any inhibition of PCR reactions. No template controls were included in the run.

Results

Denitrification activity and groundwater chemistry

Physicochemical parameters measured in sampled groundwater are shown in Table 1. Total organic carbon (TOC) and nitrite concentrations were below the quantification limit (1.1 mg L^{-1} and 0.02 mg L^{-1}, respectively) in samples for all the wells.

Denitrification rates in groundwater samples from sites C, D and H were estimated under four different conditions (endogenous, nitrate amended, nitrate plus pyrite amended and nitrate plus acetate amended). Results were

analyzed statistically to compare the endogenous denitrification potential and the effects of amendments within each site (Table 2). According to the endogenous and nitrate amended rates, denitrification is feasible at all three sites, even in a scenario of increasing nitrate concentration. At sites C and H, nitrate plus acetate amendments significantly stimulated denitrification compared to the respective endogenous rates. Conversely, neither nitrate nor nitrate plus pyrite additions changed denitrification rates significantly at any sample site. Samples revealed differential response to the added nutrients, as denitrification rates with nitrate addition showed significantly higher denitrification rates in well H compared to other wells.

Bacterial community structure in basal groundwater samples

A total of 19,844 16S rRNA gene sequences were obtained from the three groundwater samples before incubation. This number was reduced to 13,447 after quality checking and removal of chimeras. Sequences were grouped in a final OTU table composed of 936 OTUs and 11,154 reads, which were distributed as 3343, 3094 and 4717 reads for sites C, D and H, respectively. About 97% of the sequences in the final data set were between 220 and 240 bp long. The rarefaction curves (Fig S1) levelled off at the sequenced depth for all the samples, indicating adequate coverage for each site.

As shown in Fig S2, *Proteobacteria* was the major phylum in all three samples. The distribution within this phylum however varied between sampling sites. In site C, *Alpha* and *Gammaproteobacteria* were the dominant classes, while in sites D and H, sequences affiliated to *Betaproteobacteria* dominated the bacterial communities. Other phyla that were present in high proportions were *Actinobacteria* at site C (20%),

Nitrospirae at site D (22%), *Verrucomicrobia* (11%) and *Chlamydiae* (10%) at sample H.

Analysis of SIP incubations by DGGE

In order to identify the denitrifiers that would respond to an input of nitrate and an energy source to the groundwater, replicate DNA-SIP incubations were set up with 2 mmol L^{-1} of nitrate and either 1.5 mmol L^{-1} of acetate or pyrite plus carbonate (final concentration 1200 mg L^{-1} and 0.5 mmol L^{-1} respectively). These were monitored over time by measuring nitrate consumption (Fig S3); acetate consumption was measured in acetate amended treatments at the end of incubation. Flasks that were amended with pyrite did not show more than 20% decrease in nitrate concentration over four weeks of incubation and cells were not harvested from these flasks. This was similar to the observations obtained from the denitrification rates experiments, where no increase in the rates was observed with pyrite amendment. Acetate-amended flasks responded to the substrates and consumed the added nitrate and acetate, although in a time course that differed among sites. A shorter incubation time was observed for sample H (seven days), while 10 and 14 days were required for samples C and D to consume at least 90% of the added nitrate, and the stoichiometric amount of acetate, respectively.

Bacterial community composition in non-fractionated DNA and in DNA from fractions from ^{13}C and ^{12}C acetate incubations obtained after gradient centrifugation and fractionation were analysed by DGGE fingerprinting of 16S rRNA genes. Biological replicates of ^{13}C incubations and ^{12}C controls displayed reasonable similarity among non-fractionated profiles (Fig S4);

thus, replicate A was selected for further work. Heavy DNA fractions from ^{13}C incubations in wells C and D displayed different 16S rRNA gene profiles from those obtained with light fractions from ^{13}C incubations, with the presence of specific bands only in the heavy fractions (Fig 1). This suggests that selective ^{13}C acetate assimilation had occurred after nitrate addition in these wells. Moreover, these specific bands were intense in light fractions from ^{12}C incubations, and faint, or mostly absent, in the heavy fractions from these ^{12}C controls. Taken together these results reveal successful ^{13}C labelling of DNA in specific microorganisms after acetate consumption and nitrate reduction in wells C and D. Heavy fractions from ^{13}C incubations from site H also showed different profiles from light fractions, suggesting specific labelling. However, the main band observed in heavy fractions from ^{13}C incubation was not absent or faint in heavy fractions from ^{12}C incubations. Therefore, the results obtained for well H do not provide enough evidence to confirm that the main band detected in heavy fractions from ^{13}C incubations correspond to the active denitrifiers.

Identification of active denitrifiers by DNA-SIP with acetate

To facilitate preliminary identification of the active denitrifiers responding to a nutrient input, selected DGGE bands were analysed. 16S rRNA gene analysis of predominant bands in heavy fractions from ^{13}C incubations by excision from gels and sequencing (Fig 1) revealed that members of the genus *Vogesella* are the main active bacteria at site C, along with *Comamonas*, *Hydrogenophaga* and *Duganella* (all members of *Comamonadaceae* family) though in lower proportions. In site H on the other hand, bands that belong to *Dechloromonas*, *Comamonas* and *Ensifer* genera

were identified in the heavy fraction while bands excised from site D heavy DNA fractions were affiliated exclusively to the genus *Dechloromonas*.

Data sets obtained after amplicon sequencing of 16 rRNA genes of heavy and light DNA fractions from ^{13}C incubations recovered from acetate SIP incubations with groundwater samples C, D and H contained 99,581 raw reads. This dataset was reduced to 72,109 sequences after quality control and removal of chimeras, and sequences were grouped into 982 OTUs at 97% nucleotide similarity. Removal of low abundance OTUs and the sequences that failed to align resulted in 66,932 16S rRNA sequences distributed over 18 different OTUs. Ninety percent of DNA sequences in the final 16S rRNA gene dataset were between 450 and 530 bp long. A rarefaction analysis showed that at a depth of 2600 sequences per sample, the number of OTUs reached a plateau for the six samples, indicating adequate sequencing coverage (Fig S1).

The distribution of 16S rRNA gene sequences retrieved from heavy and light fractions of ^{13}C incubations and their taxonomic classification is shown in Table 3. The OTUs identified in heavy fractions showed consistent results with DGGE bands sequencing. In most cases, OTUs that arose only in heavy fractions were identified, while other OTUs were considerably enriched from light to heavy fraction. These OTUs were considered as the active consumers of the labelled acetate in the denitrifying conditions imposed, and therefore identified as active denitrifiers. Site C harboured several OTUs that occurred exclusively in the heavy DNA fraction. The predominant sequences belonged to the family *Neisseriaceae*, which comprised up to 42% of the 16S rRNA gene library at this site. Moreover, the genus *Vogesella* was the

predominant component within this family (36% of the 16S rRNA gene library at site C). Members of the family *Comamonadaceae* were also abundant (24%) in the heavy DNA fraction at site C. In heavy DNA from site D the dominant 16S rRNA genes were from the family *Rhodocyclaceae* (47%) with members of the genus *Dechloromonas* being a major component (45% of this 16S rRNA gene library). The most abundant OTU in site H was a member of the genus *Comamonas* (79% of the 16S rRNA gene library). An OTU affiliated with the *Rhizobiaceae* was also present although in a much lower proportion (7%).

Abundance and functional diversity of responding denitrifiers

PCR products were obtained with gene-specific primers targeting *nirS* and *nirK* in heavy DNA fractions from ^{13}C incubations. Interestingly, no amplification of any of the denitrification genes was observed in light DNA fractions, indicating that the only denitrifiers that were enriched during SIP incubations were able to use the labelled acetate. The abundance of both *nirS* and *nirK* genes was also determined in DNA from heavy fractions from ^{13}C incubations and the relative abundance (*nirS* copies µL^{-1}/*nirK* copies µL^{-1}) was calculated. *nirS* gene copy numbers in the heavy fractions were 1.2 - 3.6 x 10^5 copies µL^{-1} of DNA, whereas *nirK* abundance was 1.1 - 1.5 x 10^4 copies µL^{-1}. The abundance of *nirS* was at least 10 times higher than *nirK* for every site, which suggests that the microbial communities responding to nitrate and acetate input in the three sites are dominated by *nirS*-containing denitrifiers.

Amplicon sequencing of *nirS* and *nirK* was performed on DNA from heavy fractions from ^{13}C incubations. Total data sets obtained for *nirS* contained

16,334 reads, which were reduced to 13,481 (7024 for site C, 2862 for site D and 3595 for site H) after quality control, chimera checking and removal of sequences that failed to align. 95% of the sequences in the final set had between 360 and 400 bp and 21 OTUs were defined (at 97% sequence identity). Rarefaction analysis was performed and the number of OTUs reached a plateau for the three samples for both *nir* genes.

The phylogenetic relatedness among representative *nirS* and *nirK* sequences from each OTU with sequences from both uncultivated and extant strains recovered from databases was analysed (Fig 2 and Fig 3). Tree topology showed that gene sequences from this study clustered in different groups that were supported by high bootstrap values and consistently obtained with NJ as well as with ML methods. All the closest *nirS* sequences from extant bacteria found in databases were affiliated to the class *Betaproteobacteria*. The majority (89%) of representative *nirS* sequences obtained from site C belonged to the same OTU and clustered with denitrifier gene sequences found in two *Vogesella* species (*Neisseriaceae*) which were isolated from the same aquifer in a previous study (Bellini 2013). The remaining OTUs clustered with denitrifier gene sequences obtained from *Comamonadaceae* isolates (8.5%) and from the genus *Dechloromonas* (2%). All the *nirS* sequences present in the heavy DNA fraction from site D were closely related to *nirS* sequences obtained from isolates of the family *Rhodocyclaceae*. Most of these sequences (97.5%) showed maximum identity with DNA sequences retrieved directly from the environment, mainly ocean sediments, and with sequences from *Dechloromonas* genus isolates. The remaining 2.5% grouped closely with *nirS* sequences retrieved directly

from the environment. Most of the *nirS* sequences in the site H library (55%) were closely affiliated with environmental sequences, obtained mostly from aquatic environments. 32% of the sequences clustered with *nirS* sequences from *Rhodocyclaceae* isolates, which were distributed among the genera *Dechloromonas*, *Azospira*, *Azoarcus* and *Zoogloea*. The closest *nirS* sequence from extant bacteria to the remaining 13% of site H library was *nirS* from soil isolate I-Bh25-7 (Braker 2010), a member of the *Comamonadaceae* family.

In contrast to information on *nirS*, *nirK* gene sequences retrieved fromheavy fractions from ^{13}C incubations grouped mostly with *nirK* sequences from cultivated representatives of the *Alpha* and *Gammaproteobacteria*. The majority of the *nirK* OTUs from site C (90%) clustered with environmental clones and with *nirK* sequences affliated to *Alphaproteobacteria* of the genera *Sinorhizobuim*, *Ensifer*, *Rhizobium*, *Mesorhizobuim*, *Bradyrhizobium* and *Bosea (*within the *Rhizobiales)*. *Gammaproteobacteria*-affiliated *nirK* sequences represented 10% of the *nirK* sequences retrieved from site C, with one OTU clustering with *nirK* of *Pseudomonas*. All representative *nirK* sequences from site D were affiliated with *nirK* from *Alphaproteobacteria*. Seventy-two percent of the sequences clustered closely to *nirK* from extant *Rhizobium* while the remaining 28% were affiliated with *nirK* sequences from *Bosea*. Most (93%) of the *nirK* sequences from site H clustered in one OTU, most closely related to *nirK* from members of the genera *Ensifer* and *Sinorhizobiu*m. Other less well-represented OTUs clustered with *nirK* from *Rhizobium* (5%), *Ochrobactrum* and *Paracoccus* (2%).

Discussion

Our aim was to evaluate the denitrification potential of indigenous microbial communities from a low impacted aquifer that is likely face an input of nitrate and to identify the microorganisms responsible for the observed activity. The strategy combined kinetic measurements of the denitrification process together with a study of the active microbial communities using DNA-SIP, in order to better predict how denitrifiers in groundwater respond to an increase in nitrate and electron donor concentration.

Natural attenuation of dissolved nitrate was feasible in the three sampled sites at *in situ* nitrate concentrations. However, according to the denitrification rates measured an increase in the nitrate levels without the input of a carbon source would be limited either by abundance or composition of denitrifiers or by insufficient electron donors, since in most of the sampled sites only acetate plus nitrate stimulated denitrification. This suggests that the indigenous denitrifying microbes in water from the Raigón aquifer appear to be adapted to organotrophic denitrification, which is in accordance with previous observations that establish that in general carbon deprivation is the main factor limiting denitrification in aquifers (Rivett 2008). However, bacteria capable of using minerals such as pyrite could be present in the aquifer but attached to sediments. Samples reacted differently to the amendments and displayed differences in their basal bacterial communities' profiles, suggesting intrinsic differences in either the denitrifying population or in geochemical conditions. The results from this study are in agreement with previous observations involving the same aquifer wells (Bellini, 2013), where a carbon-limited nitrate consumption potential was detected. However, sites C and D did not show an equivalent response to the same amendments seen

in the previous study, which could be due to the temporal fluctuation of nutrients and/or denitrification inhibitor concentrations (Lin, 2012). On the contrary, site H responded similarly to amendments than in the previous study. Considering the activities occurring in the surroundings of each well, groundwater in site H (located in a small town) could be receiving a relatively constant input of nutrients from the surface while activities on sites D (potato processing plant) and C (dairy farm) would sporadically contribute nutrients to the subsurface.

SIP incubations performed on groundwater under the same conditions as the denitrification activity measurements enabled us to identify key denitrifiers responding to increased nitrate contamination of the aquifer. Amplicon sequencing of 16S rRNA genes from ^{13}C-labelled DNA from SIP incubations indicated that *Betaproteobacteria* play a major role in organotrophic denitrification in two sites examined. Previous molecular and/or cultivation-dependent studies have also shown that *Betaproteobacteria* are the main denitrifiers in diverse environments such as paddy soil (Saito 2008), wastewater treatment systems (Ginige 2005; Osaka 2006; McIlroy 2016) as well as aquifers where heterotrophic denitrification occurs (Ginige 2013; Calderer 2014; Zeng 2016). Although a high diversity was detected in naturally occurring communities in all three wells, *Betaproteobacteria* was the predominant class in naturally occurring communities from sites D and H, although it was less represented in site C (Fig S2). However, different denitrifying bacteria from this class respond rapidly to nitrate and carbon input in the wells.

Members of the genus *Vogesella* were the predominant denitrifiers in samples from site C. In a previous study, *Vogesella* was isolated from the same aquifer from wells C and H, and its denitrifying capability with acetate as electron donor was confirmed in the laboratory (Bellini 2013). The fact that *Vogesella* was detected by both molecular and cultivation-based methods, in both samplings suggests *Vogesella* is well adapted to this groundwater system and could be relevant in the response to carbon and nitrate input in this site. Interestingly, though *Betaproteobacteria* have been identified as important denitrifiers in different environments, *Neisseriales* have scarcely ever been reported as major actors in denitrification processes (Yoshida 2012). The ability of *Vogesella* to denitrify was observed when the genus was first described (Grimes 1997). *Vogesella* has also been shown to be prevalent in other processes that may occur in groundwater such as removal of heavy metals (Vishnivetskaya 2010) or improving resistance to disinfectants (Whiteley 2001). Together with our findings, this suggests that *Vogesella* could be relevant not only for denitrification but also for other environmental processes occurring in groundwater ecosystems. Members of the *Comamonadaceae* were also identified as active denitrifiers at site C. This is also in agreement with previous findings where *Comamonadaceae*, along with other bacteria, were found (Wakelin 2011) or enriched under denitrifying conditions in groundwater samples (Bellini 2013; Calderer 2014) and in other systems, such as paddy soils and wastewater treatment (Saito 2008; McIlroy 2016). In addition, growth and molecular-based studies have shown species of genus *Comamonas*, a member of *Comamonadaceae*, to be capable of denitrification (Etchebehere 2001; Gumaelius 2001) and that they play important roles in denitrification processes in activated sludges

(Ginige 2005), paddy soils (Osaka 2006) and groundwater systems (Calderer 2014; Wang 2014). Site D contained only one dominant active denitrifying genus, *Dechloromonas*, a member of the *Rhodocyclaceae.* Denitrifying members of this family were isolated from the same well from Raigón aquifer in a previous study (Bellini 2013), suggesting that members of this family regularly respond to denitrifying conditions in this site. *Rhodocyclaceae* have also been depicted as key denitrifiers in systems with a greater load of nitrate and/or organic matter such as activated sludge (Ginige 2005), paddy soil (Osaka 2005) and surface freshwater systems (Yu 2014). The most abundant taxon detected in site H was genus *Comamonas*, although according to DGGE results its role as key denitrifier was not confirmed.

To further characterise the diversity of active denitrifying communities from the Raigón aquifer, we analysed *nirS* and *nirK* genes in ^{13}C-labelled DNA from SIP incubations. Care must be taken when assigning taxonomical information to *nir* genes sequences since congruence between 16S rRNA and *nir* genes phylogenies is taxon dependent (Heylen 2006; Jones 2008). The *nirS* gene library from site C was dominated by two OTUs that clustered closely with *nirS* sequences obtained from two *Vogesella* species isolated from the same aquifer well (Bellini 2013). Retrieved *nirS* sequences that cluster closely with members of *Comamonadaceae* and *Dechloromonas* could also be active denitrifiers, although they were less abundant. *nirK* sequences affiliated with *Rhizobiales* also appear to represent active denitrifiers at site C.

For site D, *nirS* sequences that clustered with *nirS* from *Dechloromonas* were most abundant while most *nirK* gene sequences affiliated with *nirK* from

Rhizobium and *Bosea.* These results reinforce the findings obtained with 16S rRNA gene analyses and suggest that *Dechloromonas* is a key denitrifier in site D, with a minor contribution to the denitrification process by *Rhizobiaceae.*

Analysis of *nirS* libraries in site H showed that the dominant OTUs clustered with environmental sequences and sequences from the family *Rhodocyclaceae,* although this family was clearly underrepresented in 16S rRNA gene library. The dominant taxon observed in the 16S rRNA gene library (*Comamonas*) was not observed either in *nirS* or *nirK* libraries. However, most of *nirS* sequences from this site clustered with environmental clones and 13% of the sequences clustered closely to the *Comamonadaceae* isolate I-Bh25-7. A BLAST search using 16S rRNA gene from isolate I-Bh25-7 indicates it could belong to the genus *Simplicispira*, within the *Comamonadaceae.* Moreover, most denitrifying *Comamonas* isolates studied so far possesses *nirS* (Heylen 2006) and these *nirS* sequences should have been retrieved with the PCR primers used in this study. Accordingly, most of the *nirS* sequences found in this site could actually belong to the *Comamonas* that were detected with 16S rRNA gene sequences. Altogether, 16S rRNA, *nirS* and *nirK* data suggests that *Comamonadaceae*, *Rhodocyclaceae* and *Rhizobiaceae* were present at site H.

In summary, the three sampled sites from the Raigón aquifer differed in microbial communities and in their denitrification rates in response to nutrients amendments. However, the indigenous communities in this aquifer have the potential to remove contaminating nitrate and perform a natural

attenuation process through different and taxonomically diverse genera, mainly *Betaproteobacteria.*

Acknowledgments

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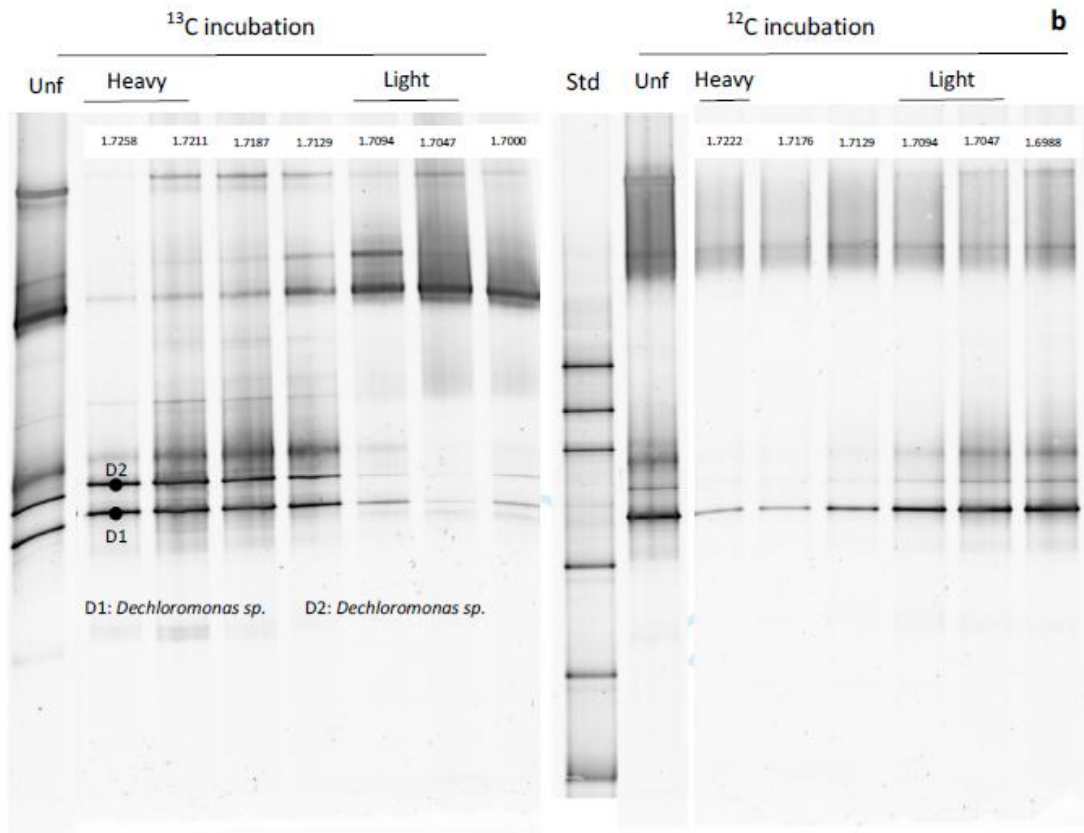
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a

^{13}C incubation — ^{12}C incubation

Unf | Heavy | Light | Std | Unf | Heavy | Light

1.7269 1.7211 1.7164 1.7129 1.7070 1.7035 | 1.7269 1.7222 1.7187 1.7141 1.7094 1.7059

C4
C3
C2
C1

C1: *Vogesella sp.*   C2: *Comamonas sp.*   C3: *Hydrogenophaga sp.*   C4: *Duganella sp.*

b

^{13}C incubation — ^{12}C incubation

Unf | Heavy | Light | Std | Unf | Heavy | Light

1.7258 1.7211 1.7187 1.7129 1.7094 1.7047 1.7000 | 1.7222 1.7176 1.7129 1.7094 1.7047 1.6988

D2
D1

D1: *Dechloromonas sp.*   D2: *Dechloromonas sp.*
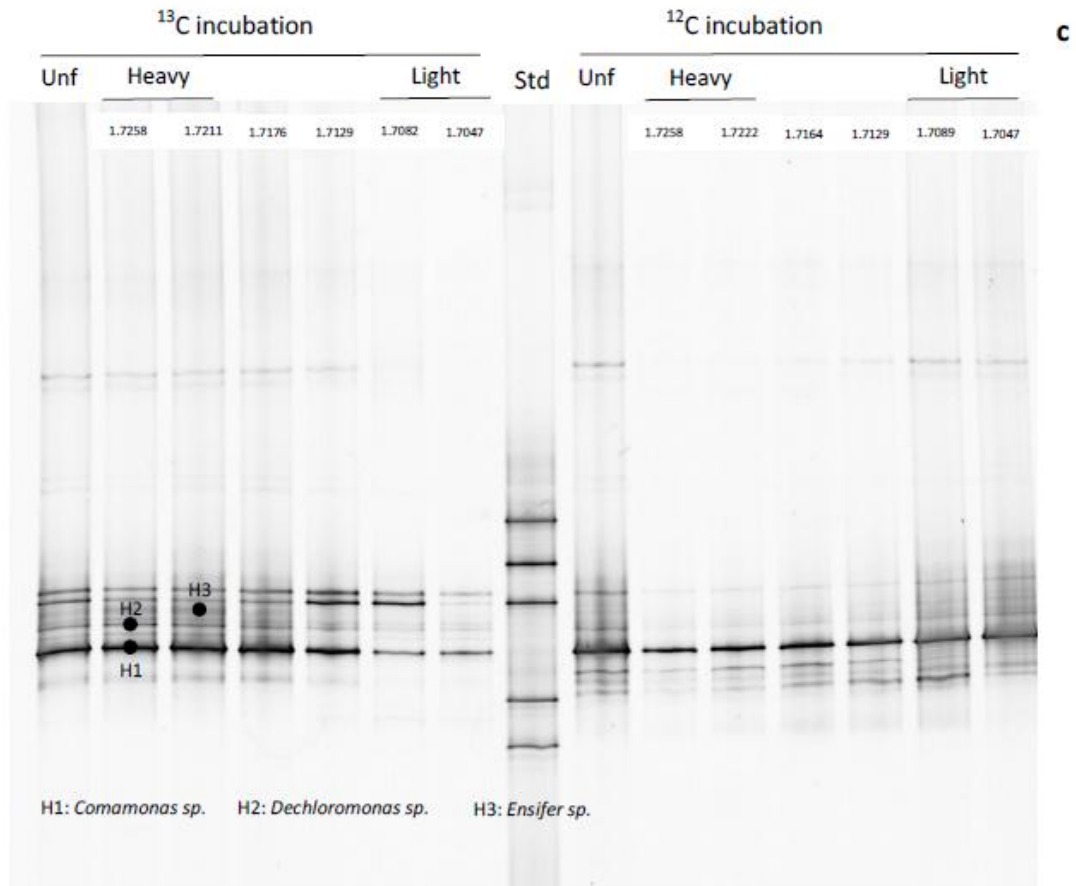
Figure 1. Denaturing gradient gel electrophoresis analysis of 16s rRNA gene fragments in unfractionated and in heavy and light fractions from ^{13}C- and ^{12}C-acetate SIP incubations. Density from each fraction is shown (g mL^{-1}). Predominant bands identification is shown. (Std: DGGE standard)

Figure 2. Unrooted neighbor-joining phylogenetic analysis of partial

nucleotide sequences of *nirS* gene fragments from SIP heavy and light

fractions from ^{13}C incubations. Squares, triangles and circles represent

sequences from sites C, D and H respectively. Percentages indicate OTU proportion in sample library. Bootstrap values greater than 70 % (1000 replicates) are shown. The scale bar indicates the number of changes per sequence position. Sequences obtained from prior culturing from this aquifer are shown in bold.
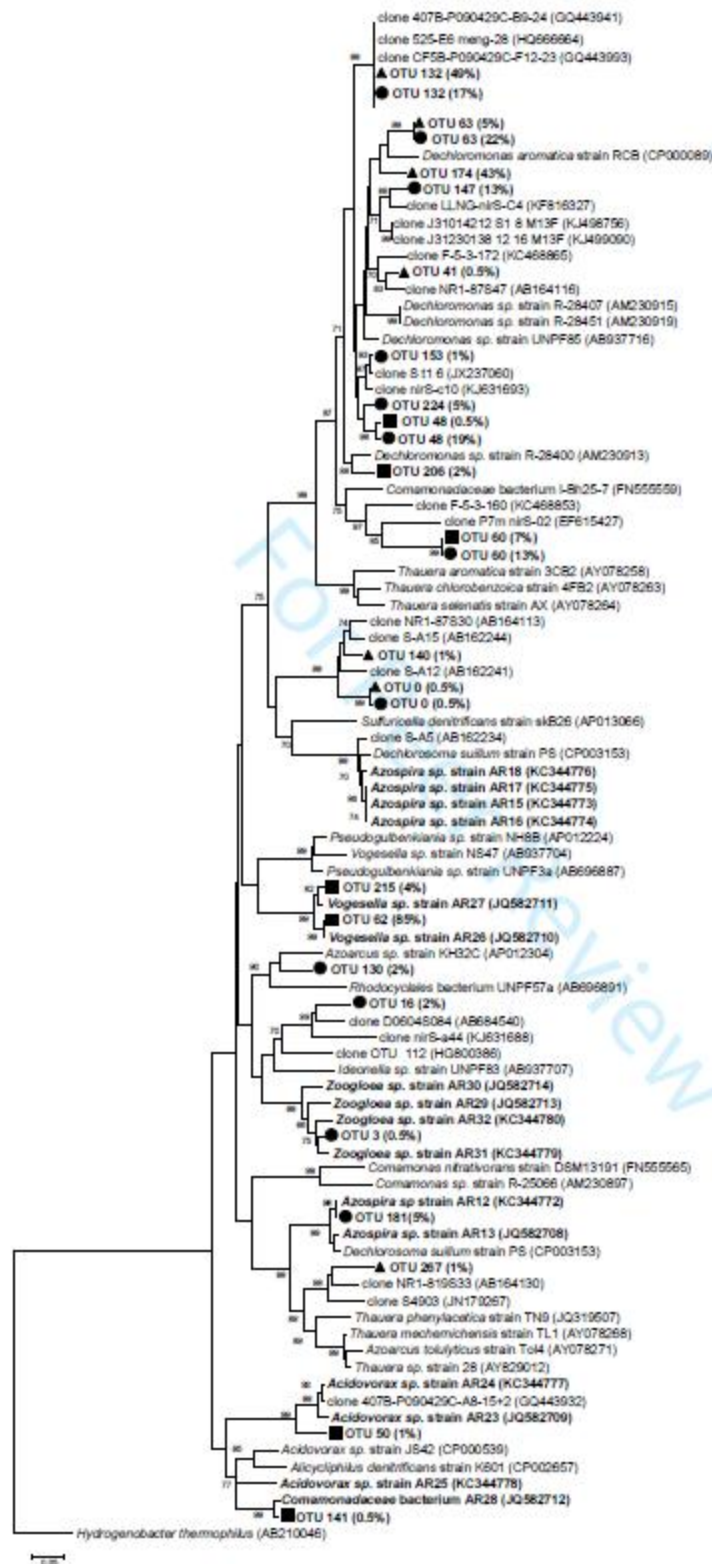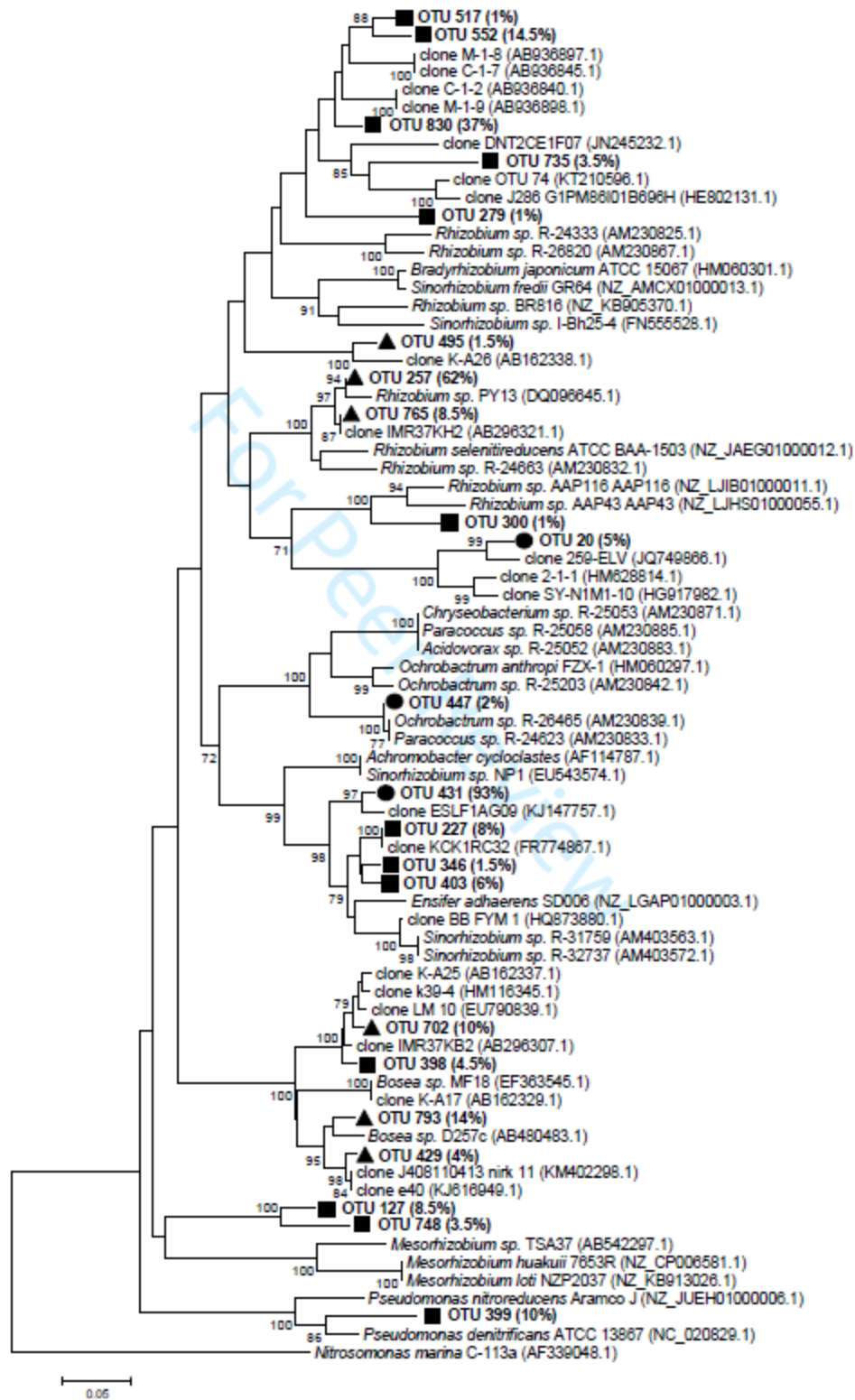
Figure 3. Unrooted neighbor-joining phylogenetic analysis of partial nucleotide sequences of *nirK* gene fragments from SIP heavy and light fractions from ^{13}C incubations. Squares, triangles and circles represent

sequences from sites C, D and H respectively. Percentages indicate OTU

proportion in sample library. Bootstrap values greater than 70 % (1000

replicates) are shown. The scale bar indicates the number of changes per

sequence position.

Table 1. Groundwater physicochemical parameters at sampling sites

Site	T (°C)	pH	NO$_3^-$ (mg L^{-1}/ mmol L^{-1})	Total Fe (ug L^{-1})	SO$_4^{2-}$ (mg L^{-1})	O$_2$ (mg L^{-1})
C	18.6	7.7	33.6 / 0.54	<17	49.0	7.4
D	18.0	7.1	10.3 / 0.17	29	33.2	3.9
H	19.2	7.4	24.9 / 0.40	65	28.8	6.4

Table 2. Mean denitrification rates of indigenous bacterial communities per well (μmol N_2O L^{-1} h^{-1})

Site		Amendment		
	Endogenous	Nitrate	Nitrate + pyrite	Nitrate + acetate
C	0.14 [a,A]	0.22 [a,A]	0.91 [a,AB]	4.43 [b,B]
D	0.07 [a,A]	0.14 [a,A]	0.07 [a,A]	0.65 [a,A]
H	1.44 [a,A]	3.09 [ab,B]	2.02 [a,B]	7.42 [b,B]

p=0.05, lowercase letters indicate significant differences between rates within one site (lines), uppercase letter indicate differences between sites (columns)

Table 3. Taxonomic composition and relative abundance (%) of bacterial communities in ^{13}C SIP incubations for the heavy and light fractions in the three studied sites

Taxon	C		D		H	
	heavy	light	heavy	light	heavy	light
Alphaproteobacteria						
Rhizobiaceae	**3**	0	**2**	0	**7**	**2**
Betaproteobacteria						
Burkholderiales						
Comamonadaceae						
Aquabacterium	0	0	0	0	0	2
Comamonas	**9**	0	0	0	**79**	39
Hydrogenophaga	**1**	0	0	0	**2**	**8**
Other *Comamonadaceae*	**14**	0	**2**	0	**9**	**15**
Neisseriales						
Neisseriaceae						
Vogesella	**36**	0	0	0	0	0
Other *Neisseriaceae*	**6**	0	0	0	0	0
Rhodocyclales						
Rhodocyclaceae						
Dechloromonas	**1**	0	**45**	0	**1**	**4**
KD1-23	0	0	**1**	0	0	0
Other *Rhodocyclaceae*	0	0	**1**	0	**1**	**2**
Other *Betaproteobacteria*	**7**	0	0	0	0	0

Gammaproteobacteria						
Aeromonadaceae	**2**	0	0	0	0	0
Pseudomonadales						
Acinetobacter	21	**96**	0	0	0	0
Pseudomonas	0	0	**4**	0	0	0
Flavobacteriia						
Flavobacteriaceae						
Flavobacterium	0	**4**	0	0	0	0
Weeksellaceae						
Chryseobacterium	0	0	**2**	**2**	0	0
Other *Weeksellaceae*	0	0	43	**98**	0	0
Bacilli						
Bacillus	0	0	0	0	1	**28**