RESEARCH ARTICLE

Structure and activity of lacustrine sediment bacteria involved in nutrient and iron cycles

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Abstract

Knowledge of the bacterial community structure in sediments is essential to better design restoration strategies for eutrophied lakes. In this regard, the aim of this study was to quantify the abundance and activity of bacteria involved in nutrient and iron cycling in sediments from four Azorean lakes with distinct trophic states (Verde, Azul, Furnas and Fogo). Inferred from quantitative PCR, bacteria performing anaerobic ammonia oxidation were the most abundant in the eutrophic lakes Verde, Azul and Furnas (4.5–16.6%), followed by nitrifying bacteria (0.8–13.0%), denitrifying bacteria (DNB) (0.5–6.8%), iron-reducing bacteria (0.2–1.4%) and phosphorus-accumulating organisms ($< 0.3\%$). In contrast, DNB dominated sediments from the oligo-mesotrophic lake Fogo (8.8%). Activity assays suggested that bacteria performing ammonia oxidation (aerobic and anaerobic), nitrite oxidation, heterothrophic nitrate reduction, iron reduction and biological phosphorus storage/release were present and active in all Azorean lake sediments. The present work also suggested that the activity of DNB might contribute to the release of phosphorus from sediments.

Introduction

Sediments shelter a complex microbial ecosystem that thrives on several organic and inorganic electron donors and acceptors and performs several biochemical reactions (e.g. nitrification, denitrification, phosphorus uptake/release, iron reduction, sulphate reduction, methanogenesis) simultaneously or sequentially, depending on the environmental conditions (Nealson, 1997; Wobus *et al.*, 2003). To date, bacteria diversity studies have revealed that *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Bacteroidetes* and *Planctomycetes* (in particular after algae bloom occurs) are the most common phyla in freshwater lake sediments (Kojima *et al.*, 2006; Musat *et al.*, 2006; Nelson *et al.*, 2007; Zeng *et al.*, 2009). The better studied groups are the ones involved in nitrogen cycling, namely ammonia-oxidizing bacteria (AOB) belonging to *Betaproteobacteria*, nitrite-oxidizing bacteria (NOB) belonging to the genera *Nitrospira* (*Nitrospira* phylum) and *Nitrobacter* (*Alphaproteobacteria*) and denitrifying bacteria (DNB), which are widespread among the bacterial domain (Altmann *et al.*, 2004; Nakamura *et al.*, 2006; Oakley *et al.*, 2007). More recently, anaerobic ammonia-oxidizing bacteria (AnAOB) belonging to the order *Planctomycetales* have been shown to play an important role in

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nitrogen cycling in sediments (Dalsgaard *et al.*, 2005). Iron-reducing bacteria (IRB) are also present in freshwater sediments, and best known is the family *Geobacteraceae* belonging to *Deltaproteobacteria* (Cummings *et al.*, 2003).

In eutrophied lakes, sediments can contribute to eutrophication through phosphorus (P) release into the water column (Ribeiro *et al.*, 2008; Martins *et al.*, 2008). This phenomenon is still poorly understood and several biogeochemical processes have been proposed to explain it (Hupfer & Lewandowski, 2008). Processes contributing to the dissolution of phosphate bound to metal oxides are microbial dissimilatory Fe(III) reduction and chemical Fe(III) reduction by hydrogen sulphide. IRB reduce Fe(III) to soluble Fe(II), with the consequent release of Fe(III) oxide-bound phosphate to pore water under anoxic conditions (Azzoni *et al.*, 2005). The pool of nonrefractory organic P contributes to phosphate dissolution both via organic matter decomposition (Golterman, 2001; Rozan *et al.*, 2002) and via biological storage/release of P as polyphosphate (Poly-P) (Hupfer *et al.*, 2008). The decomposition of organic P has been reported to occur by the activity of phosphatases excreted by some DNB (Yiyong *et al.*, 2002; White & Metcalf, 2007). Microorganisms are able to store and release P in organic-rich lake sediments, depending on the redox conditions. P-accumulating organisms (PAO) store P as Poly-P intracellularly under aerobic conditions (up to 20% of their dry weight) and release intracellular P via enzymatic hydrolysis when conditions become anaerobic. Well-known PAO belong to the genera *Rhodocyclus* (*Betaproteobacteria*), *Acinetobacter* (*Gammaproteobacteria*) and *Tetrasphaera* (*Actinobacteria*) (Gaechter & Meyer, 1993; Ahn *et al.*, 2001; Tsuneda *et al.*, 2005; Hupfer *et al.*, 2008). It is estimated that Poly-P could represent at least 10% of the total P in surface sediments (Hupfer *et al.*, 2007). In addition, under acidic conditions provided by the oxidation of ammonium to nitrite by the genera *Nitrosomonas* and *Nitrosospira*, P dissolution might occur from the pool of calcium-bound P (Altmann *et al.*, 2004; Nakamura *et al.*, 2006). Thus, it is important to study the microbial community in lake sediments in order to help to clarify the role of bacteria in P release.

However, little is known about the quantitative composition of microbial communities in sediments. The application of culture-dependent methods to quantify specific groups of bacteria in sediments led to results that deviated considerably from those obtained using culture-independent methods. The use of real-time quantitative PCR (qPCR), targeting specific functional or phylogenetic genes, has overcome the limitation of traditional culture-based quantification methods, and has recently been broadened to the quantification of bacteria in complex environments, such as biofilms (Kindaichi *et al.*, 2006), wastewater treatment plants (He *et al.*, 2007), soil (Henry *et al.*, 2004) and sediments (Nakamura *et al.*, 2006; Bedard *et al.*, 2007; Bulow *et al.*, 2008).

Previous research in Azorean lakes has focused on the study of the phytoplankton growing in the water column (Santos *et al.*, 2005; Martins *et al.*, 2008), and little attention has been paid to the effect of sediment microbial processes on lake water quality, especially those involved in the cycling of nutrients. The aim of the present work was to quantify the abundance and activity of bacteria involved in nutrient and iron cycling in sediments from the eutrophic lakes Furnas and Verde, the meso-eutrophic lake Azul and the oligo-mesotrophic lake Fogo (Azores, Portugal).

Materials and methods

Study site and sampling

The four studied lakes are in the island of S. Miguel in the archipelago of Azores (Portugal), located between the parallels 36°45′–39°43′ of latitude North and meridians 24°45′–31°17′ of longitude West. Lake Furnas is situated in the East side of the island and lake Sete Cidades in the Western side. Lake Sete Cidades is formed by two interconnected lakes: lake Azul and lake Verde. In the central and highest part of the island is situated lake Fogo. Lakes Furnas and Verde are in an advanced state of eutrophication and were classified as eutrophic. Lake Azul is in a meso-eutrophic state. From the four lakes studied, lake Fogo presents the smallest degree of eutrophication, and was classified as oligo-mesotrophic. A more detailed description can be found in Ribeiro *et al.* (2008) and Martins *et al.* (2008).

Sediments from the studied lakes were taken in July 2008 using a gravitational Uwitec-corer (6 cm diameter, 60 cm length) that enabled the collection of undisturbed sediment cores. The depth of the water column at the collection sites was 24 m for lake Verde, 25 m for lake Azul, 25 m for lake Fogo and 11 m for lake Furnas. Sediment cores were sealed *in situ* and carefully transported to the laboratory (around 1 h) at 4 °C. The water above the sediment surface was discharged. The sediment cores for DNA extraction were cut into five slices with the following depths: 0–1, 1–2, 2.5–3.5, 5–6 and 9–10 cm. Each slice was stored frozen inside a sterile plastic Petri dish. For the activity assays, the first 10 cm of the sediment cores were mixed and stored at 4 °C. Sampling conditions (temperature, dissolved oxygen and pH) were assessed *in situ* using a portable multimeter (Multi 350i, WTW). To determine the organic and water fractions, approximately 2 g of sediment was weighed, dried at 105 °C for 24 h and reweighed. Subsequently, the sediment was burned for 2 h at 550 °C and weighed for the quantification of organic components.

DNA extraction, amplification and real-time PCR assay

DNA was extracted over the vertical sediment profile according to previously published protocols (Kowalchuk

Table 1. Primers used in qPCR

Target	Primer set	Sequence (5′–3′)	Sequence position	References
BAC	1055f	ATGGCTGTCGTCAGCT	1055–1070*	Harms *et al*. (2003)
	1392r	ACGGGCGGTGTGTAC	1392–1406*	
Candidatus Accumulibacter	518f	CCAGCAGCCGCGGTAAT	518–534*	He *et al*. (2007)
	PAO-846r	GTTAGCTACGGCACTAAAAGG	846–866*	
Geobacteraceae	Geo564f	AAGCGTTGTTCGGAWTTAT	564–582*	Bedard *et al*. (2007)
	Geo840r	GGCACTGCAGGGGTCAATA	840–858*	
AOB	CTO189fA/B	GGAGRAAAGCAGGGGATCG	189–207*	Kowalchuk *et al*. (1997)
	CTO189fC	GGAGGAAAGTAGGGGATCG	189–207*	
	RT1r	CGTCCTCTCAGACCARCTACTG	283–304*	Hermansson & Lindgren (2001)
NOB (*Nitrobacter* spp.)	FGPS872f	CTAAAACTCAAAGGAATTGA	872–891*	Degrange & Bardin (1995)
	FGPS1269r	TTTTTTGAGATTTGCTAG	1269–1286*	
NOB (*Nitrospira* spp.)	Nspra-675f	GCGGTGAAATGCGTAGAKATCG	675–696*	Graham *et al*. (2007)
	Nspra-746r	TCAGCGTCAGRWAYGTTCCAGAG	746–768*	
AnAOB	Amx809f	GCCGTAAACGATGGGCACT	809–827*	Tsushima *et al*. (2007)
	Amx1066r	AACGTCTCACGACACGAGCTG	1065–1087*	
DNB (nir*K* gene)	nirK876f	ATYGGCGGVCAYGGCGA	876–892†	Henry *et al*. (2004)
	nirK1040r	GCCTCGATCAGRTTRTGGTT	1020–1039†	
DNB (nir*S* gene)	nirS4Qf	GTSAACGYSAAGGARACSGG	916–935†	Throbäck *et al*. (2004)
	nirS6Qr	GASTTCGGRTGGSGTCTTSAYGAA	1322–1345†	

K = G or T, M = A or C, R = A or G, S = C or G, W = A or T, Y = C or T.

**Escherichia coli* numbering.

†Positions in the nir*S* gene of *Pseudomonas stutzeri* ZoBell (X56813) and in the nir*K* gene of *Alcaligenes faecalis* S-6 (D13155).

et al., 1998; Griffiths *et al*., 2000). Briefly, 0.5–0.7 g (wet weight) of sediment was extracted with 1 mL solution containing one part of CTAB extraction buffer (10% w/v) and one part of phenol–chloroform–isoamyl alcohol (25 : 24 : 1). Glass beads (0.25 g of 0.1 mm plus 0.25 g of 0.5 mm diameter) were added to the sediment suspension, which was shaken in a cell disrupter (5000 g) (Disruptor Genie™, Scientific Industries Inc.) for 8 min to promote cell lyses. An equal volume of chloroform–isoamyl alcohol (24 : 1) was added to the supernatant to remove phenol. Then, DNA was precipitated with two volumes of 30% (w/v) polyethylene glycol and washed with 70% (v/v) ice-cold ethanol. Finally, the pellet was air-dried and dissolved in milli-Q water. The concentrations and purities of the extracted DNA were measured and checked at a wavelength of 260, 230 and 280 nm using a UV-vis spectrophotometer (Cary 50, VARIAN, Palo Alto, CA).

Preliminary PCR reactions were carried out to verify the amplification of the desired PCR products and, if necessary, to optimize the reaction conditions. The choice of primer sets was based on the best-available primers in the literature, i.e. the primers that covered the widest range of microorganisms for each studied group. Primers targeting the 16S rRNA gene were used to quantify total bacteria (BAC), AOB, NOB, AnAOB, *Candidatus* Accumulibacter and *Geobacteraceae*. In the case of DNB, primers targeting both functional genes nir*K* and nir*S* were mixed. Table 1 lists the primers used. Primers CTO 189fA/B and CTO 189fC were used at a 2 : 1 ratio. PCR amplification of DNA extracted from sediments and standards was performed in a 25 μL reaction mixture containing 2.5 μL DNA template, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 200 μM of each deoxynucleotide, 1.5 mM $MgCl_2$, 2.5 U mL^{-1} of Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA) and 0.4 μM each forward and reverse primers. DNA templates were diluted 100 ×, the final concentrations being in the range of 0.10–1.60 ng μL^{-1} for sediments and from 10^1 to 10^8 target copies per reaction for standards. PCR reactions were performed using a thermocycler (T3000, Biometra, Göttingen, Germany) under the conditions described in Table 2. For a subset of primers listed in Table 1, the annealing temperature, and the duration and number of cycles was optimized. PCR products were visualized by 1% (w/v) agarose gel electrophoresis with ethidium bromide staining.

The amplification of real-time PCR products, under the conditions described in Table 2, was carried out using a Chromo4 real-time PCR detector (MJ/Bio-Rad, Hercules, CA) using SYBR Green as a signal dye. PCR amplification was performed in a 25 μL reaction mixture containing 12.5 μL of master mix (iQ™ SYBR® Green Supermix, Bio-Rad 170-8882), 0.4 μM of each forward and reverse primers and 5 μL of DNA template. All samples were measured in triplicate. A final melting curve analysis was performed to check the specificity of the amplification reaction. Temperature was increased from 62 to 95 °C at 0.2 °C s^{-1} and the appearance of a single melting peak in the melting curve was indicative that no contaminating products were present in the reaction. A no-template control, that is, a control

Table 2. PCR conditions and standard curves of qPCR for the quantification of specific bacterial groups

Organism	Thermal cycling conditions	Standard curve*	Amplification efficiency (%)†	References
BAC	10 min at 94.0 °C plus 40 cycles: 30 s at 94.0 °C denaturation 1 min at 55 °C annealing 1 min at 72.0 °C extension and 10 min at 72.0 °C final extension	$y = -3.29x+36.01$ $r^2 = 0.999$	102	Harms *et al*. (2003)
Candidatus Accumulibacter	10 min at 94.0 °C plus 40 cycles: 30 s at 94.0 °C denaturation 45 s at 60.0 °C annealing 30 s at 72.0 °C extension and 10 min at 72.0 °C final extension	$y = -3.30x+35.22$ $r^2 = 0.999$	101	This study
Geobacteraceae	10 min at 94.0 °C plus 40 cycles: 30 s at 94.0 °C denaturation 30 s at 56.5 °C annealing 45 s at 72.0 °C extension and 10 min at 72.0 °C final extension	$y = -3.35x+34.85$ $r^2 = 0.999$	99	This study
AOB	5 min at 94.0 °C plus 40 cycles: 30 s at 94.0 °C denaturation 30 s at 60.0 °C annealing 1 min at 72.0 °C extension and 10 min at 72.0 °C final extension	$y = -3.24x+36.27$ $r^2 = 0.999$	104	Kowalchuk *et al*. (1997)
NOB (*Nitrobacter* spp.)	10 min at 94 °C plus 40 cycles: 30 s at 94 °C denaturation 30 s at 50 °C annealing 1 min at 72.0 °C extension and 10 min at 72.0 °C final extension	$y = -3.88x+41.12$ $r^2 = 0.999$	81	Degrange & Bardin (1995)
NOB (*Nitrospira* spp.)	10 min at 94.0 °C plus 40 cycles: 30 s at 94.0 °C denaturation 30 s at 58.0 °C annealing 1 min at 72.0 °C extension and 10 min at 72.0 °C final extension	$y = -3.14x+36.93$ $r^2 = 0.998$	108	This study
AnAOB	5 min at 94.0 °C plus 40 cycles: 30 s at 94.0 °C denaturation 30 s at 67.0 °C annealing 1 min at 72.0 °C extension and 10 min at 72.0 °C final extension	$y = -3.33x+35.24$ $r^2 = 0.999$	100	Tsushima *et al*. (2007)
DNB (nir*K*)	15 min at 95 °C plus 6 touchdown cycles: 15 s at 94 °C denaturation 30 s at 63 °C annealing 30 s at 72 °C extension The annealing temperature was progressively decreased by 1 °C down to 58 °C. Finally, a last cycle with an annealing temperature of 58 °C was repeated 40 times	nir*K*: $y = -3.28x+30.06$ $r^2 = 0.999$	102	Henry *et al*. (2004)
DNB (nir*S*)		nir*S*: $y = -3.55x+33.58$ $r^2 = 0.998$	91	

*y is the number of cycles and x is the log quantities.
†Defined as $[10^{(-1/(\text{slope}))} - 1] \times 100$.

reaction that contains all components except the template, was included in duplicate in all quantification experiments. Data were analysed using the MJ OPTICONMONITOR 3.1 analysis software (MJ/Bio-Rad). The standard curve for each primer (Table 1) was generated using a 10-fold dilution series of seven different concentrations of the standard (10^1–10^8 target copies per reaction). Each standard curve was checked for validity, the threshold line being defined in order to obtain the higher r^2 value for the relation between the logarithm of gene copy numbers and the cycle number (Terada *et al*., 2010). Standards were measured in duplicate for each concentration.

As standards in 16S rRNA gene-targeted PCR for AOB, NOB, AnAOB, *C.* Accumulibacter and *Geobacteraceae*, a plasmid DNA was used carrying the AOB 16S rRNA gene (accession no. FJ529920), *Nitrobacter* (accession no. FJ529919), *Nitrospira* (accession no. FJ529918), AnAOB (accession no. FJ710745), *C.* Accumulibacter 16S rRNA gene (accession no. HQ529690) and *Geobacter bemidjiensis* Bem^T (accession no. AY187307), respectively. For DNB functional gene-targeted PCR, plasmid DNA was used from an environmental clone (Henry *et al.*, 2004) and from *Pseudomonas flurescens* C7R12 carrying nir*K* and nir*S*, respectively. Briefly, the DNA from the standards was extracted and amplified with the specific primers listed in Table 1. The fragments obtained were cloned into a vector, pCR® II-TOPO® (TOPO TA cloning kit, Invitrogen), transformed into chemically competent *Escherichia coli* cells and plated on LB agar. The plasmid DNA was extracted (Qiagen kit, Qiagen) and purified following the manufacturer's instructions (QIAquick PCR purification kit, Qiagen). Selected clones were sequenced and the phylogeny was checked.

The copy number of a plasmid was calculated based on the concentration of DNA measured at 260 nm and the average molecular weight of the plasmid. Total bacterial cell numbers (BAC) were calculated assuming that bacteria contain 3.6 copies per cell of the 16S rRNA gene (Klappenbach *et al.*, 2001; Harms *et al.*, 2003). In the case of AOB and NOB, one copy per cell of the 16S rRNA gene was assumed (Aakra *et al.*, 1999). The number of *Geobacteraceae* and *C.* Accumulibacter was calculated assuming that both contain two copies per cell of the 16S rRNA gene (Bedard *et al.*, 2007; He *et al.*, 2007). For AnAOB, two gene copies per cell of the 16S rRNA gene were assumed because the 16S rRNA gene operon of AnAOB is not presently known (Tsushima *et al.*, 2007). The number of bacteria was normalized by the DNA concentration extracted per gram of dry sediment. Depth average gene ratios were calculated by dividing the average amount for each bacterial group by the average amount of total bacteria (BAC).

Activity assays

Batch experiments were performed to assess the activity of specific bacterial groups in the sediment of the eutrophic lake Verde. A sediment suspension, prepared by resuspending 50 g of wet sediment from a homogenized sediment core with 10 cm depth in 300 mL distilled water, was incubated in the dark, at room temperature and 150 r.p.m., for several weeks. Substrates were added periodically to stimulate the activity of specific bacterial groups: (1) AOB – $CaCO_3$ (20 mM) and NH_4Cl (52 mg L^{-1} N), (2) NOB – $CaCO_3$ (20 mM) and $NaNO_2$ (100 mg L^{-1} N), (3) DNB – Na-acetate (20 mM) and KNO_3 (140 mg L^{-1} N), (4) IRB – Na-acetate (20 mM) and $FeC_6H_5O_7 \cdot H_2O$ (200 mg L^{-1} Fe) and (5) PAO – Na-acetate (20 mM), K_2HPO_4 (20 mg L^{-1} P) and KH_2PO_4 (10 mg L^{-1} P). Table 3 presents in detail the conditions used in the activity assays. AOB and NOB assays were performed under aerobic conditions obtained through continuous air sparging. Anoxic conditions used in DNB and IRB assays were obtained through N_2 gas sparging for 20 min in the beginning of the assay and after each sampling. In the PAO assay, the aeration was regulated as described in Table 3. Two types of control assays were included in the experimental plan. One was designed to check the release of substrates from sediments (sediments plus water), while the other was planned to check abiotic activity in the sediments (autoclaved sediments plus the addition of the specific inhibitors), as shown in Table 3. To inhibit nitrite and ammonium oxidation activities, 50 mg L^{-1} chlorate (Remde & Conrad, 1991) and 10 mg L^{-1} *N*-allythiourea (Reuschenbach *et al.*, 2003) were used, respectively. Nitrate reduction activity was inhibited by the addition of 50 mg L^{-1} hydroquinone (Zhengping *et al.*, 1991). Control assays were run in parallel with the tests. To monitor microbial activity, samples were taken along the time points, centrifuged at 500 g for 15 min to remove sediment particles and analysed.

The anammox activity was assessed according to Dapena-Mora *et al.* (2007). Briefly, the assays were performed in vials with a total volume of 38 mL and a volume of liquid of 25 mL, closed with a gas-tight coated septum capable of withstanding about 2 bars of pressure. The vials were inoculated with sediment (~12 g of volatile suspended solids) and the headspace and the liquid phase was gasified with argon to remove oxygen. The vials were placed in a thermostatic shaker, at 150 r.p.m. and 30 °C, until stable conditions were reached. Then, the substrates ammonium [1.75 mg N of $(NH_4)_2SO_4$] and nitrite (1.75 mg N of $NaNO_2$) were added and the pressure was equalized to the atmospheric 1. The production of N_2 gas was tracked by measuring the overpressure in the headspace and analysing the gas composition. The anammox activity rate was calculated from the maximum slope of the curve describing the pressure increase in the vial along the time (Dapena-Mora *et al.*, 2007).

Chemical analyses were performed according to standard methods (APHA, 1998). The following spectrophotometric methods were used: the molybdenum blue/stannous chloride method for PO_4^{3-} (SMEWW 4500-P), the UV absorbance method for NO_3^- (SMEWW 4500-NO_3), the Nessler method for NH_4^+ (SMEWW 4500-NH_3 C), the sulphanilamide method for NO_2^- (SMEWW 4500-NO_2^-) and the phenanthroline method for Fe^{2+} and Fe^{3+} (SMEWW 3500-Fe). Acetate was measured by HPLC (87H – Jasco 880). Non-purgeable organic carbon was measured using a TOC analyzer (ShimadzuTOC-Vc, Kyoto). Dissolved oxygen and pH were measured using an oximeter (Oxi 538, WTW)

Table 3. Conditions used and substrates added in each phase of the activity assays of sediment bacteria from Lake Verde

Group	Phase	Test	Control – autoclaved sediments
AOB	I	NH_4^+; CO_3^{2-}	NH_4^+; CO_3^{2-}; chlorate; *N*-allythiourea
	II	NH_4^+	–
	III	NH_4^+	–
NOB	I	NO_2^-; CO_3^{2-}	NO_2^-; CO_3^{2-}; chlorate; *N*-allythiourea
	II	NO_2^-	–
	III	NO_2^-	–
PAO	I	Acetate; PO_4^{3-}; aeration	Acetate; PO_4^{3-}; aeration
	II	Acetate; aeration suppressed	–
	III	Aeration	–
	IV	Acetate; aeration suppressed	–
	V	Aeration	–
DNB	I	Acetate; NO_3^-	Acetate; NO_3^-
	II	NO_3^-	NO_3^-
	III	Acetate	Acetate
	IV	Acetate; NO_3^-	New autoclavation; acetate; NO_3^-
	V	Acetate; NO_3^-	Acetate; NO_3; hydroquinone
	VI	acetate; NO_3^-	Acetate; NO_3^-
	VII	Acetate; NO_3^-	Acetate; NO_3^-; hydroquinone
	VIII	Acetate; NO_3; hydroquinone	Acetate; NO_3^-; hydroquinone
IRB	I	Acetate; Fe(III)	Acetate; Fe(III)
	II	Acetate	–
	III	Acetate	–
	IV	Acetate; Fe(III)	Acetate; Fe(III)
	V	Acetate; Fe(III)	Acetate; Fe(III)
	VI	Acetate; Fe(III)	Acetate; Fe(III)

and a pH meter (420A, ORION), respectively. Volatile suspended solids were determined according to standard methods (SMEWW 2540-E).

Results

Bacterial community composition was studied in lacustrine sediments from four distinct lakes. Table 4 provides all measured environmental conditions in lakes Verde, Azul, Furnas and Fogo. Lakes Verde, Azul and Fogo could be classified as warm monomictic lakes because they are thermally stratified throughout summer. The density difference between the warm surface waters (the epilimnion) and the colder bottom waters (the hypolimnion) prevents these lakes from mixing in summer (Martins *et al.*, 2008). Consequently, the concentration of oxygen near the sediments is very low. The exception is lake Furnas, which is shallower than the others, and rarely stratifies. Thus, lake Furnas sediments are in contact with oxygen almost year round.

Regarding the sediment characteristics, the eutrophic lake Verde had an average (throughout the sediment depth) water content of 92% and an organic content of 18%, which were the highest values determined among the studied lakes. The lowest values were obtained for sediments from the oligo-mesotrophic lake Fogo, with an average 62% water content and 6% organic carbon content. In between are the meso-eutrophic lake Azul and the eutrophic lake Furnas, with an average water content of 77% and 78% and an organic carbon content of 12% and 10%, respectively.

Quantification of total bacteria in sediments

Bacteria involved in nitrogen, phosphorus and iron cycling were quantified by qPCR and the results are depicted in Fig. 1. For the first 10 cm of sediments collected from lakes Azul and Furnas, the bacterial density profiles were almost constant (Fig. 1). For lake Fogo, the bacterial density decreased after 6 cm sediment depth, and for lake Verde, the bacterial density profiles presented an untypical behaviour at a sediment depth of 2 cm when compared with the other lakes. The average total number of sediment bacteria ($n = 5$) quantified with the EUBAC primer set (Harms *et al.*, 2003) was one order of magnitude higher in lake Verde ($3.22 \times 10^9 \pm 2.02 \times 10^8$ cell g^{-1}) than that in the other lakes ($8.48 \times 10^8 \pm 8.93 \times 10^7$ cell g^{-1} in lake Furnas; $4.86 \times 10^8 \pm 3.77 \times 10^7$ cell g^{-1} in lake Azul; $2.33 \times 10^8 \pm 4.24 \times 10^7$ cell g^{-1} in lake Fogo).

The bacterial groups quantified with specific primers, calculated as the sum of the average number of bacteria, represented a small percentage of the total bacteria, especially in sediments from lake Verde (Table 5). The values obtained were of the same order of magnitude for lake Furnas ($2.48 \times 10^8 \pm 3.09 \times 10^7$ cell g^{-1} dry sediment), lake Verde ($1.90 \times 10^8 \pm 3.59 \times 10^7$ cell g^{-1} dry sediment) and lake Azul ($1.64 \times 10^8 \pm 2.50 \times 10^7$ cell g^{-1} dry sediment),

Table 4. Values of water quality in bottom waters and percentage of water and organic carbon content in sediments from lakes Verde, Azul, Furnas and Fogo

	Lake Bottom						Sediment		
Lake	T (°C)	O_2 (mg L^{-1})	pH	NO_3^- (mg L^{-1})	Total P (μg L^{-1})	NH_4^+ (mg L^{-1})	Sediment depth	Water content (mass %)	Organic carbon content/mass %
Verde	14.5	1.7	6.5	0.3	68.0	1.04	0–1 cm	91	18
							1–2 cm	96	18
							2.5–3.5 cm	93	18
							5–6 cm	91	17
							9–10 cm	89	16
Azul	16.3	2.0	6.7	0.6	5.0	0.90	0–1 cm	85	14
							1–2 cm	74	13
							2.5–3.5 cm	79	12
							5–6 cm	75	11
							9–10 cm	74	11
Furnas	21.0	5.3	6.7	0.5	63.0	0.02	0–1 cm	83	11
							1–2 cm	76	9
							2.5–3.5 cm	76	10
							5–6 cm	78	10
							9–10 cm	76	10
Fogo	13.8	5.0	6.7	0.2	8.0	0.02	0–1 cm	63	6
							1–2 cm	67	6
							2.5–3.5 cm	64	6
							5–6 cm	62	6
							9–10 cm	55	5

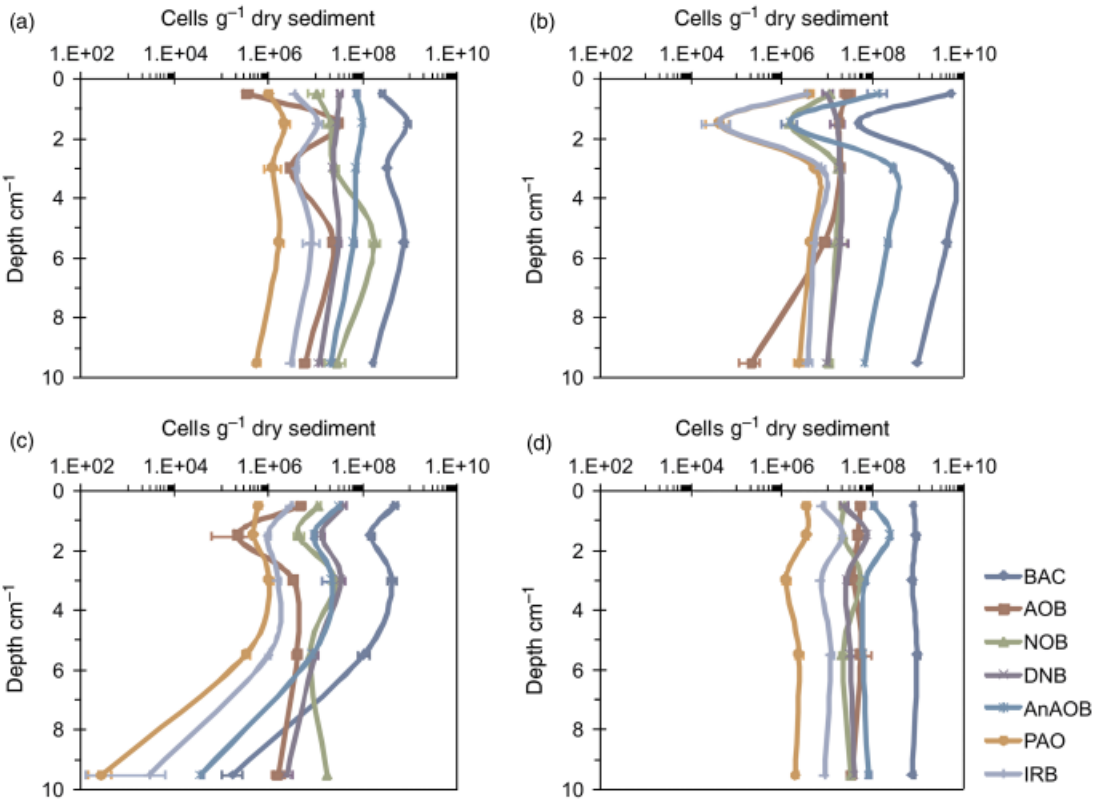

Fig. 1. Sediment bacterial profiles in volcanic Azorean lakes: (a) Azul; (b) Verde; (c) Fogo; (d) Furnas. AOB, ammonia-oxidizing bacteria; NOB, nitrite-oxidizing bacteria; DNB, denitrifying bacteria; AnAOB, anaerobic ammonia-oxidizing bacteria; IRB, iron-reducing bacteria; and, PAO, polyphosphate-accumulating organisms. Error bars represent the SDs resulting from triplicate analyses.

Table 5. Ratios of bacteria quantified with specific primers and total bacteria – BAC, unknown bacteria and BAC

	Average number bacteria/total bacteria (SD) (%)						
Lake	AOB	NOB	DNB	AnAOB	IRB	PAO	Unknown
Azul	2.3 (1.6)	10.7 (8.8)	6.8 (3.7)	16.6 (8.3)	1.4 (0.3)	0.3 (0.1)	62.0 (9.8)
Verde	0.3 (0.2)	0.5 (0.4)	0.5 (0.4)	4.5 (1.6)	0.2 (0.1)	0.1 (0.1)	93.6 (2.3)
Furnas	5.7 (1.0)	3.8 (2.0)	4.7 (2.3)	13.3 (8.2)	1.4 (0.6)	0.3 (0.1)	70.8 (10.1)
Fogo	1.4 (1.6)	4.6 (2.4)	8.8 (0.7)	6.6 (1.3)	0.9 (0.5)	0.2 (0.1)	77.7 (5.2)

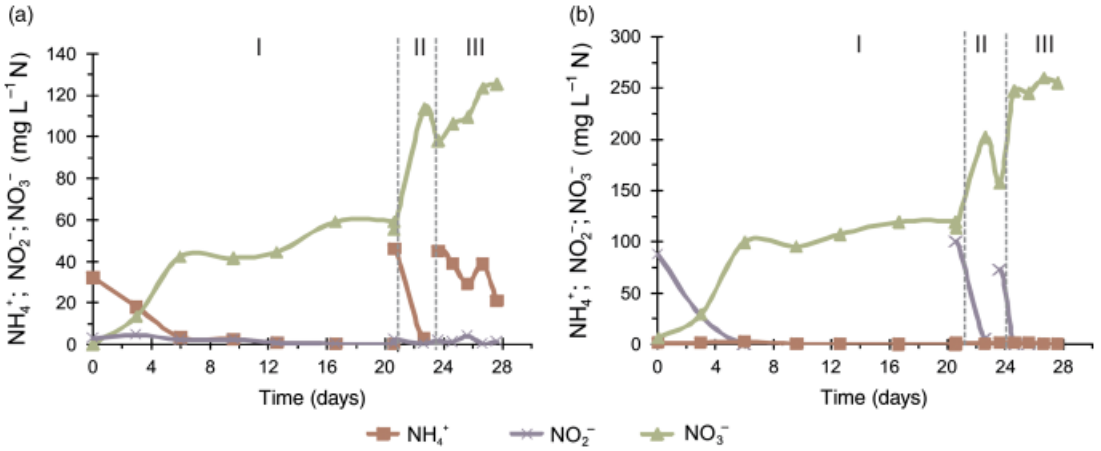

Fig. 2. Activity assays of (a) AOB and (b) NOB assessed with sediments from lake Verde: I, addition of carbonate and NH_4^+ to the AOB assay, and carbonate and NO_2^- to the NOB assay; II and III, addition of NH_4^+ to the AOB assay, and NO_2^- to the NOB assay.

but one order of magnitude lower for lake Fogo ($5.25 \times 10^7 \pm 6.88 \times 10^6$ cell g^{-1} dry sediment). In general, AnAOB were the most abundant bacterial group detected in the sediments from lakes Verde, Azul and Furnas, followed by nitrifiers (AOB and NOB), DNB, IRB and PAO. For lake Fogo, DNB were the most abundant group, followed by AnAOB, nitrifiers, IRB and PAO. Among the specific groups of bacteria investigated, the percentage of AOB, IRB and PAO was higher in lake Furnas than in the other lakes, while lake Azul presented the highest percentages of NOB and AnAOB (Table 5).

Bacteria involved in the nitrogen cycle

Aerobic bacteria performing nitrification (AOB and NOB) were also detected in anoxic/anaerobic layers of sediments from all studied lakes. The amounts of AOB and NOB obtained in the present study were in the range of 10^5–10^7 cells g^{-1} and of 10^6–10^7 cells g^{-1}, respectively. AOB were more abundant than NOB in sediments from lake Furnas; the opposite was observed in sediments from lakes Verde, Azul and Fogo (Table 5). The abundance of NOB affiliated to *Nitrobacter* spp. and *Nitrospira* spp. suggested that *Nitrobacter*-like bacteria were more abundant in sediments from lakes Azul (69%), Fogo (58%) and Furnas (55%), while in lake Verde, *Nitrospira*-like bacteria dominated (92%). In sediments from lake Azul and Fogo, the amount of NOB was slightly higher than the one of AOB, which has also been reported in other studies for river sediments (Nakamura *et al.*, 2006) and biofilms (Nogueira *et al.*, 2005).

AnAOB and DNB were the most abundant groups even in the sediment surficial layers (Fig. 1). AnAOB densities varied between 10^6 and 10^8 cells g^{-1} and were the highest in sediments from lakes Furnas, Azul and Verde, while DNB dominated in sediments from lake Fogo. Regarding DNB, the functional gene nirS (10^6–10^7 cells g^{-1}), associated with uncultured marine bacterial clones (*Pseudomonas* sp., *Marinobacter* sp. and, *Thauera*), contributed more than the gene nirK (10^4–10^6 cells g^{-1}), related to the genus *Alcaligenes* and *Hyphomicrobium* (Yoshie *et al.*, 2004; Osaka *et al.*, 2006).

Nitrifying activity of sediment bacteria from the eutrophic lake Verde was assessed in aerobic batch assays in the presence of specific substrates and inhibitors. The activity of AOB (Fig. 2a) and NOB (Fig. 2b) was stimulated by the addition of NH_4^+ and NO_2^-, respectively. A decrease in the concentrations of NH_4^+ and NO_2^- was observed in AOB and NOB assays, respectively, followed by an increase in the concentration of NO_3^- in both assays. The repeated addition of NO_2^- to the NOB assay (phases II and III) resulted in the decrease of the time needed for complete NO_2^- removal, likely due to NOB enrichment in the sediment suspension.

In the AOB assay, the decrease of the NH_4^+ concentration was also faster during phase II, but incomplete NH_4^+ oxidation and a decrease in pH (pH decreased from 7.36 to 6.46) were observed in phase III, probably due to inorganic carbon limitation. As expected, the concentrations of NH_4^+ and NO_2^- remained constant with time, in assays conducted with autoclaved sediments and in the presence of specific inhibitors for AOB and NOB, *N*-allythiourea and chlorate, respectively (data not shown). The additional control assay, conducted without the addition of substrates, suggested that N-compounds were not released to a significant extent from sediments by abiotic processes (data not shown).

In the anammox activity assay, an increase in the amount of N_2 in the gas phase with time was observed (Fig. 3). The decrease of NH_4^+ and NO_2^- in the liquid phase followed the stoichiometry of the anammox reaction. Of the 1.75 mg N of both NO_2^- and NH_4^+ initially added, around 0.41 mg N of NO_2^- and 0.74 mg N of NH_4^+ were consumed. The anammox activity determined in the present study, 2.03 ± 0.37 fmol per cell day^{-1} (assuming an average value of $8.40 \times 10^7 \pm 9.87 \times 10^6$ AnAOB cells per gram of sediment), is consistent with reported values for other natural environments (Schubert *et al.*, 2006).

Nitrate reduction, assessed in anoxic batch assays, was stimulated by the addition of acetate and NO_3^- (Fig. 4). A decrease in the concentration of both compounds was observed (phase I), and subsequent replicate assays (phases III–VII) resulted in the decrease of the time needed for complete NO_3^- removal, probably due to DNB enrichment in the sediment suspension. Without acetate (phase II), the NO_3^- concentration remained constant, suggesting that nitrate reduction in lake Verde sediments was carried out by heterotrophic bacteria. Both attempts to inactive biological activity in the sediment, by autoclaving and addition of a DNB inhibitor, did not prevent the removal of acetate and NO_3^-, but retarded nitrate reduction (data not shown).

PO_4^{3-} uptake/release was observed during the repetitive addition of acetate and NO_3^- to batch assays (phases IV–VII in Fig. 4). On average, in the anoxic phase, 213 mg L^{-1} C and 112 mg L^{-1} N were consumed with the consequent uptake of 59 μg L^{-1} P, while in the anaerobic phase (absence of NO_3^-), 74 mg L^{-1} C was consumed and 68 μg L^{-1} P was released.

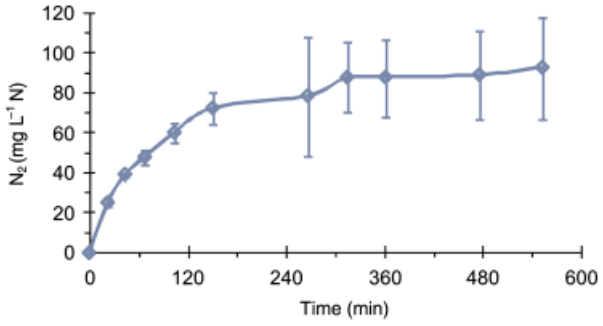

Fig. 3. Activity assays of anammox in the sediment from lake Verde.

IRB

Depth-invariant profiles of IRB belonging to the *Geobacteraceae* family were observed in sediments from all lakes. The densities, however, differed from 10^3 to 10^7 cells g^{-1}. Lake Furnas had the highest density of IRB ($2.20 \times 10^7 \pm 7.80 \times 10^5$ cells g^{-1}, sediment depth of 1–2 cm), which was expected because it also had the highest concentration of total iron (241 μg L^{-1}) in the water column (Cruz *et al.*, 2006).

Iron-reducing activity of the sediment from lake Verde was assessed in anaerobic batch assays in the presence of acetate and soluble Fe(III) (Fig. 5). A decrease in the concentration of both acetate and soluble Fe(III) was observed in phase I. The subsequent addition of acetate (phases II and III) resulted in the complete reduction of Fe(III); however, the concentration of Fe(II) resulting from the reduction of Fe(III) did not increase. Two exceptions included phases V and VI, where a slight increase in Fe(II) was detected between days 29 and 31 and days 32 and 36, respectively. One possible explanation for this result is that Fe(II)-containing minerals were formed, but not accounted for, by the method used in the present study. As suggested in the literature, magnetite might be an end product of the activity of Fe(III)-reducing microorganisms in sedimentary environments (Lovley, 1991). In the presence of an excess of acetate (phases IV–VI), the time needed for complete Fe(III)

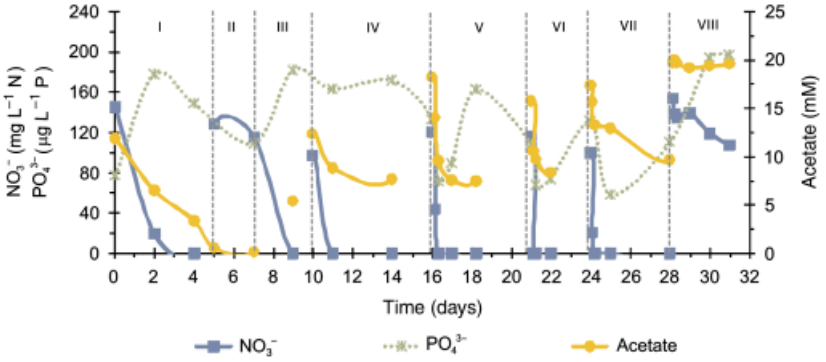

Fig. 4. Activity assays of DNB in the sediment from lake Verde: I, addition of acetate and NO_3^-; II, addition of NO_3^-; III, addition of acetate; IV, addition of acetate and NO_3^-; V, addition of acetate and NO_3^-; VI, same as IV; VII, same as V; VIII, addition of acetate, NO_3^-, and hydroquinone.

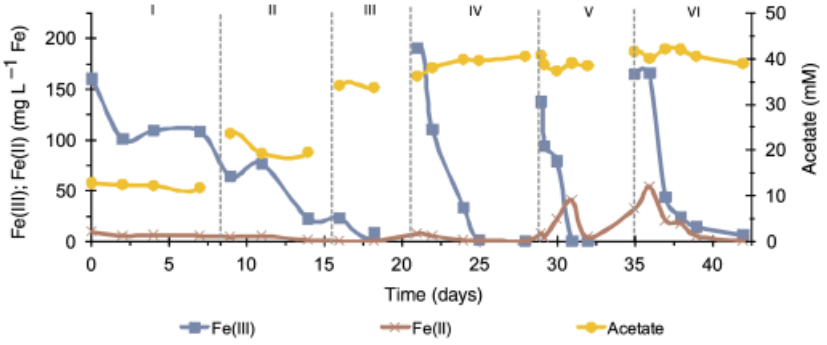

Fig. 5. Activity assays of IRB in the sediment from lake Verde: I, addition of acetate and soluble Fe(III); II and III, addition of acetate; IV–VI, addition of acetate and soluble Fe(III).

removal decreased probably due to the enrichment of the sediment suspension in IRB. Assays conducted with a sediment suspension previously autoclaved (Table 3) also exhibited the removal of acetate and soluble Fe(III), but at a much lower rate.

PAOs

The density of *C.* Accumulibacter-related PAO in sediments from Azorean lakes was invariant with depth, ranging between 10^2 and 10^6 cells g^{-1} (Fig. 1, Table 5). Among the Azorean lakes, lake Verde contained the highest density of PAO ($5.40 \times 10^6 \pm 2.2 \times 10^5$ cells g^{-1}). The activity of PAO in sediments from lake Verde was checked in batch assays by alternating aerobic/anaerobic conditions and periodic acetate addition (Table 3). During the aerobic phase, the uptake of P was not observed (data not shown), while in the presence of nitrate (Fig. 4; phases V, VI and VII) the soluble P concentration decreased. These results suggested that PAO present in sediments from lake Verde prefer nitrate over oxygen as the final electron acceptor.

Discussion

This study, for the first time, documents the abundance of several microbial groups, namely AOB, AnAOB, NOB, DNB, IRB and PAO, in lacustrine sediments of Azorean lakes. The values found were in the same range as those previously reported for soils and freshwater sediments (Henry *et al.*, 2004; Nakamura *et al.*, 2006; Bedard *et al.*, 2007; Gloess *et al.*, 2007; Smith *et al.*, 2007; Bulow *et al.*, 2008; Himmelheber *et al.*, 2009). The exception was the relative abundance of AnAOB (4.5 ± 1.6–$16.6 \pm 8.3\%$ of the total bacteria), which was higher than the previously reported values (Tal *et al.*, 2005; Schubert *et al.*, 2006; Schmid *et al.*, 2007). Cell counts based on FISH reported that AnAOB amounted to 1.4% of all bacteria in freshwater sediments using the probe Amx820 (Schubert *et al.*, 2006) and between 0.4% and 8.6% in sea sediments, obtained with both Amx820 and BS820 probes (Tal *et al.*, 2005; Schmid *et al.*, 2007). To our knowledge, this is one of the first studies reporting high densities of AnAOB in lacustrine sediments.

Relating the results on bacterial group densities with the trophic state of the lakes, it suggests that AnAOB are more competitive than DNB in sediments from lakes Verde (eutrophic), Furnas (eutrophic) and Azul (meso-eutrophic). The lower amount of AnAOB in the sediment from lake Fogo (oligo-mesotrophic) might be due to the very low concentration of NH_4^+ in this lake (0.02 mg L^{-1} N) compared with the others. Also, the total bacteria density and PAO densities seem to be related to the trophic state of the lakes, being higher in the eutrophic lake Verde than in the oligo-mesotrophic lake Fogo.

Only for lakes Azul and Furnas did the sediments present bacterial density stratification. Surface sediments of lake Azul were dominated by anaerobic groups (DNB and AnAOB), while in lake Furnas sediments, aerobes (AOB, NOB and PAO) dominated in the upper layers and anaerobes downward. Nevertheless, bacterial stratification never exceeded one order of magnitude over the examined 10 cm depth. This bacterial distribution might be explained by the fact that lake Azul presented anoxic conditions near the sediments (2% of oxygen saturation), while aerobic conditions were observed in lake Furnas (81% of oxygen saturation). For the other two lakes, bacterial density stratification was not observed. The growth of AnAOB and DNB in the upper sediment layers might have been enhanced by the prevalence of anoxic/anaerobic conditions due to the low oxygen penetration, which is generally < 1 cm (Altmann *et al.*, 2004; Himmelheber *et al.*, 2009). On the other hand, the presence of AOB and NOB deeper in the sediment was expected because nitrifiers can grow under oxygen-limited conditions, as reported in previous studies (Pynaert *et al.*, 2002).

Contrary to our expectation, the bacterial density profiles were almost homogeneous with depth. Similar results have been reported for stream sediments (Altmann *et al.*, 2004; Nakamura *et al.*, 2006) and anoxic sediments from the Black Sea, where the total bacterial cell number was almost constant until a depth of 100 cm (Leloup *et al.*, 2007). Several phenomena occurring in sediments, namely physical resuspension and bioturbation, as well as the formation of gas bubbles, feeding tubes and burrows from benthic fauna,

might explain the mixing between the different zones (Kemp *et al.*, 1990; Stockdale *et al.*, 2009). The bacterial profiles for lake Verde presented an atypical pattern at a sediment depth of 2 cm. A possible explanation for this result might be the very high amount of water (96%) in this layer that hindered DNA extraction and consequently PCR quantification (Bürgmann *et al.*, 2001; Sagova-Mareckova *et al.*, 2008). For lake Fogo, the bacterial density decreased mainly after a sediment depth of 6 cm, which might be the result of the very low organic carbon content present in the deepest layers of this sediment (5% at depth between 6 and 10 cm) (Miller *et al.*, 1999; Sagova-Mareckova *et al.*, 2008). The amount of organic carbon might also explain the differences in the total bacterial density found in the sediments from the different lakes, because it has been related to DNA yields (Miller *et al.*, 1999). In the present study, the amount of total bacteria correlated positively with the content of organic matter in sediments.

The main limitation of the present study was related to the oligonucleotide primer choice used for the qPCR-based detection of IRB and PAO, which may have led to an underestimation of these bacterial groups. A previous fingerprinting study using PCR-DGGE-cloning methods suggested the presence of members of the *Shewanelanaceae* and *Ferrimonadaceae* families as well as the *Actinobacteria* and *Gemmatimonadetes*-related sequences in sediments from lake Furnas that were not covered by the chosen primers (Martins *et al.*, 2010). Real-time qPCR is an emerging tool to quantify bacteria in environmental samples. However, at present, two main constraints hinder the general use of this technique: the availability of suitable primers is still limited and PCR is easily inhibited by humic substances that are coextracted with DNA. Humic substances are very common in lake sediments (Wolfe *et al.*, 2002) and they inhibit the restriction endonucleases and Taq DNA polymerase (Porteous & Armstrong, 1991). Two published methods based on phenol/chloroform extractions (Kowalchuk *et al.*, 1998; Miller *et al.*, 1999) and subject to various pre- and post-treatment [$CaCl_2$, $AlNH_4(SO_4)_2$ and $CaCO_3$ incubations] were compared with a commercially available method (UltraClean Soil DNA kit) for the recovery of DNA from sediments. Ultimately, the method of Kowalchuk without any pre-/post-treatment was chosen in view of DNA quantity and amplifiability.

Batch assays confirmed the activity of all the bacterial groups quantified. The more interesting result was the apparent capacity of DNB to accumulate and release P, depending on the presence of acetate and NO_3^-. This behaviour suggests the presence of denitrifying PAOs (DPAO) in the sediments (Ahn *et al.*, 2001; Tsuneda *et al.*, 2006). The activity of DPAO has often been demonstrated, both in lab-scale and full-scale enhanced biological P removal systems (Carvalho *et al.*, 2007). To our knowledge, this is the first study that indicates the accumulation/release of P in sediments with the activity of these bacteria.

Eutrophication of freshwater reservoirs and lakes is mainly due to inputs of P from sediments to the water column (Martins *et al.*, 2008). The present work suggested that the activity of DNB might also contribute to the release of P from sediments. In this regard, an evaluation of the sediment microbial composition and activity is certainly useful to design requalification strategies towards attainment of the good ecological status prescribed by the Water Framework Directive (European Community, 2000). Thus, future measures towards lake restoration should include, in addition to the classical procedure, an evaluation of the contribution of biological processes in sediments to the eutrophication problem. Besides, the results of the present study suggest the improvement/calibration of lake water quality models. Existing models often describe the processes occurring in the sediments in less detail than those occurring in the water column (Martins *et al.*, 2008). Extended models including sediment processes contributing to the uptake/release of N and P from sediments to the water column need a considerable amount of data for calibration.

Future studies characterizing the relationship between the activity of specific bacterial groups (PAO, DNB, IRB and sulphate-reducing bacteria) and P cycling may reveal the limiting factors that control P release from sediments. In addition to microcosm activity assays, *in situ* activities should also be investigated. Other tools like microelectrodes (Himmelheber *et al.*, 2009), the use of radio labelled elements or the quantification of expression of genes that encode key enzymes could aid in the determination of microbial activities and bioprocess rates (Dar *et al.*, 2007).

In conclusion, the combination of phylogenetic and fractional gene-targeted qPCR and activity assays suggested that bacteria performing anaerobic ammonium oxidation were the most abundant in sediments from the eutrophic lakes Verde, Furnas and Azul, while DNB dominated in sediments from the oligo-mesotrophic lake Fogo. Lake Furnas sediments presented bacterial stratification (aerobes in upper layers and anaerobes downward) due to the presence of oxygen in the hypolimnion all year round. The present work also suggested that the activity of DNB might contribute to the release of phosphorus from sediments.

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References

Aakra A, Utaker JB & Nes LF (1999) RFLP of rRNA genes and sequencing of the 16S–23S rDNA intergenic spacer region of ammonia-oxidizing bacteria: a phylogenetic approach. *Int J Syst Bacteriol* **49**: 123–130.

Ahn J, Daidou T, Tsuneda S & Hirata A (2001) Metabolic behavior of denitrifying phosphate-accumulating organisms under nitrate and nitrite electron acceptor conditions. *J Biosci Bioeng* **92**: 442–446.

Altmann D, Stief P, Amann R & Beer DD (2004) Distribution and activity of nitrifying bacteria in natural stream sediment versus laboratory sediment microcosms. *Aquat Microb Ecol* **36**: 73–81.

APHA, AWWA & WPCF (1998) *Standard Methods for the Examination of Water and Wastewater*, 20th edn. American Public Health Association, Washington, DC.

Azzoni R, Giordani G & Viaroli P (2005) Iron–sulphur–phosphorus interactions: implications for sediment buffering capacity in a Mediterranean eutrophic lagoon (Sacca di Goro, Italy). *Hydrobiologia* **550**: 131–148.

Bedard DL, Ritalahti KM & Löffler FE (2007) The Dehalococcoides population in sediment-free mixed cultures metabolically dechlorinates the commercial polychlorinated biphenyl mixture Aroclor 1260. *Appl Environ Microb* **73**: 2513–2521.

Bulow SE, Francis C, Jackson G & Ward BB (2008) Sediment denitrifier community composition and nirS gene expression investigated with functional gene microarrays. *Environ Microbiol* **10**: 3057–3069.

Bürgmann H, Pesaro M, Widmer F & Zeyer J (2001) A strategy for optimizing quality and quantity of DNA extracted from soil. *J Microbiol Meth* **45**: 7–20.

Carvalho G, Lemos PC, Oehmen A & Reis MA (2007) Denitrifying phosphorus removal: Linking the process performance with the microbial community structure. *Water Res* **41**: 4383–4396.

Cruz JV, Antunes P, Amaral C & Nunes JC (2006) Volcanic lakes of the Azores archipelago (Portugal): geological setting and geochemical characterization. *J Volcanol Geoth Res* **156**: 135–157.

Cummings DE, Snoeyenbos-West L, Newby DT, Niggemyer AM, Lovley DR, Achenbach LA & Rosenzweig RF (2003) Diversity of Geobacteraceae species inhabiting metal-polluted freshwater lake sediments ascertained by 16S rDNA analyses. *Microb Ecol* **46**: 257–269.

Dalsgaard T, Thamdrup B & Canfield DE (2005) Anaerobic ammonium oxidation (anammox) in the marine environment. *Res Microbiol* **156**: 457–464.

Dapena-Mora A, Fernández I, Campos JL, Mosquera-Corral A, Méndez R & Jetten MSM (2007) Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. *Enzyme Microb Tech* **40**: 859–865.

Dar SA, Yao L, van Dongen U, Kuenen JG & Muyzer G (2007) Analysis of diversity and activity of sulfate reducing bacterial communities in sulfidogenic bioreactors using 16S rRNA and dsrB genes as molecular markers. *Appl Environ Microb* **73**: 594–604.

Degrange V & Bardin R (1995) Detection and counting of *Nitrobacter* populations in soil by PCR. *Appl Environ Microb* **61**: 2093–2098.

European Community (2000) Directive 2000/60/EC of October 23 2000 of the European Parliament and of the Council establishing a framework for community action in the field of water policy. *Off J Eur Comm* **L327**: 1–72.

Gaechter R & Meyer JS (1993) The role of microorganisms in mobilization and fixation of phosphorus in sediments. *Hydrobiologia* **253**: 103–121.

Gloess S, Hupfer M, Ratering S & Grossart HP (2007) Detection and phylogenetic characterization of polyphosphate accumulating bacteria in lake sediments. *Annual Report* 2006. Berichte der IGB.

Golterman HL (2001) Phosphate release from anoxic sediments or 'What did Mortimer really write?'. *Hydrobiologia* **450**: 99–106.

Graham DW, Knapp CW, Van Vleck ES, Bloor K, Lane T & Graham CE (2007) Experimental demonstration of chaotic instability in biological nitrification. *ISME J* **1**: 385–394.

Griffiths RI, Whiteley AS, O'Donnell AG & Bailey MJ (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl Environ Microb* **66**: 5488–5491.

Harms G, Layton AC, Dionisi HM *et al.* (2003) Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. *Environ Sci Technol* **37**: 343–351.

He S, Gall DL & McMahon KD (2007) *Candidatus* Accumulibacter' population structure in enhanced biological phosphorus removal sludges as revealed by polyphosphate kinase genes. *Appl Environ Microb* **73**: 5865–5874.

Henry S, Baudoin E, López-Gutiérrez JC, Martin-Laurent F, Brauman A & Philippot L (2004) Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. *J Microbiol Meth* **59**: 327–335.

Hermansson A & Lindgren PE (2001) Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. *Appl Environ Microb* **67**: 972–976.

Himmelheber DW, Thomas SH, Loffler FE, Taillefert M & Hughes JB (2009) Microbial colonization of an *in situ* sediment cap and correlation to stratified redox zones. *Environ Sci Technol* **43**: 66–74.

Hupfer M, Gloess S & Grossart H (2007) Polyphosphate-accumulating microorganisms in aquatic sediments. *Aquat Microb Ecol* **47**: 299–311.

Hupfer M, Glöss S, Schmieder P & Grossart H (2008) Methods for detection and quantification of polyphosphate and polyphosphate accumulating microorganisms in aquatic sediments. *Int Rev Hydrobiol* **93**: 1–30.

Hupfer M & Lewandowski J (2008) Oxygen controls the phosphorus release from lake sediments - a long-lasting paradigm in limnology. *Internat Rev Hydrobiol* **93**: 415–432.

Kemp WM, Sampou P, Caffrey J & Mayer M (1990) Ammonium recycling versus denitrification in Chesapeake Bay sediment. *Limnol Oceanogr* **35**: 1545–1563.

Kindaichi T, Kawano Y, Ito T, Satoh H & Okabe S (2006) Population dynamics and *in situ* kinetics of nitrifying bacteria in autotrophic nitrifying biofilms as determined by real-time quantitative PCR. *Biotechnol Bioeng* **94**: 1111–1121.

Klappenbach JA, Saxman PR, Cole JR & Schmidt TM (2001) rrndb: the Ribosomal RNA Operon Copy Number Database. *Nucleic Acids Res* **29**: 181–184.

Kojima H, Koizumi Y & Fukui M (2006) Community structure of bacteria associated with sheaths of freshwater and brackish Thioploca species. *Microb Ecol* **52**: 765–773.

Kowalchuk GA, Stephen JR, DeBoer W, Prosser JI, Embley TM & Woldendorp JW (1997) Analysis of ammonia oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Appl Environ Microb* **63**: 1489–1497.

Kowalchuk GA, Bodelier PLE, Heilig GH, Stephen JR & Laanbroek HJ (1998) Community analysis of ammonia-oxidizing bacteria, in relation to oxygen availability in soils and root-oxygenated sediments using PCR, DGGE and oligonucleotide probe hybridization. *FEMS Microbiol Ecol* **27**: 339–350.

Leloup J, Loy A, Knab NJ, Borowski C, Wagner M & Jørgensen BB (2007) Diversity and abundance of sulfate-reducing microorganisms in the sulfate and methane zones of a marine sediment, Black Sea. *Environ Microbiol* **9**: 131–142.

Lovley DR (1991) Dissimilatory Fe(III) and Mn(IV) reduction. *Microbiol Rev* **55**: 259–287.

Martins G, Ribeiro DC, Pacheco D, Cruz JV, Cunha R, Gonçalves V, Nogueira R & Brito AG (2008) Prospective scenarios for water quality and ecological status in Lake Sete Cidades (Portugal): the integration of mathematical modelling in decision processes. *Appl Geochem* **23**: 2171–2181.

Martins G, Peixoto L, Ribeiro DC, Parpot P, Brito AG & Nogueira R (2010) Towards implementation of a benthic microbial fuel cell in lake Furnas (Azores): phylogenetic affiliation and electrochemical activity of sediment bacteria. *Bioelectrochemistry* **78**: 67–71.

Miller DN, Bryant JE, Madsen EL & Ghiorse WC (1999) Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl Environ Microb* **65**: 4715–4724.

Musat N, Werner U, Knittel K, Kolb S, Dodenhof T, van Beusekom JEE, de Beer D, Dubilier N & Amann R (2006) Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Rømø Basin, Wadden Sea. *Syst Appl Microbiol* **29**: 333–348.

Nakamura Y, Satoh H, Kindaichi T & Okabe S (2006) Community structure, abundance, and *in situ* activity of nitrifying bacteria in river sediments as determined by the combined use of molecular techniques and microelectrodes. *Environ Sci Technol* **40**: 1532–1539.

Nealson KH (1997) Sediment bacteria: who's there, what are they doing, and what's new? *Annu Rev Earth Pl Sc* **25**: 403–434.

Nelson DM, Ohene-Adjei S, Hu FS, Cann IKO & Mackie RI (2007) Bacterial diversity and distribution in the Holocene sediments of a northern temperate lake. *Microb Ecol* **54**: 252–263.

Nogueira R, Elenter D, Brito AG, Melo LF, Wagner M & Morgenroth E (2005) Evaluating heterotrophic growth in a nitrifying biofilm reactor using fluorescence *in situ* hybridization and mathematical modelling. *Water Sci Technol* **52**: 135–145.

Oakley BB, Francis C, Roberts KJ *et al.* (2007) Analysis of nitrite reductase (nirK and nirS) genes and cultivation reveal depauperate community of denitrifying bacteria in the Black Sea suboxic zone. *Environ Microbiol* **9**: 118–130.

Osaka T, Yoshie S, Tsuneda S, Hirata A, Iwami N & Inamori Y (2006) Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing. *Microb Ecol* **52**: 253–266.

Porteous LA & Armstrong JL (1991) Recovery of bulk DNA from soil by a rapid, small-scale extraction method. *Curr Microbiol* **22**: 345–348.

Pynaert K, Sprengers R, Laenen J & Verstraete W (2002) Oxygen-limited nitrification and denitrification in a lab-scale rotating biological contactor. *Environ Tech* **23**: 353–362.

Remde A & Conrad R (1991) Role of nitrification and denitrification for NO metabolism in soil. *Biogeochemistry* **12**: 189–205.

Reuschenbach P, Pagga U & Strotmann U (2003) A critical comparison of respirometric biodegradation tests based on OECD 301 and related test methods. *Water Res* **37**: 1571–1582.

Ribeiro DC, Martins G, Nogueira R, Cruz JV & Brito AG (2008) Phosphorus fractionation in volcanic lake sediments (Azores - Portugal). *Chemosphere* **70**: 1256–1263.

Rozan TF, Taillefert M, Trouwborst RE, Glazer BT, Ma S, Herszage J, Valdes IM, Price KS & Luther GW III (2002) Iron–sulphur–phosphorus cycling in the sediments of a shallow coastal bay: implications for sediment nutrient release

and benthic macroalgal blooms. *Limnol Oceanogr* **47**: 1346–1354.

Sagova-Mareckova M, Cermak L, Novotna J, Plhackova K, Forstova J & Kopecky J (2008) Innovative methods for soil DNA purification tested in soils of widely differing characteristics. *Appl Environ Microb* **74**: 2902–2907.

Santos M, Pacheco D, Santana F & Muelle H (2005) Cyanobacteria blooms in Sete-Cidades lake (S. Miguel Island – Azores). *Algol Stud* **117**: 393–406.

Schmid MC, Risgaard-Petersen N, van de Vossenberg J *et al.* (2007) Anaerobic ammonium-oxidizing bacteria in marine environments: widespread occurrence but low diversity. *Environ Microbiol* **9**: 1476–1484.

Schubert CJ, Durisch-Kaiser E, Wehrli B, Thamdrup B, Lam P & Kuypers MM (2006) Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika). *Environ Microbiol* **8**: 1857–1863.

Smith CJ, Nedwell DB, Dong LF & Osbourn AM (2007) Diversity and abundance of nitrate reductase genes (*narG* and *napA*), nitrite reductase genes (*nirS* and *nrfA*), and their transcripts in estuarine sediments. *Appl Environ Microb* **73**: 3612–3622.

Stockdale A, Davison W & Zhang H (2009) Micro-scale biogeochemical heterogeneity in sediments: a review of available technology and observed evidence. *Earth Sci Rev* **92**: 81–97.

Tal Y, Watts JE & Schreier HJ (2005) Anaerobic ammonia-oxidizing bacteria and related activity in Baltimore Inner Harbor sediment. *Microbiology* **71**: 1816–1821.

Terada A, Lackner S, Kristensen K & Smets BF (2010) Inoculum effects on community composition and nitritation performance of autotrophic nitrifying biofilms reactors with counter-diffusion geometry. *Environ Microbiol* **12**: 2858–2872.

Throbäck IN, Enwall K, Jarvis A & Hallin S (2004) Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiol Ecol* **49**: 401–417.

Tsuneda S, Miyauchi R, Ohno T & Hirata A (2005) Characterization of denitrifying polyphosphate-accumulating organisms in activated sludge based on nitrite reductase gene. *J Biosci Bioeng* **99**: 403–407.

Tsuneda S, Ohno T, Soejima K & Hirata A (2006) Simultaneous nitrogen and phosphorus removal using denitrifying phosphate-accumulating organisms in a sequencing batch reactor. *Biochem Eng J* **27**: 191–196.

Tsushima I, Kindaichi T & Okabe S (2007) Quantification of anaerobic ammonium-oxidizing bacteria in enrichment cultures by real-time PCR. *Water Res* **41**: 785–794.

White AK & Metcalf WW (2007) Microbial metabolism of reduced phosphorus compounds. *Annu Rev Microbiol* **61**: 379–400.

Wobus A, Bleul C, Maassen S, Scheerer C, Schuppler M, Jacobs E & Röske I (2003) Microbial diversity and functional characterization of sediments from reservoirs of different trophic state. *FEMS Microbiol Ecol* **46**: 331–347.

Wolfe AP, Kaushal SS, Fulton JR & Mcknight DM (2002) Spectrofluorescence of Sediment Humic substances and historical changes of lacustrine organic matter provenance in response to atmospheric nutrient enrichment. *Environ Sci Technol* **36**: 3217–3223.

Yiyong Z, Jianqiu l & Min Z (2002) Temporal and spatial variations in kinetics of alkaline phosphatase in sediments of a shallow Chinese eutrophic lake (Lake Donghu). *Water Res* **36**: 2084–2090.

Yoshie S, Noda N, Tsuneda S, Hirata A & Inamori Y (2004) Salinity decreases nitrite reductase gene diversity in denitrifying bacteria of wastewater treatment systems. *Appl Environ Microb* **70**: 3152–3157.

Zeng J, Yang L, Li J, Liang Y, Xiao L, Jiang L & Zhao D (2009) Vertical distribution of bacterial community structure in the sediments of two eutrophic lakes revealed by denaturing gradient gel electrophoresis (DGGE) and multivariate analysis techniques. *World J Microb Biot* **25**: 225–233.

Zhengping W, Liantie L, Van Cleemput O & Baert L (1991) Effect of urease inhibitors on denitrification in soil. *Soil Use Manage* **7**: 230–233.