

Coupling of bacterial nitrification with denitrification and anammox supports N removal in intertidal sediments (Arcachon Bay, France)

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ABSTRACT

Microbial activity measurements and molecular approaches were combined to study temporal variation in nitrogen (N) removal mechanisms from muddy and sandy intertidal sediments (Arcachon Bay, France). Stable isotopes ($^{15}\text{NH}_4^+ / ^{15}\text{NO}_3^-$) were used to measure oxidation of NH_4^+ to $\text{NO}_3^- / \text{NO}_2^-$ and its subsequent reduction to N_2 via denitrification and/or anammox. We found that denitrification mainly fuelled N loss in both sediment types. However in sandy sediments, anammox accounted for relatively higher N_2 production (45%). Nitrification-denitrification (D_n) and nitrification-anammox coupling was observed particularly in muddy sediments. Temporal variations in prokaryotic abundance (Bacteria, Archaea) and of functional groups responsible for nitrification and denitrification were studied by qPCR and for anammoxifiers by cloning. Bacterial density in both sediment types varied between 10^{7-8} equivalent cell per g^{-1} dry weight sediment and were overall more abundant in muddy sediments. Archaeal equivalent cell varied between 0.5 to 6.5% of the total prokaryotes, with extreme values observed in muddy sediment during October and January respectively. Denitrifier abundance was stable over time than betaproteobacterial or archaeal nitrifiers. Furthermore, nitrifiers exhibited greater fluctuations in sandy sediments compared to muddy ones. Anammoxifiers were mainly restricted to the “*Candidatus Scalindua*” group, and were detected in both sediment types and all sampling periods providing an indirect proof on the occurrence of anammox. In muddy sediments, N_2 production presented overall higher rate over sandy sediments and exhibited the same trend as nitrifier abundance. This finding suggests a central role of bacterially-mediated benthic nitrification in supporting dissimilatory processes through generation of $\text{NO}_3^- / \text{NO}_2^-$ and preventing eutrophication in the Arcachon Bay.

Keywords: Anammox; denitrification; diversity; nitrification; nitrogen; sediments

1. Introduction

Denitrification has long been regarded as the major step in removing nitrogen (N) from aquatic systems. The first experimental proof of a second N_2 producing process viz., anammox was published by Mulder et al. (1995). Later, anammox was found to be responsible for ~ 24 – 67% of N loss in marine sediments (Thamdrup and Dalsgaard 2002) while in sub-oxic waters of the Black Sea and Golfo Dulce it contributed to about 20 – 40% (Dalsgaard et al., 2003, Kuypers et al., 2003). Without NO_3^-/NO_2^- or NH_4^+ , anammoxifiers cannot be sustained. They must compete with denitrifiers for dissolved NO_3^- or NO_2^- and with ammonium oxidizers for NH_4^+ . Potentially beneficial interactions amongst these organisms via the supply of critical substrates, such as NO_2^- should also be considered. The control of anammox by environmental parameters in sediments is not well understood. Both increased organic matter (Trimmer et al., 2003) and NO_3^- availability (Risgaard-Petersen et al., 2004) have been suggested to enhance anammox rates. Concentration of NO_3^- and/or NO_2^- have been reported to significantly affect denitrification (Seitzinger, 1988) as well as anammox (Risgaard-Petersen et al., 2004, Rich et al., 2008). In marine sediments, NO_3^- and NO_2^- can originate from overlying water or from the oxidation of NH_4^+ to NO_3^- via nitrification (aerobic oxidation of NH_4^+ to NO_2^- or NO_3^-) (Jenkins and Kemp, 1984; Middelburg et al., 1996). The isotope pairing method (IPM) (Nielsen, 1992), which involves the use of the stable nitrogen isotope ($^{15}NO_3^-$) and the production of single-labelled ($^{14}N^{15}N$) and double-labelled ($^{15}N^{15}N$) di-nitrogen, allows the distinction between the potential origins of denitrified NO_3^- via diffusion from the water column (D_w) and the end product of nitrification (D_n). Like nitrification-denitrification coupling, a possible coupling between nitrification and anammox for the supply of NO_2^- could be envisaged. This hypothesis is strengthened by the finding that ammonium oxidizers and anammoxifiers can share the same habitat. Co-existence of nitrification-denitrification and anammox has been reported in constructed wetland dedicated to water treatment (Jetten et al., 2001). Marine sediments are perfect models with which to understand the coupling between nitrification and denitrification due to the presence of a stable oxic/anoxic interface. In this context, intertidal sediments with an oxic/anoxic interface and redox oscillations resulting from tidal forcing (Deborde et al., 2008) are likely to provide an interesting base for the estimation of anammox rates and for the consideration of a putative coupling between nitrification and denitrification as well as with anammox (Kristensen, 1988).

Di-nitrogen production originates from microbial communities whose responses to different environmental parameters are still under investigation. Depending whether the functioning group is

monophyletic or not, functional or ribosomal genes are used to analyse microbial communities responsible for a specific process. Ammonium oxidation is considered as the limiting step of nitrification. It is carried out by ammonium oxidizing bacteria which are restricted to relatively few genera in beta- and gamma-Proteobacteria and ammonium oxidizing Archaea belonging to phylum Thaumarchaeota. The key enzyme involved is ammonium monooxygenase which is encoded by the *amoA* gene. The denitrification process is widespread among prokaryotes. A variety of functional genes like *narG*, *nirS*, *nirK*, *norC* or *nosZ* have been used to study the structure of a denitrifying community on the basis of genes coding for the various steps of the denitrification pathway (Ward, 2005). The *nosZ* gene encoding for nitrous oxide reductase, an enzyme catalyzing the last step of denitrification, is largely unique to denitrifying bacteria (Scala and Kerkhof, 1998). The *nosZ* gene has been used to determine the diversity of denitrifiers (Horn et al., 2006, Rontani et al., 2010). Anammox bacteria constitute a monophyletic order (*Candidatus* Brocadiales) of anaerobic chemolithoautotrophs in phylum Planctomycetes. The 16S rDNA gene has been used as a biomarker for detecting anammox-mediating bacteria in the environment (Schmid et al., 2005; Penton et al., 2006; Dang et al., 2013). More recently, the *hzs* and *hzo* genes have been proposed as functional biomarkers in ecological studies pertaining to anammox bacteria (Schmid et al., 2008; Dang et al., 2010; Li et al., 2010; Hirsch et al., 2011; Harhangi et al., 2012; Dang et al., 2013).

The focus of this study was N removal in sediments of a coastal mesotidal shallow lagoon located along the Atlantic Ocean French coast (Arcachon Bay, France). Lagoons are among the most productive environments of the ocean (Falkowski et al., 1998). Intertidal areas could favor coupling between aerobic and anaerobic phases exacerbating complex interactions between chemical and biological processes (Hirsch et al., 2011; Harhangi et al., 2012; Dang et al., 2010; 2013). Therefore, we specifically aimed to (i) quantify the processes involved in N₂ production in muddy and sandy intertidal sediments on a temporal scale (ii) highlight the influence of ammonium oxidation on N₂ production via nitrification-denitrification, anammox and nitrification-anammox and (iii) to examine the link between the key players of the prokaryotic community and N₂ producing processes.

2. Methods

2.1. Study area

Arcachon Bay is a shallow meso-tidal lagoon on the south-west (S.W.) coast of France (Atlantic Ocean; 44°40' N, 1°10' W). The total lagoon surface can be divided in two parts: the channels (57 km²), and the intertidal area (117 km²) (Plus et al., 2010) which is made up of large tidal flats (Deborde et al., 2008). The lower part of the intertidal zone consists of muddy sediments (grain size:

15–40 μm), whereas the upper parts are made of permeable, sandy sediments (grain size: $\sim 650 \mu\text{m}$). Only 40 km^2 remains submerged at low tide (De Wit et al., 2005). Two sampling stations with contrasting sediment types were selected according to their granulometric properties (*Mastersizer S*; Malvern, UK). The muddy station was located on a sloping mud flat in the southeast part of the Arcachon Bay (Fernandes et al., 2015). This area remained exposed to the atmosphere for $\sim 12 \text{ h d}^{-1}$ (6 h per tide) and was composed of fine sediments of grain size $\approx 27 \mu\text{m}$. The “sandy station” was located in the intertidal region of the beach.

2.2. Sampling and sample processing

Sediment sampling was carried out at low tide during March 2005, June 2006, October 2007 and January 2008. Ambient temperature in March 2005 varied between -2 and 7°C . June 2006 and October 2007 were warm periods, with maximum temperatures of 30°C and 21°C , respectively. The mean temperature during day time in January 2008 was about 10°C . Five replicate sediment cores from an area of $< 5 \text{ m}^2$ were collected using PVC push cores (8 cm inner diameter, 15–20 cm length). Sediment from 0–2 cm was sectioned, pooled and mixed. Each homogenized sample was further sub-divided into (i) 3 replicates (1 mL) stored at -20°C for molecular biology experiments and (ii) duplicates (10 mL) for pore water nutrient analyses. The coefficient of variation was consistently lower for laboratory replicates than for samples from any given sample location. Hence, the level of replication reported here is for the homogenized samples obtained at each site.

2.3. Analysis of nutrients and total organic matter

For pore water NO_3^- and NO_2^- analysis, 5 mL of NO_x free synthetic seawater (Baumann and Baumann, 1981) was added to 5 mL of sediment. The samples were homogenised and centrifuged for 10 min at 5000 rpm ($\times 1803 g$) under di-nitrogen flux. The supernatant was filtered through a 0.2 μm filter. NO_3^- plus NO_2^- corresponding to NO_x in the extracted pore water were analyzed using a continuous flow analyzer (AA3 autoanalyzer, SEAL Analytical, UK; precision: $\pm 0.01 \mu\text{mol L}^{-1}$) according to Tréguer and Le Corre (1975). The dilution due to addition of synthetic seawater has been accounted for in the calculation of nutrient concentrations. For determination of NH_4^+ content (pore water + sediment extractable), sediment samples were subjected to KCl extraction (Laima, 1993) and analysed using the fluorometric method (Kérouel and Aminot, 1997). Weight of the sediment used in the nutrient analyses was estimated by drying samples in a hot air oven at 60°C for 48 h i.e. until the weight had stabilised.

To estimate total organic matter content, known volumes of sediment samples were dried at 110°C overnight and then at 550°C for 12 h.

2.4. Measurement of denitrification and anammox activity

Determination of anammox and denitrification activities followed the procedure of Thamdrup and Dalsgaard (2002) and (Risgaard-Petersen et al., 2003) with some modifications as described by Minjeaud et al. (2008). Five millilitres of sediment sub-samples were extruded from the first top two centimetres using sterile syringe cores. Extracted sediment samples were then transferred into 22 mL headspace vials and overlaid with 5 mL of filter-sterilized seawater collected from the sampling site. This step was carried out in a flexible glove box under N₂ flow. For stabilisation, open vials containing sediment were pre-incubated for 2 h. The vials were then capped with butyl rubber stoppers and aluminium crimps. Air was maintained in the headspace. Positive pressure (0.15 MPa) was added to the headspace to prevent unintentional contamination with ambient air during the incubation and the gas sampling. During the incubation period, oxygen level in the headspace was monitored by mass spectrometry and was found to be never lower than 180000 ppmv (86% of the amount found in air). Natural Argon concentration (9340 ppmv) was used as internal standard for mass spectrometry (Minjeaud et al., 2008). Total N₂ production rates were quantified using ¹⁵NO₃⁻ amendments of 80 μM, 125 μM and 250 μM. Supplementary time series with ¹⁵NH₄⁺ (200 μM) and ¹⁴NO₃⁻ (150 μM) were carried out under strict anaerobic conditions to confirm the occurrence of anammox.

Stock solution of K-¹⁵NO₃⁻ (1 mM, 97.4 atom%), K-¹⁴NO₃⁻, Cl-¹⁵NH₄⁺ (1 mM) were prepared, N₂-flushed and added to the vials with a gas tight syringe at beginning of the incubation. The incubation vials (prepared in triplicates) were gently shaken by hand to homogenise the distribution of added ¹⁵NO₃⁻ or ¹⁵NH₄⁺. The microcosms were incubated for up to 8 h (0, 2, 4, 6 and 8 h) in the dark at *in situ* temperature. A triplicate set of vials was sacrificed at each point of the time series by adding HgCl₂ at a final concentration of 10 mM. The headspace gas samples were analysed by quadrupole mass spectrometry (Quadrupole mass spectrometer *Anagaz* 100, MKS, Cheshire, UK) (Minjeaud et al., 2008) to quantify each N₂ isotope (²⁸N₂, ²⁹N₂ and ³⁰N₂) and Ar as internal standard. We obtained the production of ²⁹N₂ and ³⁰N₂ in vials over time to quantify potential anammox and denitrification. The concentrations of ²⁹N₂ and ³⁰N₂ were then plotted against time and fitted to the linear model ($A(t) = A_0 \pm m \times t$) using the method of least squares where: t = incubation time; A₀ = concentration at t = 0; m = slope of linear curve. Rate uncertainties were calculated from the errors in the linear regressions. For calculation, see details in the supplementary section.

The dependency of N₂ production based on NO₃⁻ produced in the sediment via nitrification (P_n) or coming from the overlying water (P_w) was quantified using the comprehensive method as outlined by Risgaard-Petersen et al. (2003). The ratio between ¹⁴NO_x/¹⁵NO_x in the NO₃⁻ reduction zone (r14) was calculated. In the microcosm, ¹⁴N-NO₃⁻ would be produced only from ¹⁴N-NH₄⁺ via nitrification and P_n reflected the subsequent ¹⁴N-NO₃⁻ reduction to N₂.

The following additional combinations of incubation conditions were included in most experiments: ¹⁵NH₄⁺ (final concentration corresponding to 10% of total *in situ* NH₄⁺ concentration) + ¹⁴NO₃⁻ (150 μmol L⁻¹ final concentration) i.e. (1) without He flushing (to confirm the influence of aerobic oxidation of NH₄⁺ on N₂ production processes or (2) with He flushing (in order to avoid NO₃⁻ production from aerobic nitrification and to confirm anammox). Under anaerobic conditions, P₂₉ corresponds to anammox activity from ¹⁵NH₄⁺ and ¹⁴NO₂⁻. Under aerobic condition (without He flushing), P₂₉ can come from anammox and also from the coupling between nitrification. P₃₀ reveals oxidation of ¹⁵NH₄⁺ into ¹⁵NO₂⁻ or ¹⁵NO₃⁻ by nitrification prior to the occurrence of anammox (¹⁵NH₄⁺ + ¹⁵NO₃⁻).

Production of ¹⁴NH₄⁺ during incubations can occur via mineralization. There are uncertainties in recovery of total NH₄⁺ with the KCl extraction method to measure exchangeable NH₄⁺ (Laima, 1993). Thus, it may be noted that we have not reported actual levels of anammox rates in the ¹⁵NH₄⁺ + ¹⁴NO₃⁻ treatments but used rates of ²⁹N₂ and ³⁰N₂ production (P₂₉ and P₃₀) as a proxy.

2.5. Prokaryotic gene quantification

DNA was extracted from 0.25 to 0.75 g dry weight (dw) of triplicate sediment sub-samples using the Mo Bio UltraClean™ Soil DNA isolation kit (Mo Bio Laboratories Inc., CA, USA). For all PCR amplifications, reactions were performed in a volume of 20 μL with primers and annealing temperature as indicated in Table 1. The absolute number of gene was quantified by qPCR. DNA standards were created by PCR either from strains or amplification of genomic DNA from sediment and corresponding fragment was cloned after purification into pGEMT vector (Promega, WI, USA). After purification, using the Wizard® Plus SV Minipreps Start-Up Kit (Promega), the concentration of plasmids was determined using NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, DE, U.S.A). The target abundance for standards were calculated using the following formula: gene abundance = 6.023 10²³ (copies mol⁻¹) × standards concentration (g mL⁻¹) / molecular mass (g mol⁻¹) assuming that double stranded DNA has a molecular mass of 660 Da.

For bacterial 16S rDNA, quantifications were performed with GML5F and -Uni 516R primer sets (Takai and Horikoshi, 2000; Michotey et al., 2012). Templates for standard curve were made by 10-

fold serial dilution of recombinant plasmid harboring one copy of 16S of *Pseudomonas stutzeri* 16S rDNA in order to reach 10^3 to 10^8 gene/ μ L. Efficiency of the qPCR reaction was $97 \pm 4\%$ with a R^2 of 0.99.

For archaeal 16S rDNA quantification, primers 931F and m1100R were used (Jackson et al., 2001; Einen et al., 2008). Templates for standard curve were made by 10-fold serial dilution of recombinant plasmid harboring one copy of 16S rDNA gene fragment of Euryarchaeota (KT351355) in order to reach 10^2 to 10^6 gene/ μ L. Efficiency of the qPCR reaction was $99 \pm 5\%$ with an R^2 of 0.99.

For *nosZ* gene quantification, primers *nosZ* 2F and *nosZ* 1897R were used (Henry et al., 2006; Rösch et al., 2002). Templates for standard curve were made by 10-fold serial dilution of recombinant plasmid harboring one copy *nosZ* of *Marinobacter* sp BC38 fragment (KM816604) in order to reach 10^2 to 10^5 genes/ μ L. Efficiency of the qPCR reaction was 96.5 ± 3 with a R^2 of 0.99.

For bacterial *amoA* gene quantification, primers *amoA*-1 and *amoA*-2R were used (Rotthauwe et al., 1997; Neef et al., 1998). Templates for standard curve were made by 10-fold serial dilution of recombinant plasmid harboring one copy *amoA* fragment of an environmental clone presenting 95% similarity with the corresponding gene of *Nitrosomonas nitrosa* (KT351402) in order to reach 10^2 to 10^6 genes/ μ L. Efficiency of the amplification was 90.6 ± 2 with a R^2 of 0.99.

For archaeal *amoA* gene quantification, primers Arch *amoA*-F and Arch *amoA*-R were used (modified from Wuchter et al., 2006). Templates for standard curve were made by 10-fold serial dilution of recombinant plasmid harboring one copy *amoA* fragment of uncultured clone retrieved from sediment and that present 90% similarity on nucleotide level with *Nitrososphaera gargensis* (KT351403) in order to reach 10^1 to 10^6 genes/ μ L. Efficiency of the amplification was 101 ± 2 with a R^2 of 0.95.

All quantifications by qPCR were made in triplicates. For bacterial and archaeal 16S ribosomal genes, the PCR reaction was performed with LightCycler[®] FastStart DNA Master SYBR Green kit (Roche Diagnostics, CA, USA). Conditions were similar to those previously described (Michotey et al., 2012).

The *nosZ* and *amoA* quantification was performed with SsoAdvanced[™] SYBR[®] Green supermix (Bio-Rad Laboratories, CA, USA) using a CFX96 Real Time System (C1000 Thermal Cycler, Bio-Rad Laboratories) (Rontani et al., 2010; Fernandes et al., 2012). At the end of the qPCR reactions, the specificity of the amplification was checked from the first derivative of their melting curves and

also analyzed by agarose gel electrophoresis. Data was analyzed using the comparative threshold cycle (C_T) method (Schmittgen and Livak, 2008). Regression equation was calculated on the graph, plotted between C_T value and copy number of the standard. The correspondence between gene number and cellular abundance was performed with the copy number of each gene per genome available according to literature. Average copy numbers for bacterial and archaeal 16S were set at 4 and 1.63 according to <https://rrndb.umms.med.umich.edu> (Stoddard et al., 2015), whereas the average of copy number for beta ammonium-oxidizing and denitrifying bacteria were set at 2.5 and 1 respectively (Arp et al., 2007; Philippot, 2002)

Direct amplification of 16S rDNA genes with Amx 368F/Amx 820R was not successful to assess the diversity of the anammox community (data not shown). Therefore, a nested PCR strategy was used. The first step consisted of an increase in ribosomal number of planctomycetales using Pla46F/1390R (Neef et al., 1998). The product of the initial PCR was used as template for second step amplification using Amx 368F/Amx 820R (Schmid et al., 2005). The nested procedure did not allow quantifying anammox community and only the diversity aspect was studied to confirm presence of this functional group.

2.6. Cloning and sequencing for evidence and biodiversity of anammox bacteria

PCR-amplified DNA fragments were cloned after purification (*MinElute* PCR Purification Kit; Qiagen, Germany) following the manufacturer's instructions. Purified DNA fragments were introduced into a pGEM[®]-T *Easy Vector* (Promega) and transformed into *Escherichia coli*, as instructed by the manufacturer. Cloned inserts were verified by PCR amplification and plasmids were sequenced after purification using the Wizard[®] Plus SV Minipreps Start-Up Kit (Promega). Sequencing was performed by GATC Company (Edelberg, Germany) using the Sanger method. Chimera absence was checked with *Decipher* (<http://decipher.cee.wisc.edu/FindChimeras.html>; Wright et al., 2012). The partial sequences (447 bp) were aligned with the same region of the closest relative strains available in the GenBank database by using the BLAST facility (<http://www2.ncbi.nlm.nih.gov/BLAST/>). Sequence alignment was achieved using ClustalW (Thompson et al., 1994). Phylogenetic tree was constructed in mega 5.0 using the neighbor-joining model, Jukes-Cantor parameters (Saitou and Nei, 1987) and 1,000 bootstrap replicates (Tamura et al., 2007). Sequences obtained in this study are available from NCBI sequence database under the accession numbers KT351356 to KT351401 for anammox clones.

2.7. Statistical analysis

Student's t-test was performed on the dataset to evaluate differences between N cycle processes. Pearson rank correlation (*P*) was performed on the data matrix (bacterial activities, organic matter content and nitrogen compounds) using XLSTAT software 2008 (Addinsoft, Paris, France).

3. Results

3.1. Sediment characteristics

Exchangeable NH_4^+ concentration in sandy sediments varied between 122.5 to 298.4 $\mu\text{mol L}^{-1}$ during the studied period (Table 2). In sandy sediments, a minimum of 38.3 $\mu\text{mol NH}_4^+\text{-N L}^{-1}$ was recorded during March. In October, NH_4^+ content in these sediments increased to 224.1 $\mu\text{mol L}^{-1}$. Concentration of NO_2^- in both sediments types was $\leq 0.8 \mu\text{mol L}^{-1}$ (Table 2). The concentration of NO_3^- in sandy sediments was maximum in January (7.5 $\mu\text{mol L}^{-1}$) while in muddy sediments a maximum of 11.5 $\mu\text{mol L}^{-1}$ was recorded during March (Table 2). Low organic matter content was observed in sandy sediments (0.1 – 0.8%) in comparison to muddy sediments where up to 7.7% was recorded during January (Table 2).

3.2. Potential denitrification and anammox rates

Denitrification (D_{tot}) appeared to be the main process responsible for N_2 production in the Arcachon sediments with anammox contributing between 14 and 44% (Fig. 1a and b). Potential denitrification rates (D_{tot}) in sandy sediments were of the same order of magnitude as in muddy sediments ($n = 14$, $t = -0.547$, $p > 0.05$), with the lowest values observed in March ($87.3 \pm 39.9 \mu\text{mol L}^{-1} \text{d}^{-1}$) and highest in October ($847.1 \pm 234.8 \mu\text{mol L}^{-1} \text{d}^{-1}$) (Fig. 1a). In muddy sediments, D_{tot} ranged from $136.7 \pm 105.1 \mu\text{mol L}^{-1} \text{d}^{-1}$ to $725.8 \pm 26.8 \mu\text{mol L}^{-1} \text{d}^{-1}$ in June and January respectively (Fig. 1b). A positive correlation between D_{tot} and NO_2^- concentration was observed ($n = 11$, $P = 0.69$, $p < 0.05$). Both sediment types showed similar trend with the lower denitrification rates in March and June.

The occurrence of anammox in both sediment types (Fig. 1a and b) was confirmed by substantial $^{29}\text{N}_2$ production (P_{29}) in incubations amended with $^{15}\text{NH}_4^+$ and under anaerobic conditions (Table 3). In sandy sediments, $^{15}\text{NO}_3^-$ labelling revealed anammox rates ranging from $14.8 \pm 3.6 \mu\text{mol L}^{-1} \text{d}^{-1}$ in June to $238.1 \pm 156.8 \mu\text{mol L}^{-1} \text{d}^{-1}$ in October (Fig. 1a). Significantly high anammox values corresponded to October and January ($n = 3$, $t = 8.253$, $p < 0.001$). Anammox was significantly lower than D_{tot} during March ($n = 3$, $t = 2.785$, $p < 0.05$) and June ($n = 3$, $t = 8.519$, $p < 0.001$). On the contrary, in muddy sediments, anammox was significantly lower than D_{tot} in October ($n = 3$, $t = 20.029$, $p < 0.001$) and January ($n = 3$, $t = 10.913$, $p < 0.001$). The ratio between anammox and total

N₂ potential production rate (R_a) was relatively constant throughout the study period, with anammox accounting for 8-21% of total N₂ production (Fig. 1b). In contrast, in sandy sediments, R_a fluctuated and N₂ originating via anammox peaked at 45% in January (Fig. 1a). A positive relationship between anammox and NH₄⁺ concentration was observed (n = 10, P = 0.641, p < 0.05).

3.3. Coupling between nitrification, denitrification and anammox

Di-nitrogen production at the expense NO₃⁻ from overlying water (P_w) or resulting from the coupling between nitrification and denitrification (P_n) were measured. P_n was responsible for 83 to 99% of total potential N₂ production (P_{tot}) at both sites (Fig. 2a and b) and time except during June in sandy sediments, where no P_n was measured and 100% of P_{tot} corresponded to *in situ* P_w (Fig. 2a). In muddy sediments, higher *in situ* P_n rates were recorded in January and October measuring $435.1 \pm 29.3 \mu\text{mol L}^{-1} \text{d}^{-1}$ (n = 3, t = 7.450, p < 0.05) and $362.7 \pm 33.4 \mu\text{mol L}^{-1} \text{d}^{-1}$ (n = 3, t = 7.337, p < 0.05) respectively (Fig. 2b). The highest value of *in situ* P_w rate was measured in sandy sediments ($29.8 \pm 6.4 \mu\text{mol L}^{-1} \text{d}^{-1}$) whereas in muddy sediments, values never exceeded $3.9 \pm 1.4 \mu\text{mol L}^{-1} \text{d}^{-1}$.

The coupling between nitrification and anammox was investigated by comparing ³⁰N₂ production under aerobiosis and anaerobiosis using ¹⁵NH₄⁺ labelling (Table 3). Under these conditions, ²⁹N₂ production (P₂₉) corresponded to anammox activity from ¹⁵NH₄⁺ and ¹⁴NO₂⁻, whereas ³⁰N₂ production (P₃₀) revealed an oxidation of ¹⁵NH₄⁺ into ¹⁵NO₂⁻ via nitrification prior to the anammox reaction. Nitrification largely being an aerobic process, absence of oxygen minimized coupling. For these samples, although P₂₉ was little affected by the absence of oxygen, anaerobic conditions led to 85% increase in P₃₀ production during January and a total disappearance in October (Table 3).

3.4. Quantification of Archaea and bacteria

Taking into account the mean number of ribosomal operons per genome, bacterial abundance varied between 10⁷⁻⁸ cell equivalent per g⁻¹ dw and outnumbered Archaea that represented between 0.5 and 6.5% of the total prokaryotic equivalent cell (Fig. 3a). In muddy sediments, bacterial abundance remained constant through the sampling times at around 1.5×10^8 cell equivalent per g⁻¹ dw except in March for which a 2 fold decrease was observed. In contrast, in sandy sediments, more fluctuations were recorded between 3.9×10^7 to 5.5×10^8 equivalent cell per g⁻¹ dw (Fig. 3a). Abundance of Archaea presented opposite tendencies between muddy and sandy sediment. Minimal values were recorded in March and June for muddy sediment whereas for sandy sediment, it was during October and January. The highest values were 10⁷ cell equivalent per g⁻¹ dw in muddy sediment and 5.6×10^6 cell equivalent per g⁻¹ dw in sandy sediment which were recorded for January and June respectively.

Nitrifiers belonging to betaproteobacteria and Archaea were quantified. Archaeal *amoA* genes in the qPCR reaction were below the detection threshold (47 genes in the PCR reaction) for all samples (data not shown) although this protocol was successful to amplify Archeal *amoA* from other marine sediments. In contrast, betaproteobacterial *amoA* gene could be quantified for all samples. In sandy sediments, bacterial ammonium oxidizers decreased from March (1.5×10^5 cell equivalent per g^{-1} dw) to January (4.6×10^3 cell equivalent per g^{-1} dw). In contrast, in muddy sediments relative low abundance was recorded for March and June (about 5×10^3 cell equivalent per g^{-1} dw) whereas higher values were observed for October and January (2.2×10^4 cell equivalent per g^{-1} dw) (Fig. 3b). The denitrifiers were 1-2 orders higher as compared to ammonium oxidizers in these sediments (Fig. 3b). In sandy sediments, number of denitrifiers was higher during the colder period i.e. January and March and minimal in June. However, in muddy sediments, denitrifier abundance was minimal in March and June whereas maximum values were recorded in October and January. This latter trend is similar to that observed for nitrifiers in this type of sediment (Fig. 3b). No correlation was found between number of *nosZ* and either denitrifying activities (D_{tot} ($n = 11$, $P = -0.235$, $p > 0.05$) or D_w *in situ* ($n = 11$, $P = 0.207$, $p > 0.05$)), however it should be noted that in muddy sediment rates of D_{tot} , Anx and D_n are about 3 fold higher in October and January compared to March and June in a manner commensurate with increase of nitrifier numbers between these periods.

3.5. Evidence for the presence of anammoxifiers

All sediment samples were tested for the presence of Planctomycetales and anammoxifiers. Several primers described in literature were tested (data not shown) but they did not allow amplification of anammoxifiers with specificity. Only a nested approach was successful enabling to detect their presence and diversity but not their abundance. Anammox PCR products of expected size were detected in sandy and muddy sediment whatever the sampling time. In order to confirm the presence of anammox-mediating bacteria and to determine their phylogenetic affiliation, 89 clones were sequenced. Phylogenetic analysis revealed that about 50% of the clone sequences were closely related to known anammoxifiers belonging to the “*Candidatus Scalindua*” group (Fig. 4). All the remaining environmental clones were related to Planctomycetes 16S rRNA gene sequences branching outside the known anammox bacterial cluster (data not shown).

4. DISCUSSION

In this study, we attempted to investigate the occurrence of N_2 producing processes viz., denitrification and anammox in sandy and muddy intertidal sediments of the Arcachon Bay. Though denitrification has been widely studied, studies on anammox have focused mainly on its detection in

different geographic locations from the marine environment. Less is known on temporal trends of the anammox process. Our observations showed that rate of N_2 production was in the same range than that reported from the Mediterranean Sea but lower than values from shallow sediments (< 10 m) from the North Sea or Arafusa Sea (Table 4) (Bonin et al., 1998; Thamdrup and Dalsgaard, 2002; Trimmer et al., 2003; Risgaard-Petersen et al., 2004; Dalsgaard et al., 2005; Trimmer et al., 2006; Minjeaud et al., 2009). Occurrence of both N_2 producing pathways was observed at all sampling periods at both sites (Fig. 1a and b). This observation contrasts with results previously recorded during an annual study in the Mediterranean wherein anammox was only occasionally detected (Minjeaud et al., 2009). The Mediterranean and Atlantic sites differ in sediment NO_2^- and NH_4^+ content and mean water temperature which was higher at the Mediterranean coast.

Previous studies pertaining to N_2 removal processes in shallow marine sediments have revealed denitrification to be the main process for N loss (Fernandes et al., 2012). Our results are in good agreement with this previous finding. Earlier studies have also reported anammox contribution to be less than 10% of total N_2 fluxes (Bonin et al., 1998, Thamdrup and Dalsgaard, 2002; Trimmer et al., 2003; Risgaard-Petersen et al., 2004; Dalsgaard et al., 2005; Trimmer et al., 2006; Minjeaud et al., 2009). However, our results showed much higher contribution ranging from 8 to 45%, with the greater percentage recorded in sandy sediments.

The intensity of the activities could be a result of changes in the corresponding microbial communities. Whatever the site or season, no significant change in denitrifier abundance was observed (Fig. 3a and b). No statistical correlation between denitrifier abundance and denitrifying activity could be observed. As suggested by Fernandes et al. (2012), this indicates that denitrification rates are not limited by the presence of the population able to denitrify but by the availability of NO_3^- as substrate. Furthermore, denitrifiers being facultative anaerobic prokaryotes, their presence detected by gene amplification is not an indication of their level of activity as would be indicated by active community (cDNA) quantification.

The presence of anammox clones in the total bacterial community (from sediment extracted genomic DNA) was also investigated with different PCR primer sets following the findings of several authors that specificity and adequacy of each primer set is site dependant (Li et al., 2010). Several ribosomal clones were retrieved from the sediment samples that belonged to putative anammox bacterial cluster which groups anammox sequences from other marine environments such as coastal sediments or deep-sea hydrothermal vents (Tal et al., 2005; Byrne et al., 2009; Li et al., 2010; Fig. 4). Anammox bacteria are ubiquitous in O_2 -limited marine environments, such as oxygen minimum zones and

sediments (Jetten et al., 2009; Op den Camp et al., 2006; Hu et al., 2011). Most studies show that the marine anammox bacterial communities are composed exclusively of “*Candidatus Scalindua*” group usually with high microdiversity (Hu et al., 2011; Woebken et al., 2008, Dang et al., 2013). The halophilic *Ca. Scalindua* sp. exhibits higher affinity for NO_2^- than other known anammox species (Awata et al., 2013). Therefore, these authors suggested that such a physiological characteristic enables *Ca. Scalindua* sp. to predominate in marine sediments. The results obtained in Arcachon Bay, strengthen previous findings of Awate et al. (2013) and suggest the prevalence of *Ca. Scalindua* not only in muddy but also in sandy sediments of Arcachon Bay for with the oxygen penetration is relatively high compared to deeper sediments. Despite their detection at a DNA level, (Op den Camp et al., 2006; Woebken et al., 2008; Jetten et al., 2009; Hu et al., 2011) the occurrence of anammox indicates that at least a substantial amount of anammoxifiers are active. The optimal temperature for activity of “*Candidatus Scalindua* sp.” varies from 10 to 30 °C (Awata et al., 2013). This explains the relatively higher contribution of anammox towards total N_2 production in January where mean daytime temperature was about 10 °C.

In sediments, denitrifiers and anammoxifiers require NO_3^- or NO_2^- as substrates that can either diffuse from overlying water or can be produced through nitrification. In the Arcachon Bay, high productivity (Deborde et al., 2008) and nitrification rates of about $3.3 \text{ mmol m}^{-2} \text{ d}^{-1}$ have been reported (Rysgaard et al., 1996) as compared to other shallow marine sediments (Nishio et al., 1983; Rysgaard et al., 1993; Gilbert et al., 1997). The apparent low NO_3^- and NO_2^- concentrations previously observed (Bartoli et al., 1996; Rysgaard et al., 1996) as in this study were rather surprising. These results are suggestive of a strong coupling between the different processes that produce and consume NO_3^- and/or NO_2^- . In Arcachon Bay, whatever the sampling site or season, over 83% of substrate for denitrification (D_n) originated from nitrified NH_4^+ (Fig. 2a and b), which is much higher than the coupling recorded in Colne estuary (25%; Dong et al., 2000). This result along with the correlation between denitrification rates and NH_4^+ concentrations indicates that nitrification supported denitrification in this area. This finding corroborates a similar observation by Minjeaud et al. (2009) from studies carried out in the Gulf of Fos.

Because anammox requires NH_4^+ and NO_2^- , a coupling between nitrification and anammox could not be excluded and was investigated in the present study. Incubation of sediment samples with addition of $^{15}\text{NH}_4^+ + ^{14}\text{NO}_3^-$ and without He bubbling resulted in substantial $^{29}, ^{30}\text{N}_2$ production. Incubations under strict anaerobic conditions led to 85 to 100% decrease in $^{30}\text{N}_2$ production with unchanged $^{29}\text{N}_2$ production demonstrating a substantial coupling between nitrification and anammox. Correlation between availability of NO_2^- produced by NO_3^- reduction and anammox activity has been reported in

shallow coastal (Trimmer et al., 2003) or mangrove sediments (Meyer et al., 2005). However, our results showed that in Arcachon Bay, nitrification was a NO_2^- supplier for anammox bacteria. The strong coupling between nitrification and denitrification and/or anammox was probably favoured by the receding tide wherein exposure of sediment to the ambient air enhanced organic matter degradation and consequently NH_4^+ production via ammonification. Deborde et al. (2008) have reported an accumulation of NH_4^+ in creek water for all seasons together with NO_3^- and NO_2^- production, which was clearly visible at low tide during autumn and winter. In our study, D_n and anammox activities were significantly higher in October. Seasonal tendency of bacterial activity could be the result of several parameters leading to apparent site specific response (Bonin et al., 1998; Tuominen et al., 1998; Risgaard-Petersen et al., 2004; Dalsgaard et al., 2005; Hietanen and Kuparinen, 2008; Minjeaud et al., 2009). In this study, the apparent seasonal pattern of D_n and anammox with a significant maximum in October and January seemed one of the consequences of coupling between nitrification and denitrification or anammox. Previous studies of marine sediments showed different patterns of either bacterial or archaeal nitrifier dominance. It has been suggested that their niches are defined by NH_4^+ concentration. Archaeal nitrifiers are more competitive at low NH_4^+ concentration but may be outcompeted by bacterial counterparts (Ogilvie et al., 1997; Papaspyrou et al., 2014; Li et al., 2015). Our result affirm previous findings since Archaeal *amoA* gene were under detection limit in Arcachon Bay sediments. Furthermore in muddy sediments, the abundance of bacterial nitrifiers showed a similar trend as the denitrifiers, anammox and D_n activities suggesting a more efficient coupling.

5. CONCLUSION

A temporal evolution in the activities of denitrification and anammox was observed in intertidal sediments of Arcachon Bay with maximum N_2 production observed in October and January. Members showing taxonomic affinities to *Ca. Scalindua* group were the dominant anammoxifiers in the study area. *Ca. Scalindua* sp. are known to sustain anammox under low temperature conditions of about 10 °C which could explain the relatively higher activity (up to 45% of total N_2 production) during the colder period. Anammox values recorded in the present study were also found to be greater than previously reported from other marine systems. The limiting factor for denitrification and anammox processes was most likely the oxidation of NH_4^+ which acts as an essential process in fuelling both N_2 producing processes. Archaeal nitrifiers appeared insignificant in these intertidal sediments and bacterial nitrifiers seemed to be the main suppliers of substrates for denitrification and anammox. The coupling between nitrification and denitrification/anammox could be even more important than previously thought. From this study it appeared to be more effective in muddy

sediments in contrast to sandy ones that presented greater range of fluctuations on activity as well as within the microbial community.

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Legend to figures

Fig. 1. Total potential denitrification (D_{tot}), potential anammox activity (AnX) and ratio between potential anammox and total N_2 production rate ($P_{\text{tot}} = R_a$) in sandy (a) and muddy (b) sediments. Incubations were carried out in slurries after $^{15}\text{NO}_3^-$ amendment under aerobic conditions.

Fig. 2. N_2 production rates from NO_3^- originating from the overlying water at *in-situ* concentration of the nutrient (P_w), N_2 production from NO_3^- produced through nitrification (P_n) and proportion of denitrification through coupled nitrification-denitrification (P_n/P_{tot}) in sandy (a) and muddy (b) sediments. Incubations were carried out in slurries after $^{15}\text{NO}_3^-$ amendment in the presence of ambient air in the headspace.

Fig. 3. Abundance of bacteria, archaea (a) ammonium oxidizers and denitrifiers (b) in sandy (S) and muddy (M) sediments. The number of equivalent cells were obtained by dividing the absolute number of gene g^{-1} dry weight with each mean number of gene per genome (i.e. 4 for bacterial 16S rDNA, 1.63 for 16S archaeal rDNA, 2.5 for *amoA* and 1 for *nosZ*, refer methods section for references).

Fig. 4. Distance neighbor-joining phylogenetic tree of 16S rDNA clones. The bootstrap numbers are indicated at the node. Branch length corresponds to sequence differences as indicated on the scale bar.

Table 1

PCR primers used in this study.

Primers	Target gene	Sequences (5'-3')	Hybridization	Fragment size (pb)	References
GML5F	16S rDNA Bacteria	CCGCCTACGGGAGGCAGCAG	55°C, 10 s	257	Muyzer et al. (1993); Michotey et al. (2012)
UNIV 516R	16S rDNA Bacteria/Archaea	GTDTTACCGCGGCKGCTGRCA			Takai and Horikoshi (2001)
931F	16S rDNA Archaea	AGGAATTGGCGGGGAGCA	62°C, 10 s	207	Jackson et al. (2001)
m1100R		BTGGGTCTCGCTCGTTRCC			Einen et al. (2008)
<i>amoA</i> -1F	<i>amoA</i> Beta Bacteria	GGGGHTTYTACTGGTGGT	58°C, 30 s	491	Rotthauwe et al. (1997)
<i>amoA</i> -2R		CCCCTCKGSAAAGCCTTCTTC			
Arch <i>amoA</i> -F	<i>amoA</i> Archaea	CWGAYTGGRYDTGGACHTC	62°C, 30 s	256	Modified from Wuchter et al. (2006)
Arch <i>amoA</i> -R		TTYKYTYTGTKDCCCAGTA			
Pla46F	16S Plantomycetales	GACTTGCATGCCTAATCC	59°C, 30 s	1350	Neef et al. (1998)
Univ bact 1390R	16S rDNA Bacteria	GACGGGCGGTGTGTRCAA			Olsen et al. (1986)
Amx 368F	16S rDNA Anammox Bacteria	CCTTTCGGGGCATTGCGAA	62°C, 50-s	452	Schmid et al. (2005)
Amx 820R		AAWRCCCCTCTACTTAGTACCC			
<i>nosZ</i> 2F	<i>nosZ</i> Bacteria	CGCRACGGCAASAAGTSMSSG	64°C, 15 s	280	Henry et al. (2006)
<i>nosZ</i> 1897R		T CATGTGCAGNGCRTGGCAGAA			

Table 2

Physico-chemical parameters in sandy and muddy sediments.

Sediment type	Sampling time	NH ₄ ⁺ (μmol L ⁻¹)	NO ₂ ⁻ (μmol L ⁻¹)	NO ₃ ⁻ (μmol L ⁻¹)	TOM (%)
Sandy	March 2005	ND	0.4	6.4	0.8
	June 2006	122.5	0.8	0.4	ND
	October 2007	298.4	0	0.4	0.3
	January 2008	128.8	0.5	7.5	0.1
Muddy	March 2005	38.3	0.4	11.5	0.8
	June 2006	155.5	0.6	0.2	3.3
	October 2007	224.1	0.7	0.2	6.5
	January 2008	99.1	0.8	0.9	7.7

Note: ND = Not done

Table 3

Rates of production of $^{29}\text{N}_2$ (P₂₉) and $^{30}\text{N}_2$ (P₃₀) labelled with $^{15}\text{NH}_4^+$ under ambient oxygen level and anaerobic condition ($\mu\text{mol L}^{-1} \text{d}^{-1}$).

Sampling time	Sediment type	Aerobic conditions		Anaerobic condition	
		P29	P30	P29	P30
January 2008	Sandy	74.16	18.24		
	Muddy	65.28	12.96	25.68	1.92
March 2005	Muddy	4.80	1.20		
October 2007	Sandy	105.12	5.28		
	Muddy	32.40	3.60	29.28	0

Table 4

Denitrification and anammox values from various sediment types and locations.

Coastal marine sediment	Location	Sediment type	Mean denitrification activity $\text{nmol cm}^{-3} \text{h}^{-1}$	Mean anammox activity $\text{nmol cm}^{-3} \text{h}^{-1}$	Reference
Arcachon sediment	East coast Atlantic Ocean	Muddy; intertidal	17.5^a (Min-max:-5.6-30.2)	2.8^a (Min-max:0.8-5.2)	This study
Arcachon sediment	East coast Atlantic Ocean	Sandy; intertidal	13.8^a (Min-max: 3.6-35.2)	6.2^a (Min-max: 0.4-16.2)	This study
Gulf of Fos	Mediterranean Sea	Muddy; intertidal	3.7^a (Min-max:0.9-8.8)	0.3^a (Min-max:0-1.1)	Minjeaud et al. (2009)
Gulf of Fos	Mediterranean Sea	Muddy; permanently inundated	5.7^a (Min-max:1.6-10.0)	0.1^a (Min-max: 0-1.0)	Minjeaud et al. (2009)
Gulf of Fos	Mediterranean Sea	Sandy	3^a (Min-max: 0-6.3)	0.2^a (Min-max: 0-1.5)	Minjeaud et al. (2009)
Chesapeake Bay	West coast Atlantic Ocean	nd	24.2^b (Min-max: 17.4-31)	5.5^b (Min-max:3-8)	Rich et al. (2008)
Thames estuary	North Sea	Sandy	71^c	1^c	Trimmer et al. (2003)
Randers Fjord	North Sea	nd	88^b (Min-max:31-137)	6^b (Min-max:3.8-11)	Risgaard-Petersen et al. (2004)
Logan/Albert River, Queensland, Australia	Arafusa Sea	Mangrove; intertidal	45^d (Min-max: 4-85)	4^d (Min-max: 0-8.7)	Meyer et al. (2005)

a: mean of annual measures, b: mean of spring and summer measures, c: November measures, d: sampling times not indicated

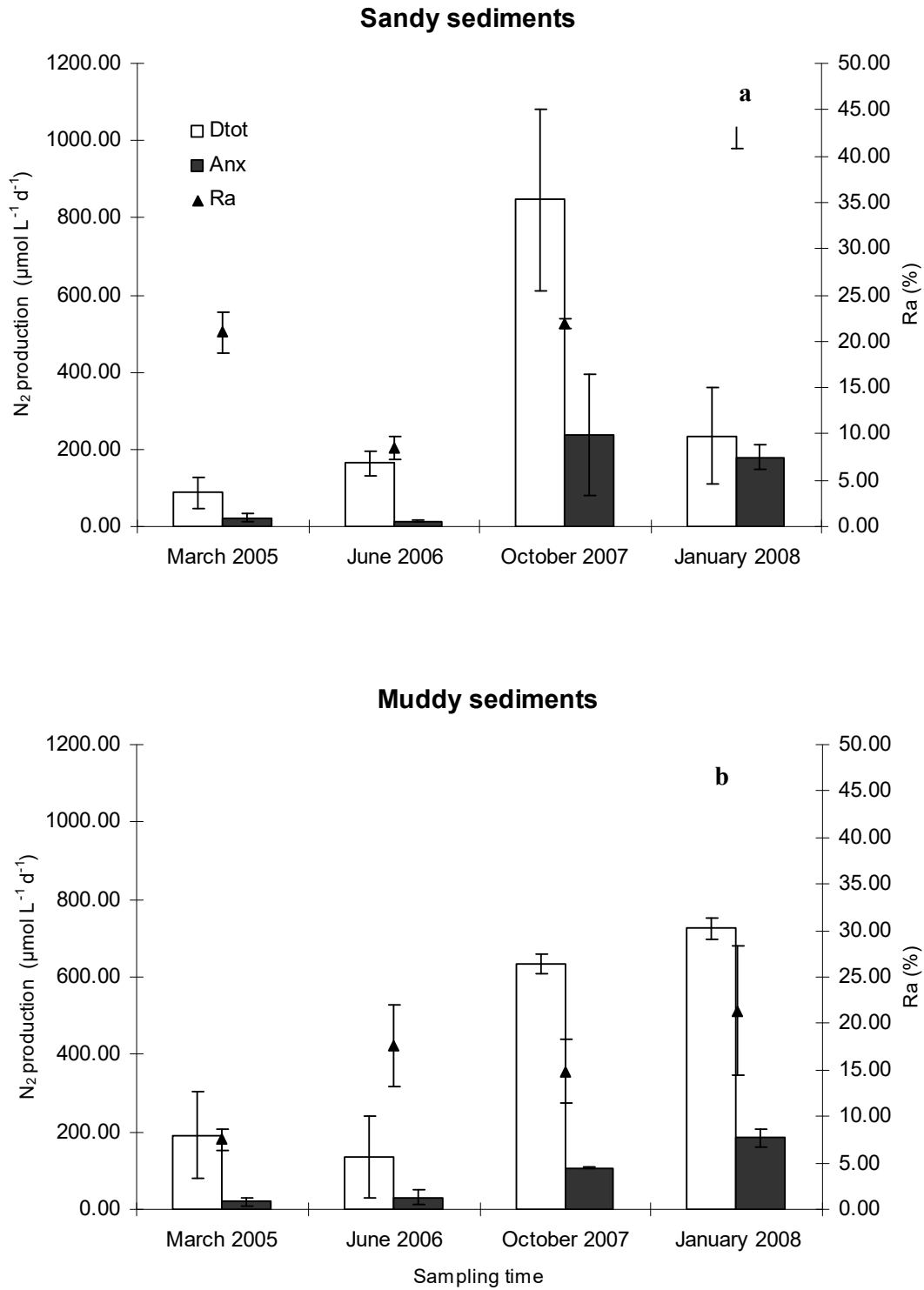


Fig. 1

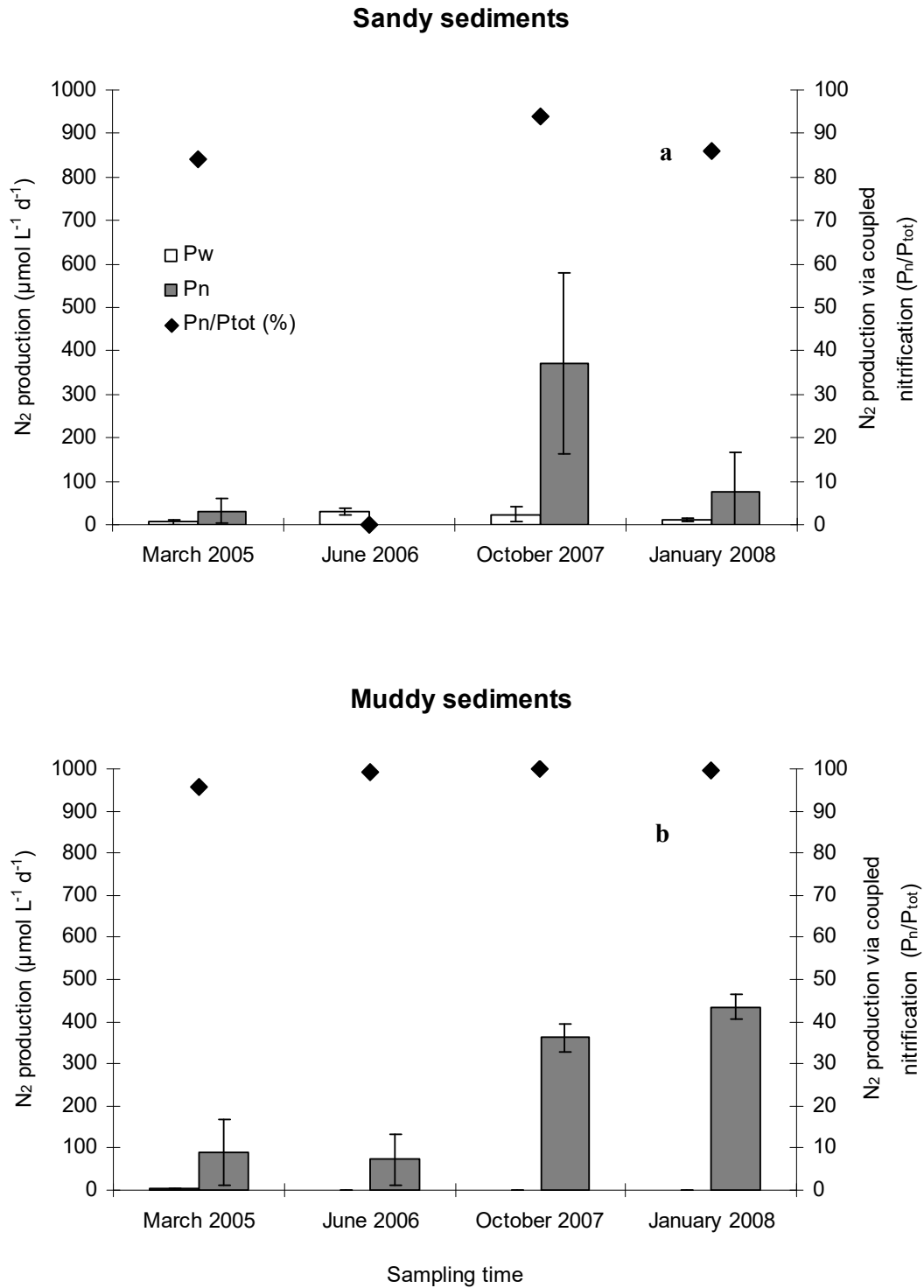


Fig. 2

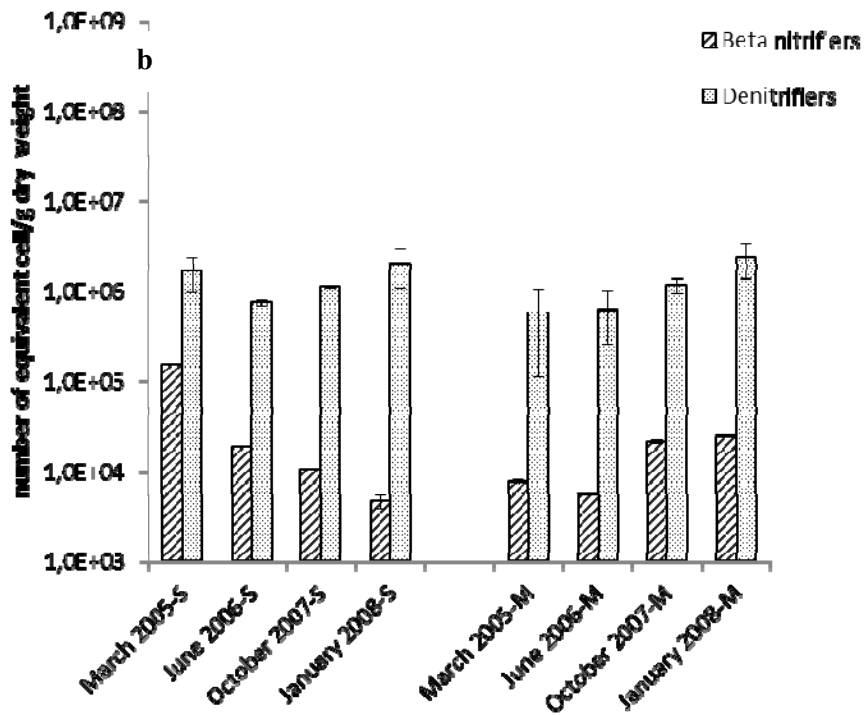
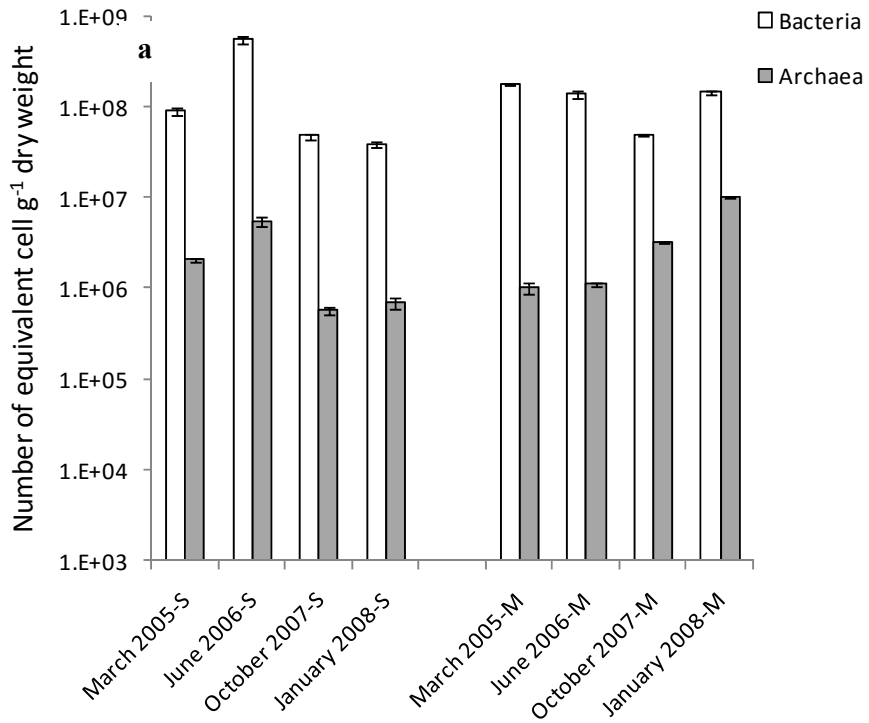


Fig. 3

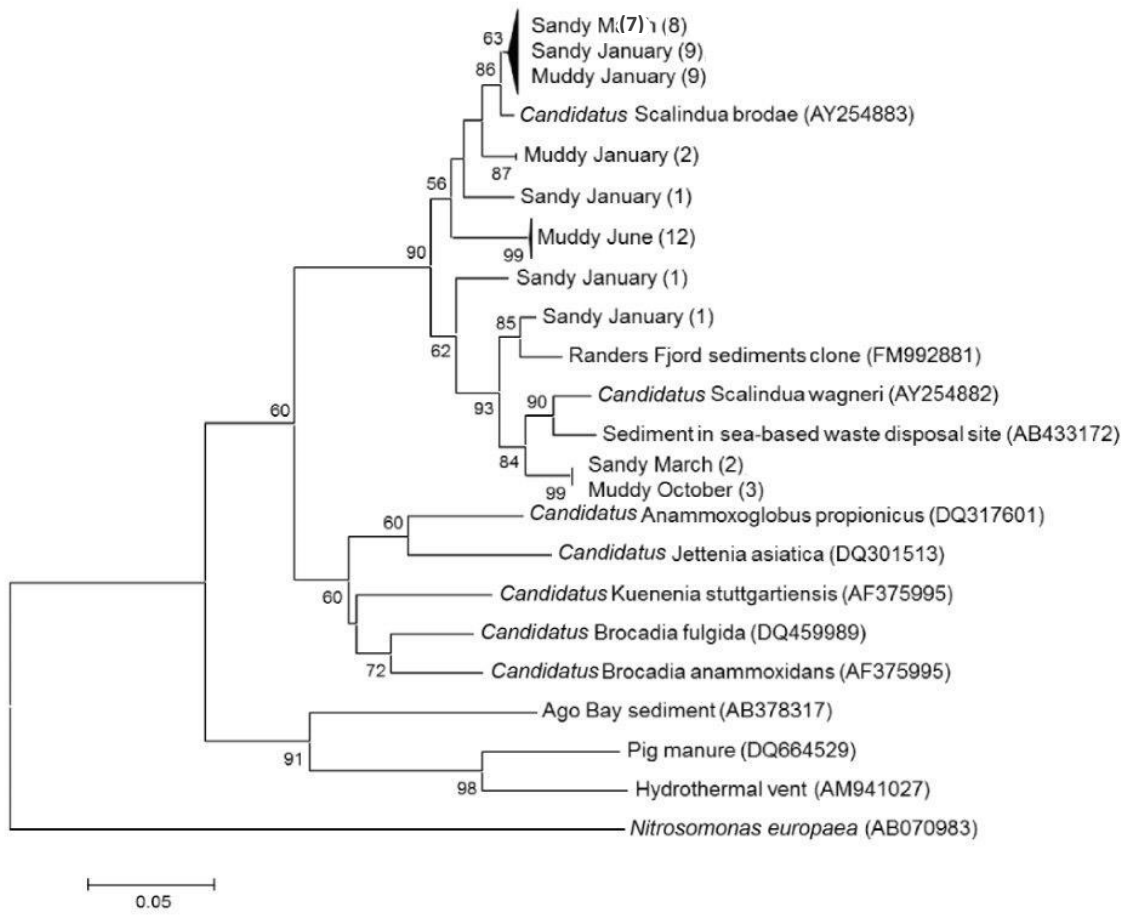


Fig. 4

Supplementary information

Total denitrification (D_{tot}) can be estimated from the production of N_2 molecules of different isotopic composition ($^{29}N_2 = ^{14}N + ^{15}N$, $^{30}N_2 = ^{15}N + ^{15}N$) upon enrichment with stable isotope tracer ($^{15}NO_3^-$) (Nielsen, 1992). The natural abundance of nitrogen isotopes is 99.64% of ^{14}N and 0.36% of ^{15}N (Steingruber et al., 2001). The tracer is added to the overlying water which mixes with the $^{14}NO_3^-$ present in the water column and in the upper sediment layer. The production rates of N_2 with masses of 28, 29, and 30 are represented by P_{28} , P_{29} , and P_{30} respectively.

The total denitrification rate in the sediment is therefore calculated as:

$$D_{\text{tot}} = D_{14} + D_{15} \quad (1)$$

Denitrification of $^{15}NO_3^-$ (D_{15}) can be calculated from the production rate of $^{29}N_2$ (P_{29}) and $^{30}N_2$ (P_{30}) as follows:

$$D_{15} = P_{29} + 2 P_{30} \quad (2)$$

To calculate denitrification rate (D_{14}) of unlabeled $^{14}NO_3^-$:

$$D_{14} = P_{29} + 2 P_{28} \quad (3)$$

P_{28} cannot be measured directly. Nielsen (1992) used the approximation of the binomial law to express D_{14} as function of $^{29}N_2$ and $^{30}N_2$. When denitrification is the sole process producing D_{14} and D_{15} , the ratio of D_{14} versus percentage of $^{14}NO_3^-$ is equal to ratio of D_{15} versus percentage of $^{15}NO_3^-$

$$\frac{D_{14}}{\%^{14}NO_3^-} = \frac{D_{15}}{\%^{15}NO_3^-} \quad (4)$$

Consequently, from equation 4:

$$D_{14} = \frac{P_{29}}{2 \times P_{30}} \times D_{15} \quad (5)$$

$$D_{14} = \frac{P_{29}}{2P_{30}} \times (P_{29} + 2P_{30}) \quad (6)$$

D_{15} = rate of denitrification of $^{15}NO_3^-$

D_{14} = rate of denitrification of $^{14}NO_3^-$

The part of D_{14} that is based on NO_3^- from the water phase (D_w) is calculated from D_{15} and the $^{14}\text{N}:^{15}\text{N}$ ratio of the water column NO_3^- :

$$D_w = D_{15} \frac{[^{14}\text{NO}_3^-]_w}{[^{15}\text{NO}_3^-]_w}$$

where,

$[^{14}\text{NO}_3^-]_w$ = concentration of $^{14}\text{NO}_3^-$ in the water column

$[^{15}\text{NO}_3^-]_w$ = concentration of $^{15}\text{NO}_3^-$ in the water column

In situ denitrification of NO_3^- produced by nitrification (D_n) is calculated as:

$$D_n = D_{14} - D_w$$

If anammox is present, the traditional isotope pairing method (IPM) (Nielsen, 1992) is erroneous and must be modified. In the presence of $^{15}\text{NO}_3^-$, the production of N_2 from anammox modifies the proportion of $^{28}\text{N}_2$, $^{29}\text{N}_2$ and $^{30}\text{N}_2$ production compared to the case where denitrification alone is implied (Risgaard-Petersen et al., 2003; Trimmer et al., 2006; Minjeaud et al., 2008). The total production of $^{28}\text{N}_2$ and $^{29}\text{N}_2$ depends on the ratio of intensities between the reactions of denitrification and anammox. The relative contribution of anammox and denitrification in the total N_2 fluxes was calculated according to the equations described by Minjeaud *et al.* (2008).

The percentage of Anammox is represented by R_a .

$$R_a = \frac{A_{28}}{P_{14}} = \frac{R_{14} (P_{29} - 2R_{14} \times P_{30})}{R_{14} (P_{29} + 2P_{30})} = \frac{P_{29} - (2R_{14} \times P_{30})}{P_{29} + 2P_{30}}$$

A_{28} is $^{28}\text{N}_2$ coming from anammox; P_{14} can be expressed as function of P_{15} and R_{14} .

$$P_{14} = R_{14} \times P_{15} = R_{14} \times (P_{29} + P_{30})$$

The calculation of R_{14} is thus essential for the calculation of P_{14} . R_{14} is the ratio between the quantities $^{14}\text{NO}_3^-$ and $^{15}\text{NO}_3^-$ in the zone of expression of the activities, this ratio is different from the initial percentage of labelling

where,

D_{29} and D_{30} represent the productions of the $^{29}\text{N}_2$ and $^{30}\text{N}_2$ coming from denitrification respectively. A_{29} is the production of $^{29}\text{N}_2$ coming from anammox.

According to Risgaard-Petersen et al. (2003), R_{14} can be experimentally determined from measurements of P_{29} and P_{30} in the presence of different $^{15}\text{NO}_3^-$ concentrations. Measurements of P_{29} and P_{30} with different concentrations of $^{15}\text{NO}_3^-$ allow the calculation of R_{14} and consequently the determination of the production of N_2 formed from $^{14}\text{NO}_3^-$.

Therefore,

$$R_{14_v} = \frac{P_{29_1} - V \times P_{29_2}}{2 \times (P_{30_1} - V^2 \times P_{30_2})}$$

The classical IPM allows quantification of and distinction between denitrification of bottom-water NO_3^- and that produced by sedimentary nitrification during incubation (Nielsen, 1992).

In sediments where anammox and denitrification co-exist, Risgaard-Petersen et al. (2003) showed that it is possible to estimate r_{14} , the ratio between $^{14}\text{NO}_x^-$ and $^{15}\text{NO}_x^-$, in the NO_x reduction zone. Using this information, estimation of N_2 production based on NO_3^- produced in the sediment (p_{14n}) or coming from the overlying water (p_{14w}) become a simple task. To quantify the dependency between p_{14} and the concentration of NO_x^- in the water column (p_{14w}) and the dependency between p_{14} and nitrification (p_{14n}), the following equation was used:

$$P_w = p_{14} \frac{r_{14w}}{r_{14}}$$

$$P_n = p_{14} - P_w = p_{14} \left(1 - \frac{r_{14w}}{r_{14}} \right)$$

Where, r_{14w} is the ratio between $^{14}\text{NO}_x^-$ and $^{15}\text{NO}_x^-$ in the water column.